日本組織培養学会第85回大会
JTCA85 2012 Kyoto

シンポジウム２　細胞接着と細胞機能制御の最先端

# 形態制御が与える機能面の変化 肝細胞初代培養の経験から
# Functional changes induced by morphological regulation in primary hepatocyte culture


絵野沢 伸
Shin Enosawa
国立成育医療研究センター　臨床研究センター　先端医療開発室
Division for Advanced Medical Sciences,
National Center for Child Health and Development


平成24年5月18日　京都

# 謝辞・利益相反・共同研究者
# Acknowledgment・COI・Collaborators

本研究において肝組織・肝細胞ならびに手術摘出がん組織を提供してくださいました患者さま、ご家族にまずは深く御礼申し上げます。

Authors would like to express sincere gratitude to people who donate hepatocytes and surgically resected tumor tissues for scientific research.

本研究は厚生労働科学研究費政策創薬マッチング官民共同研究事業「創薬研究における人由来初代細胞および幹細胞の利用円滑化に向けた研究」の補助金およびトランスパレント社の委託研究費により行いました。

Supported by Grant for Public-private sector joint research on Publicly Essential Drugs by Ministry of Health, Labour and Welfare and contract research with Transparent.

＜共同研究者＞

高橋由里子（株式会社トランスパレント）

Yuriko Takahashi, Transparent

城村友子　（株式会社トランスパレント）

Tomoko Jomura, Transparent

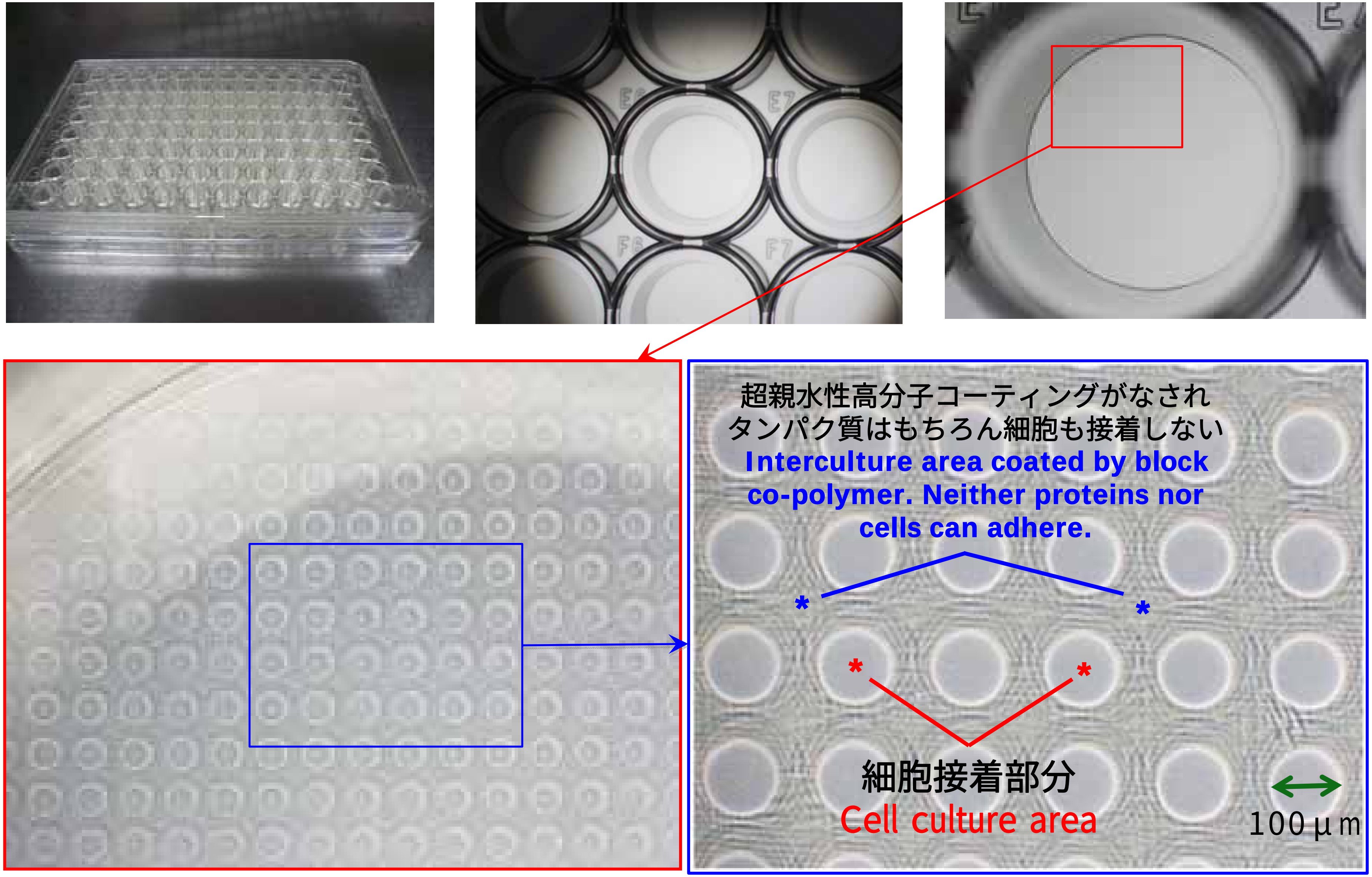
池谷武志　（株式会社トランスパレント）

Takeshi Ikeya, Transparent

宮本義孝　（国立成育医療研究センター）

Yoshitaka Miyamoto, National Center for Child Health and Development

# セルエイブル概観　Outline of Cell-Able



# ふたつの技術要素
## Two Technological factors in Cell-able System

✧培養表面のポリマー加工によって規格化されたスフェロイドを作ることができる.

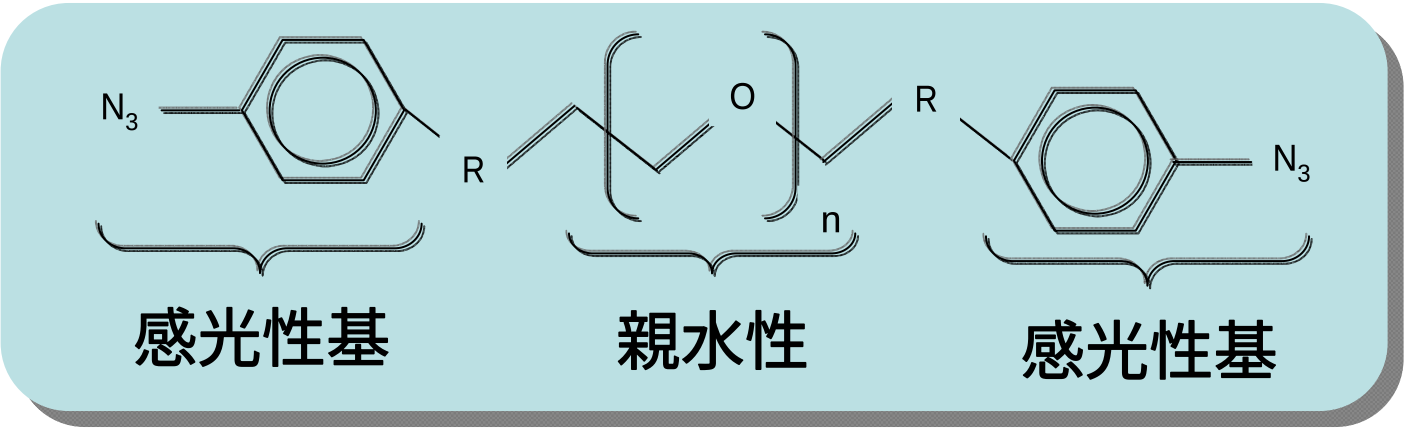
Formation of uniform-size spheroids by microfabrication of culture surface.

✧フィーダー細胞を利用すると細胞の接着がよくなり、機能維持にも優れる.

Use of feeder cells promotes hepatocytes attachment and long-term maintenance of functions.

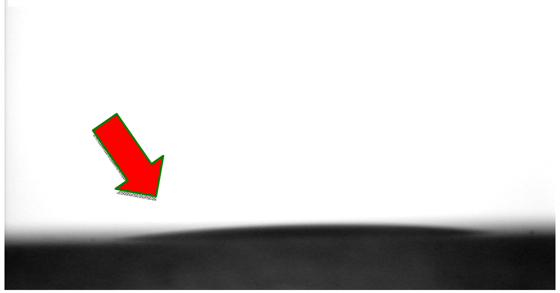
# 細胞非接着面を形成する感光材料の特徴

## 感光材料: **PEG誘導体**（感光化）

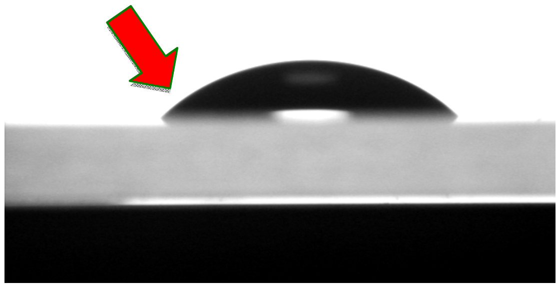



## 光硬化後の感光材料表面の性質（水の静的接触角）

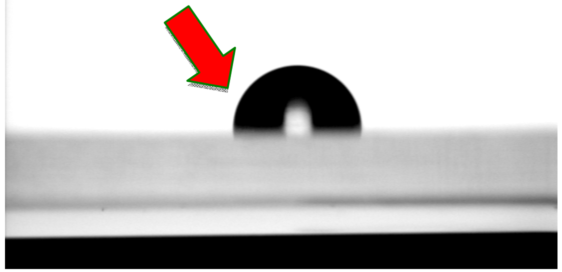
感光材料（光硬化後）
**8°**



コントロール **(TCPS)**
**42°**

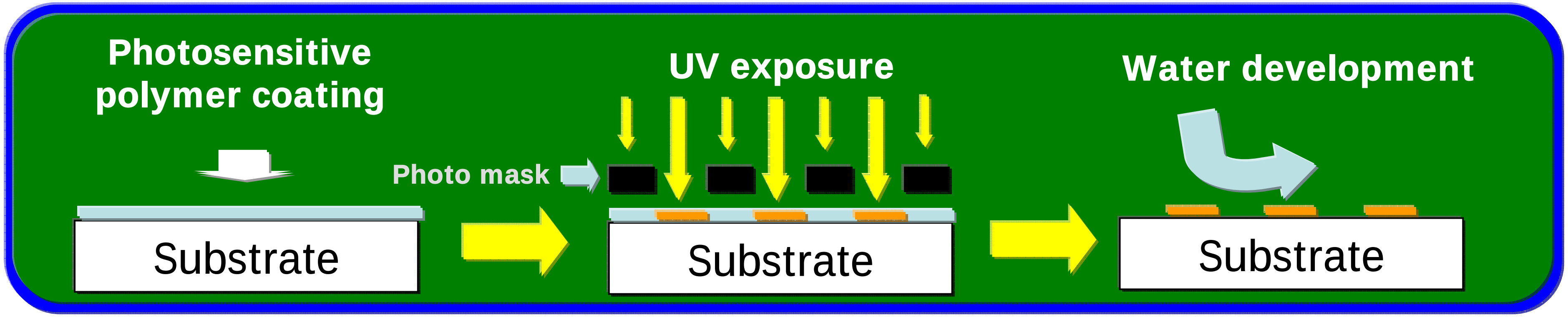


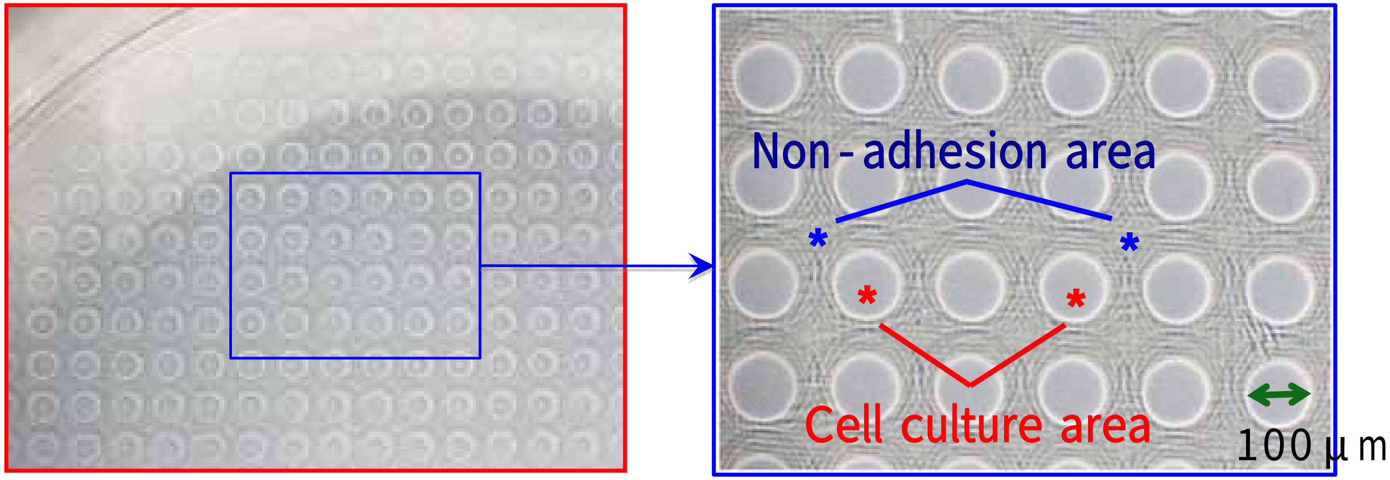
コントロール **(PSt)**
**92°**



Ultra - hydrophilic

# Production of Cell-able™



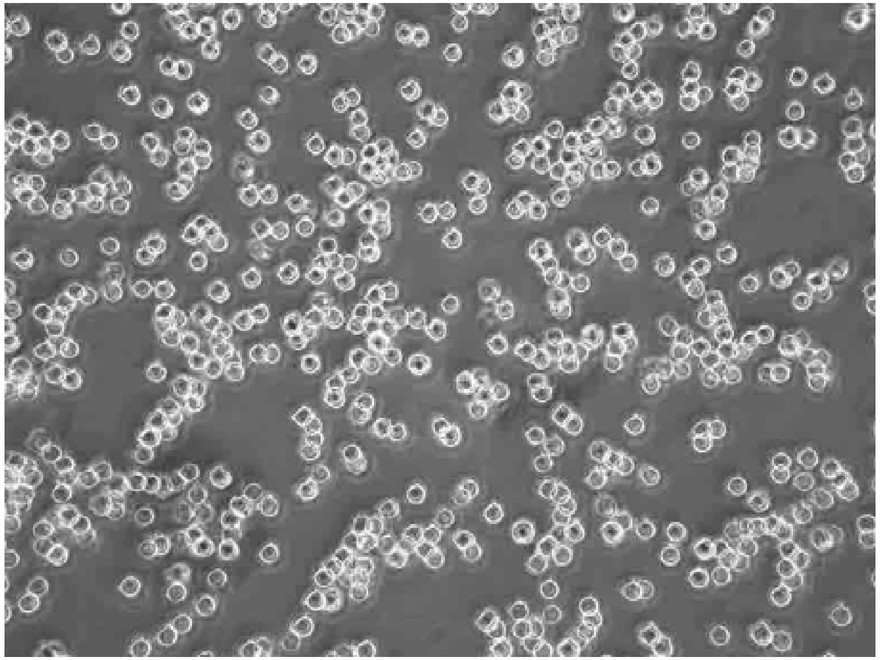




開発者　　大塚英典、長崎幸夫、片岡一則、池谷武志

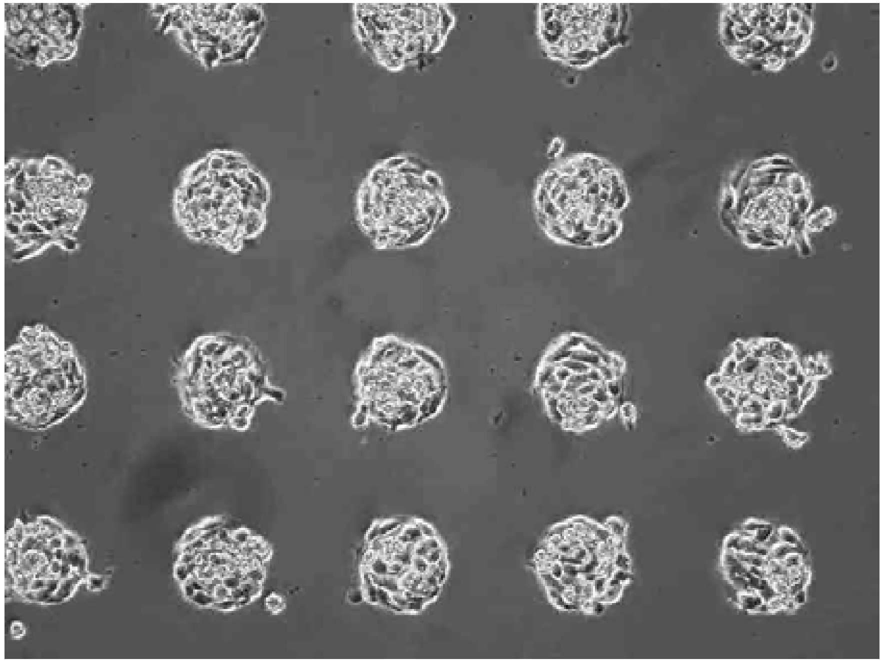
Developed by Hidenori Ootsuka, Yukio Nagasaki, Kazunori Kataoka, Takeshi Ikeya

# 播種細胞が接着するまで
# Migration of seeded cells

**Day 0**

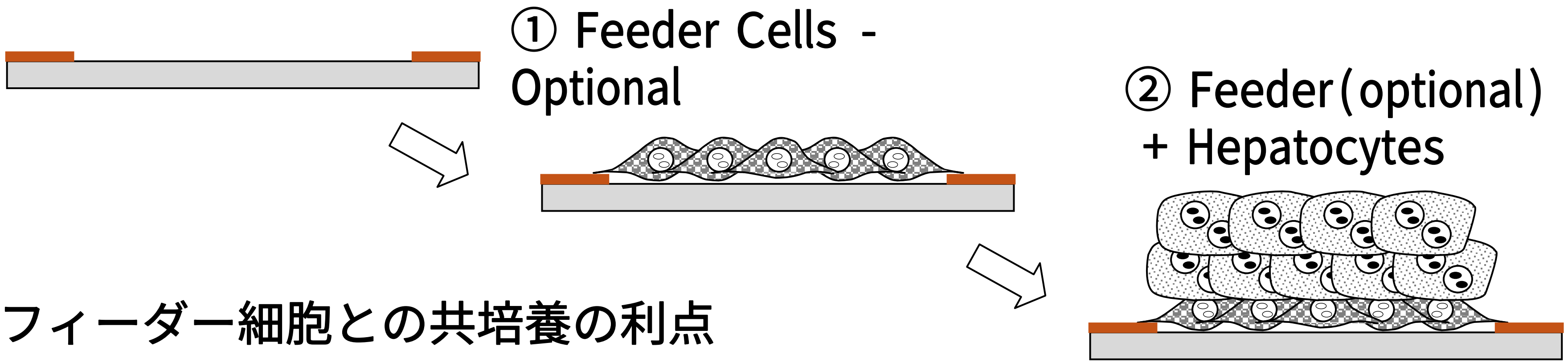


**Day 1**



# Cell-ableによる肝細胞初代培養の標準プロトコール

# Standard Protocol of Primary Hepatocyte Culture on Cell-able




フィーダー細胞との共培養の利点

Advantage of co-culture with feeder cells

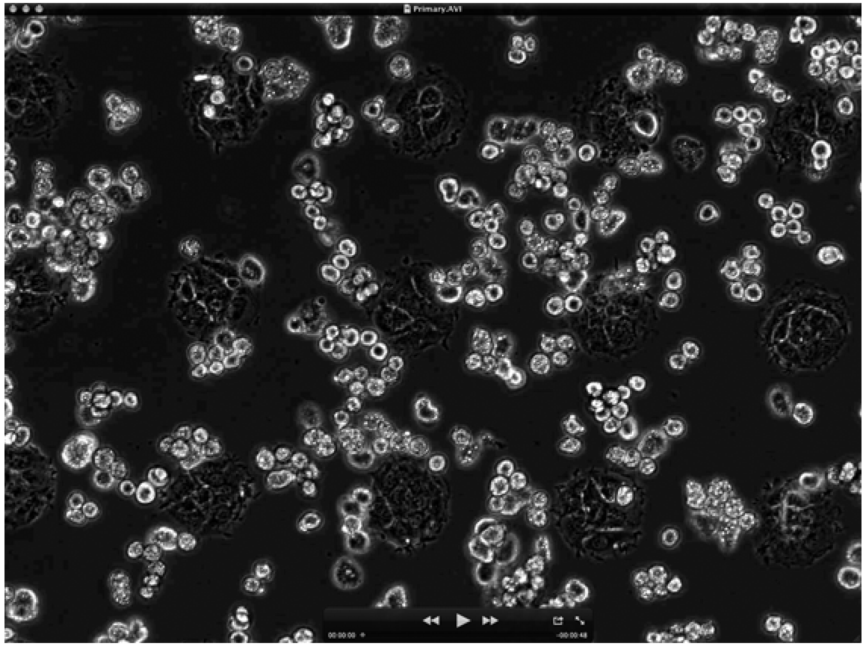
- CYP活性の長期維持

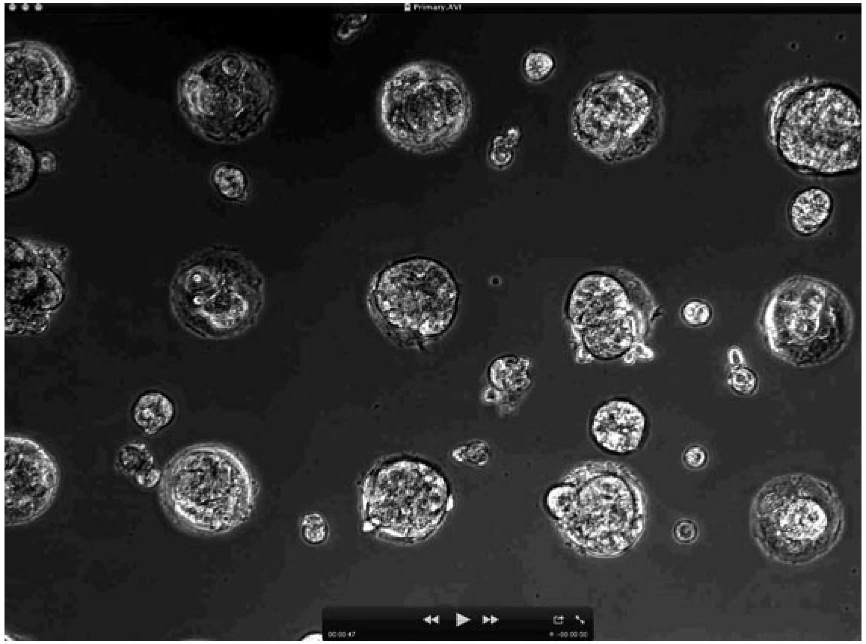
  Long-lasting CYP activities

- 低接着性凍結肝細胞の培養実験使用が可能

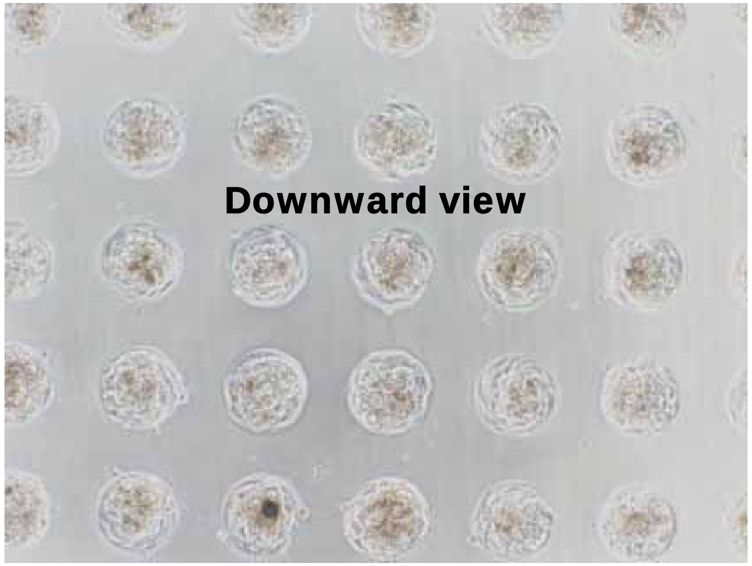
  Cryopreserved hepatocyte with low attaching capability can be cultured

# 肝細胞スフェロイドが完成するまで
# Formation of hepatocyte spheroids

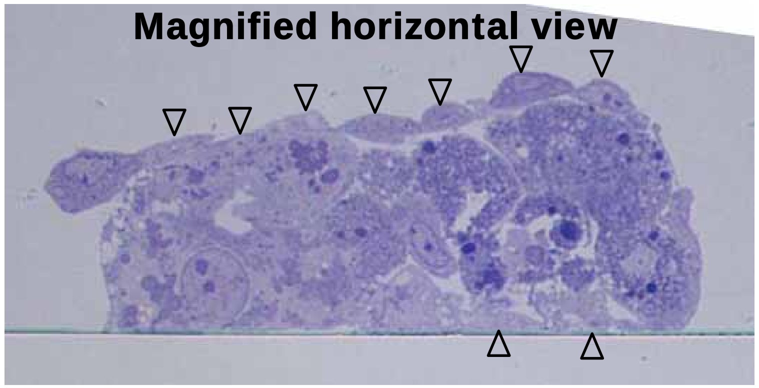


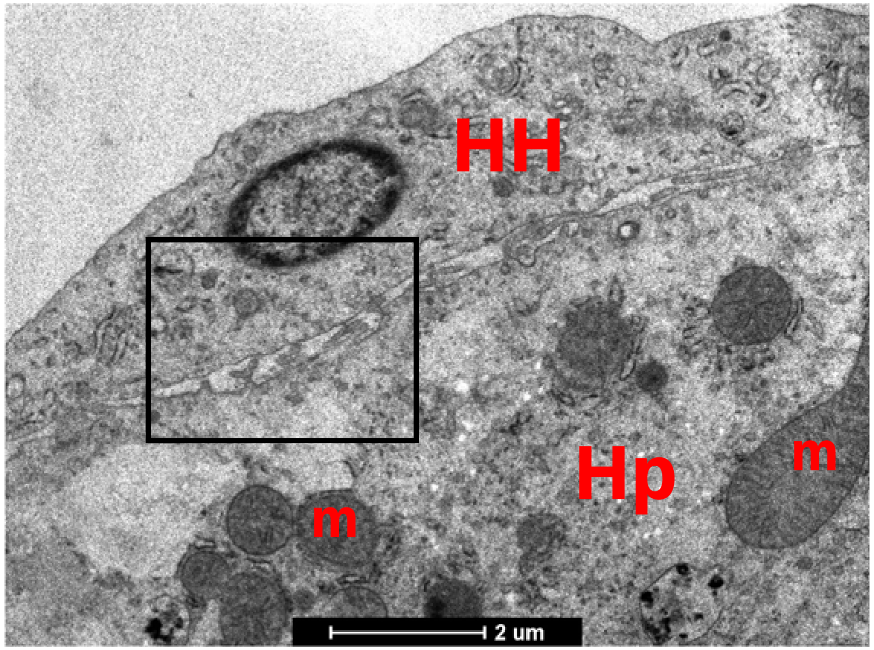


# Microstructure of Hepatocyte-feeder cell heterospheroids formed on the Cell-able








**Diesse space-like structure was observed between hepatocyte (Hp) and feeder (HH) cell with microvilli rooted from hepatocyte was observed. m; mitochondria.**

**Arrow heads indicate HH cells that migrate from the culture plate and enwrap spheroidal hepatocyte mass.**

# 材料と方法　Materials and Methods

ヒト肝細胞　[Human Hepatocytes]

Fresh; isolated from surgically resected liver in National Center for Child Health and Development (IRB permission No.385, 396)

Cryopreserved; Xenotech, IVT

フィーダー細胞　[Feeder cells]

HH bovine aortic epithelial cells (JCRB0099), Mouse 3T3 fibroblasts (ATCC CCL-92, ATCC CCL-163), Rhesus monkey retinal epithelial cells (ATCC CRL-1780)

培地　[Culture medium]

RM100; medium for rat hepatocytes (Transparent)

RM101; Medium for human hepatocytes (Transparent)

SE & YY; Williams E-base Matrigel-containing medium (reported by Enosawa and Yamada in JSSX2009)

IVT;　InVitroGRO HI Medium

XENOTECH; Hepatocyte culture media

BD; BD Hepatocyte Culture Medium Kit

培養方法　[Culture]

2x10^4 human hepatocytes /one well of 96-well

When feeder cells were used, 8x10^3 cells / one well were seeded two days before hepatocytes inoculation.

機能評価（CYP活性測定）　[CYP activity]

Testosterone 6 beta hydroxylation, Testosterone glucronidation. CYP induction; rifampicin

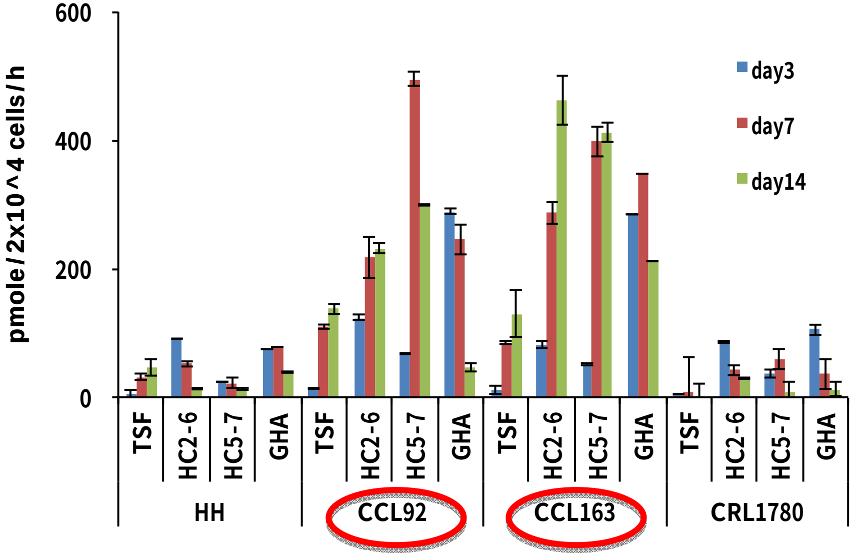
トランスポーター活性　[Transporter activities]

Influx; tritiated ([6,7-3H(N)]-estrone sulfate, Efflux; carboxy-dichlorofluorescein diacetate (CDF-DA)

# 各種フィーダー細胞を用いた場合のCYP活性

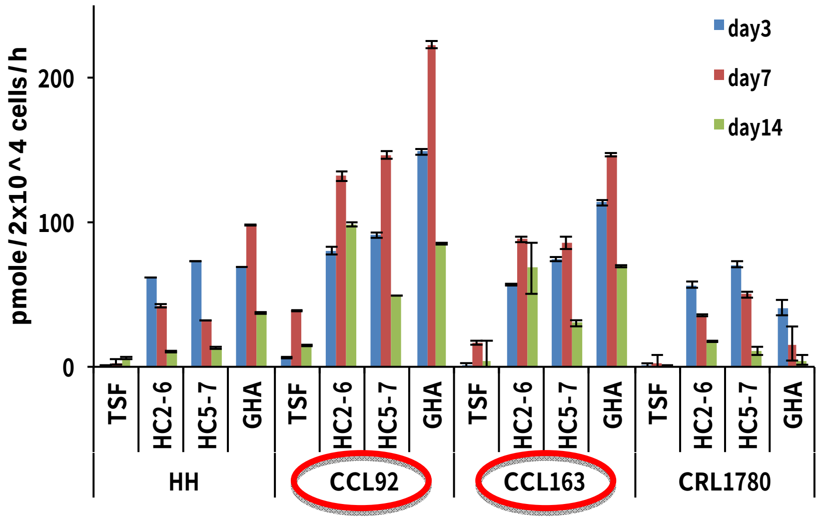
## Optimum feeder cell

Comparison of feeder cells (basal activity)
Testosterone→6βHydroxytestosterone




RECOMMENDED

Comparison of feeder cells (basal activity)
Testosterone→Testosterone glucronide

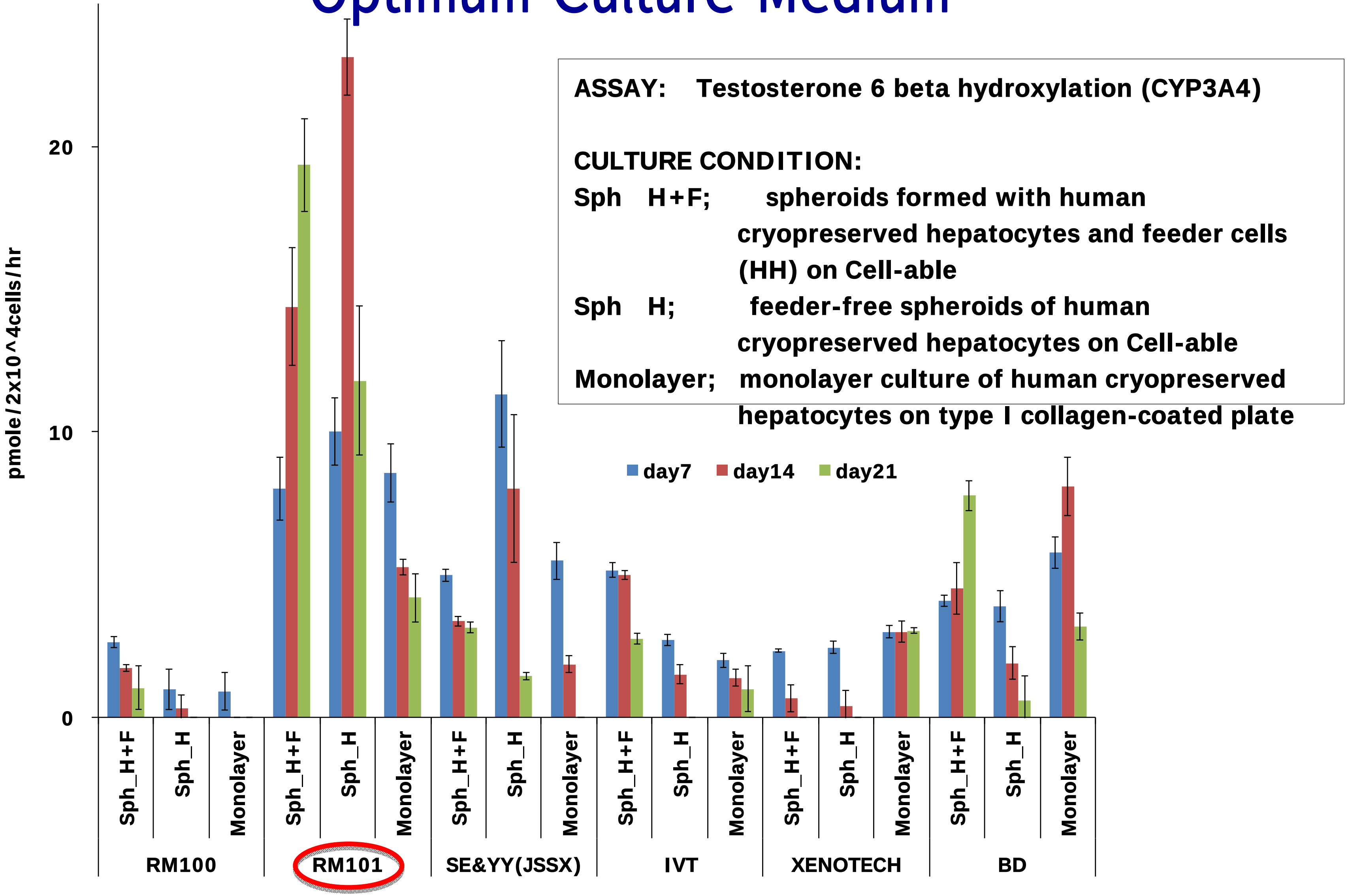



RECOMMENDED

| Cell lines examined | | |
|---|---|---|
| Designation | Code No. | Origin |
| HH | JCRB0099 | Bovine aortic epithelium |
| 3T3-Swiss albino | ATCC CCL-92 (JCRB9019*) | Mouse fibroblast |
| BALB/3T3 clone A31 | ATCC CCL-163 | Mouse fibroblast |
| RF/6A | ATCC CRL-1780 | Rhesus monkey retinal epithelium |

*ATCC CCL-92 is also distributed by JCRB as JCRB9019 in Japan

# フィーダー細胞の有無と培地比較
## Optimum Culture Medium

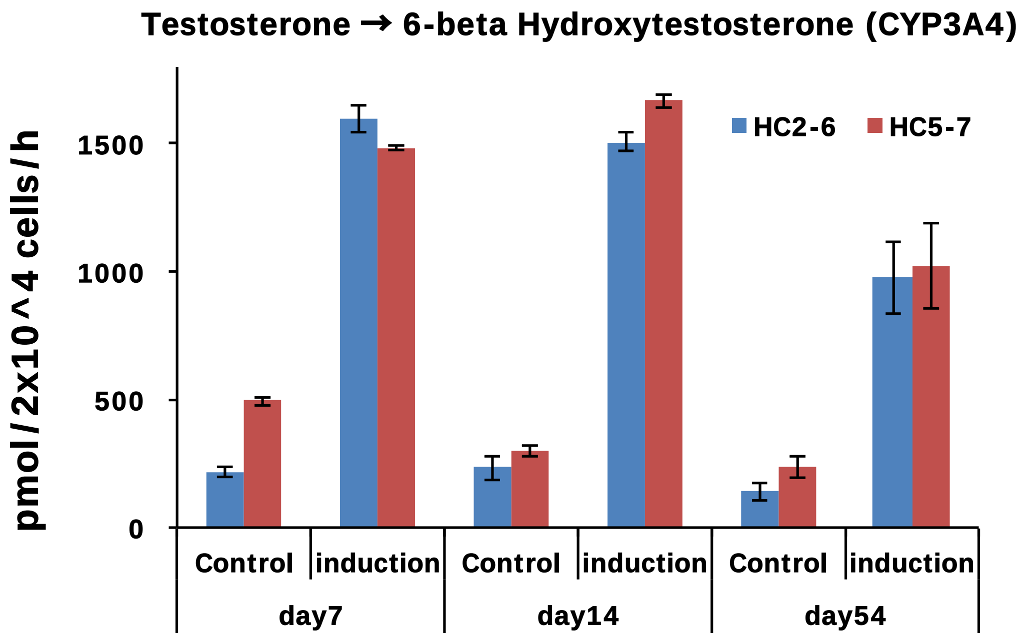



**RECOMMENDED**

**RM101 (Transparent) showed Excellent CYP activities even with feeder-free culture**

# CYP活性および誘導能の長期維持

## Long-lasting CYP Activity of Cryopreserved Human Hepatocytes Cultured on Cell-able

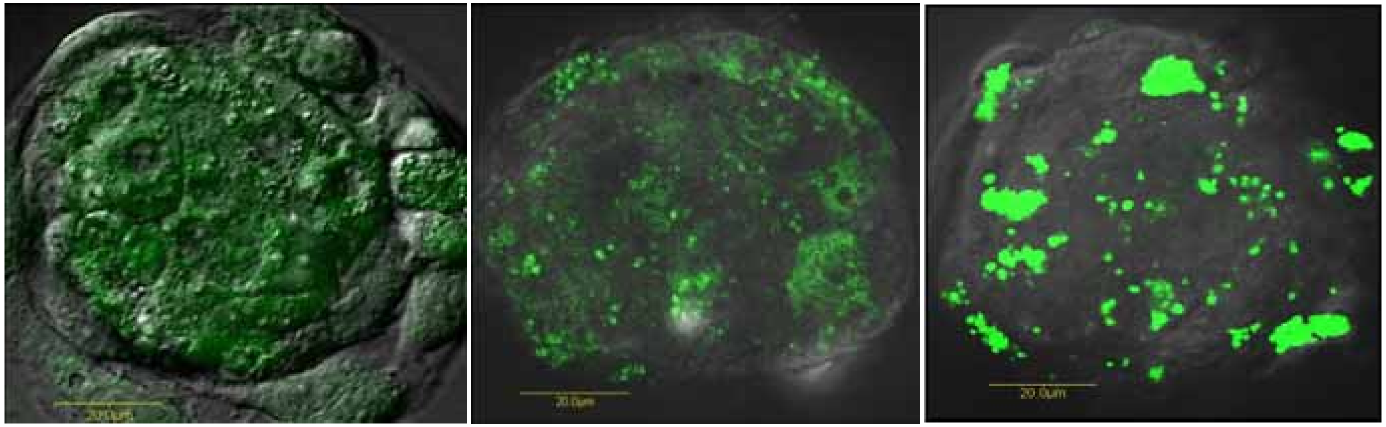



The initial activity of each lot was 549.6 and 214.8 pmol/2x10^4/h, respectively.

# CDF-DAをプローブとして見た肝細胞スフェロイドが示す排出トランスポーター活性

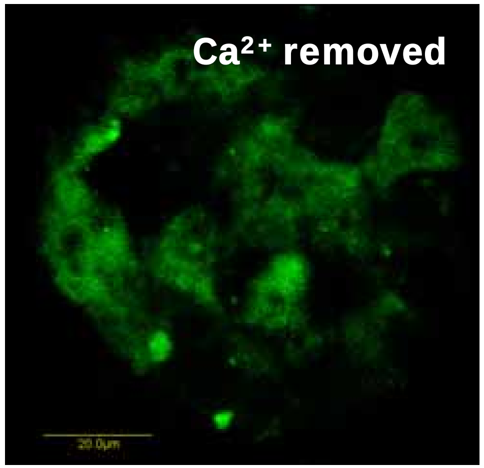
## Hepatocyte spheroids show efflux transporter activities examined by CDF-DA exclusion into intercellular bile pools

**Culture day 2** **day 4** **day 7**



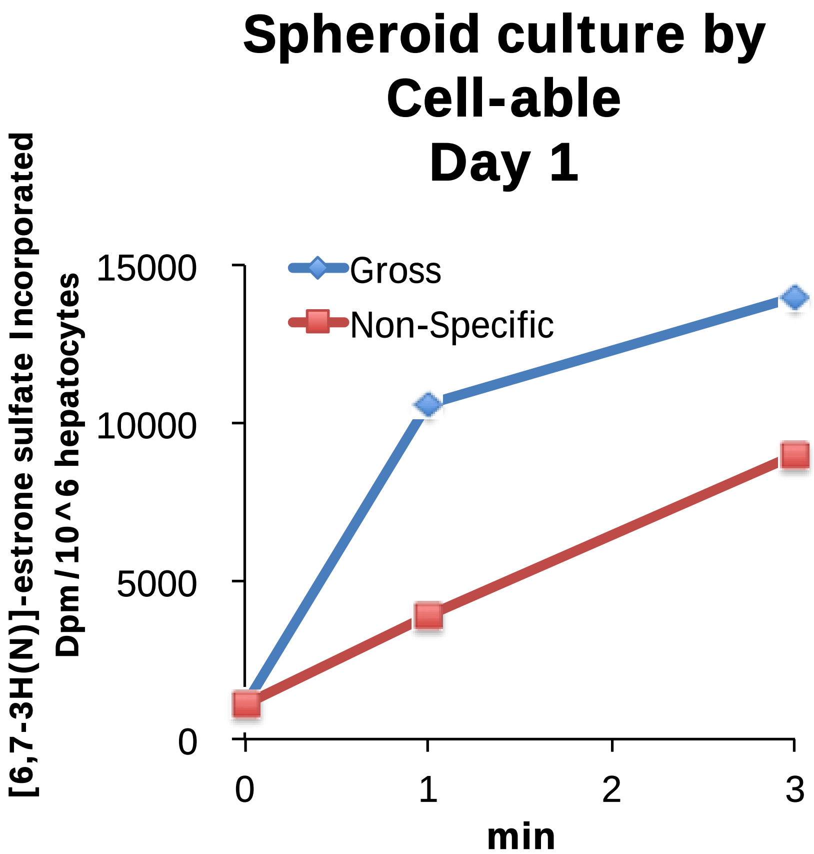
**Bile pool formation and CDF exclusion were becoming marked with the increase of culture days or maturation of spheroid. (Above)**

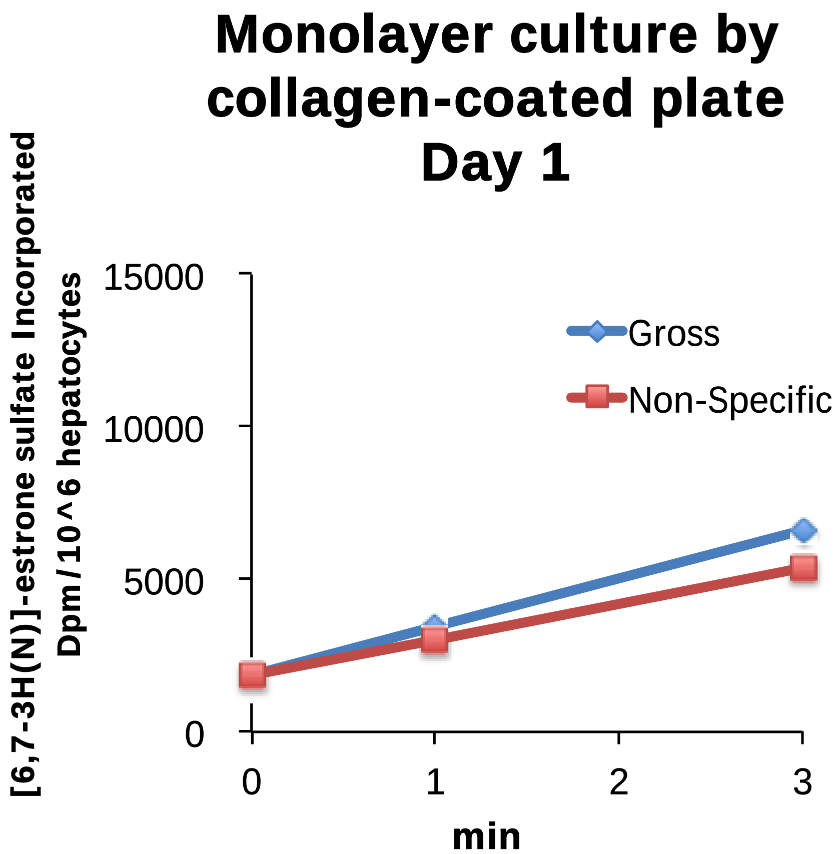
**Bile pools almost disappeared by removal of $Ca^{2+}$ ions.**



# [6,7-3H(N)]-estrone sulfateをプローブとして見た肝細胞スフェロイドが示す取込トランスポーター活性

## Hepatocyte spheroids show influx transporter activities examined by [6,7-3H(N)]-estrone sulfate exclusion into intercellular bile pools




**Hepatocyte spheroids showed good influx transporter activity.**

**Non-specific incorporation was determined under the existence of inhibitor (taurocholate).**

# 小　括　Brief Summary

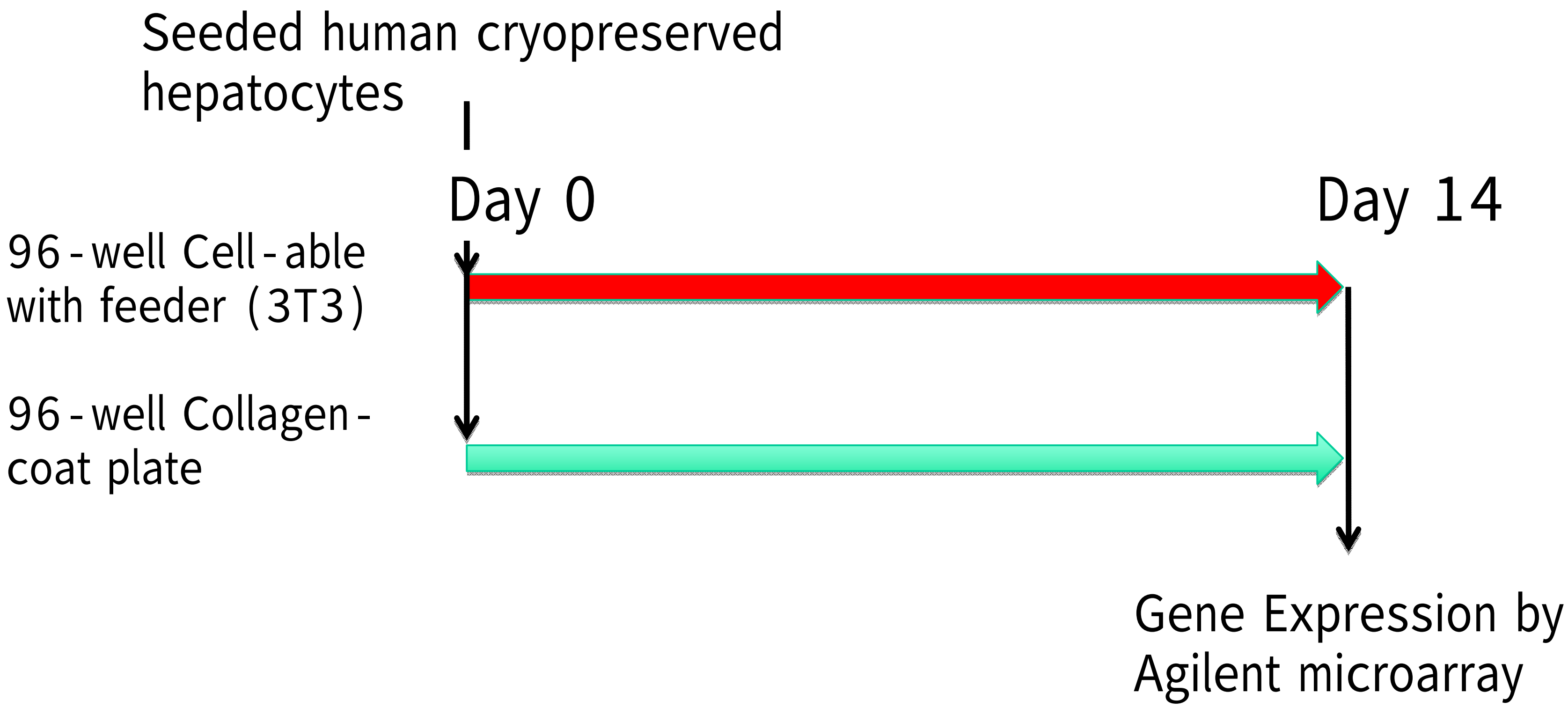
✧ Cell-able培養においてマウス3T3細胞はヒト肝細胞長期培養の際のフィーダー細胞として優れることがわかった。

Mouse 3T3 fibroblasts are more effective on long-term hepatocyte culture as feeder cells than bovine endothelial of monkey epithelial cells on Cell-able.

✧ Cell-able培養においてヒト肝細胞スフェロイドは成熟とともに取り込み、排泄トランスポーター活性を示すことがわかった。

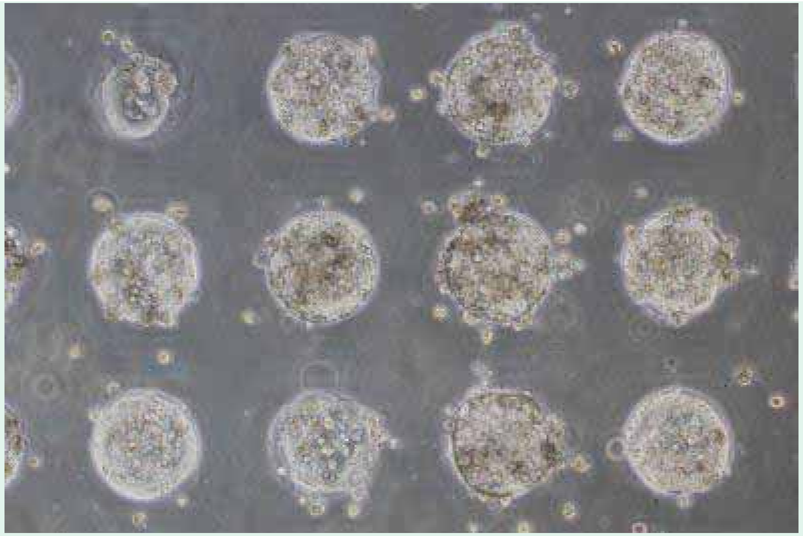
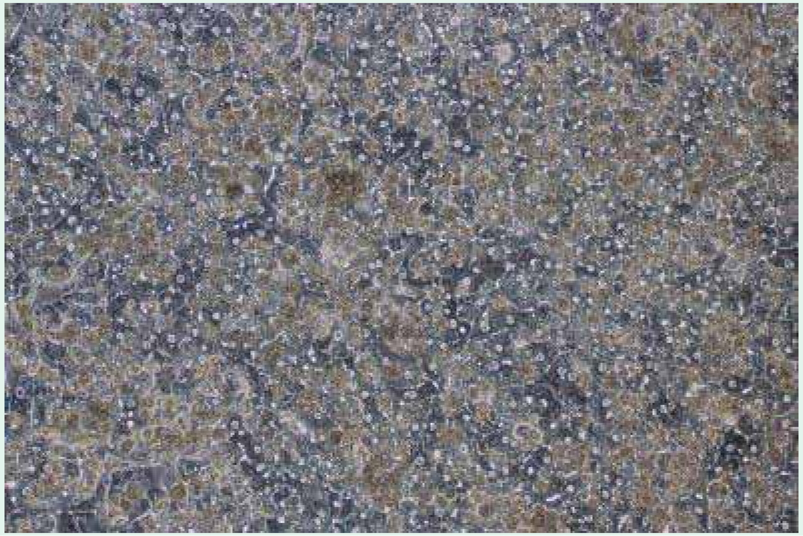
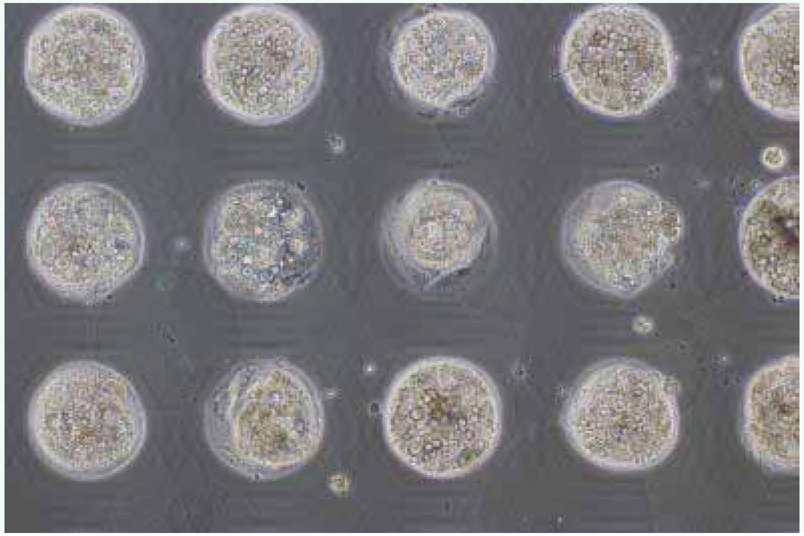
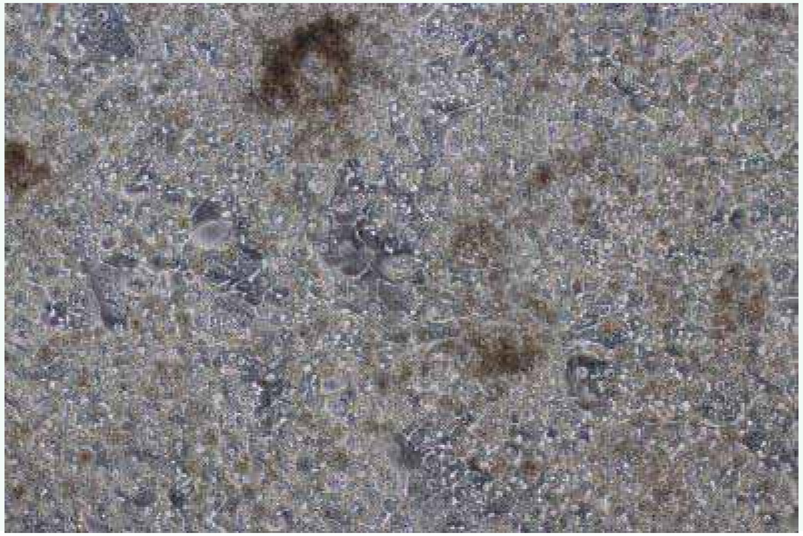
The human hepatocyte spheroids formed on Cell-able showed influx and efflux transporter activities.

# Cell-able培養と通常プレート培養における遺伝子発現状態の相違
## Difference in Gene Expression between Cell-able and Conventional Plate




1. Well間差　Inter-well difference
2. Day14/Day 0発現比　Day14/Day 0 Ratio

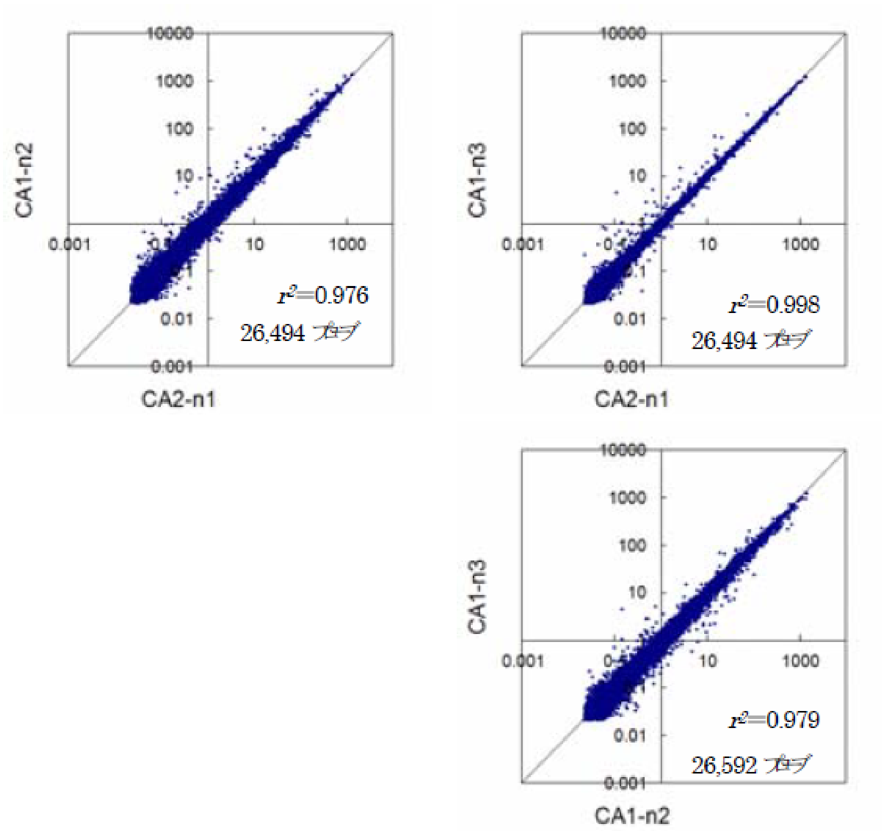
# Cell-able培養と通常プレート培養における遺伝子発現状態の相違
## Difference in Gene Expression between Cell-able and Conventional Plate

| | Cell-able | Collagen coat plate |
|---|---|---|
| Day7 |  |  |
| Day14 |  |  |

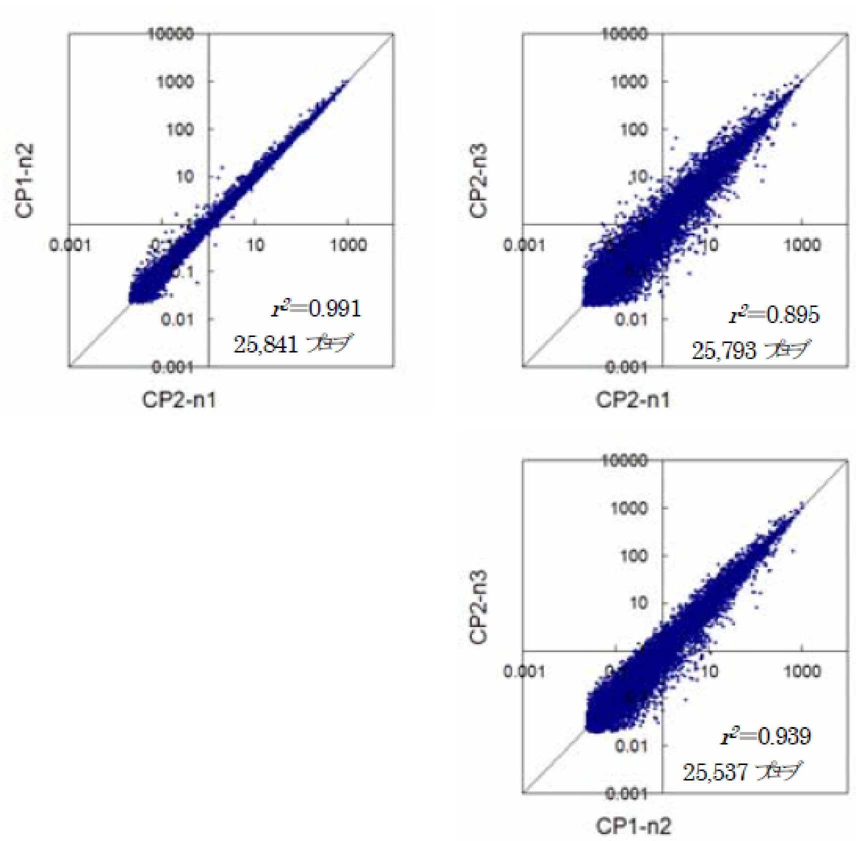
# Cell-able培養と通常プレート培養における遺伝子発現状態の相違
# Difference in Gene Expression between Cell-able and Conventional Plate

## Cell-ableの方がWell間差が少ない
## Lower difference in Cell-able than conventional plate

a)　CA 群




b)　CP 群



# Cell-able培養と通常プレート培養における遺伝子発現状態の相違
## Difference in Gene Expression between Cell-able and Conventional Plate

＜以後のデータ表示＞
Global normalizationののち、Day14／Day0比で表示しCell-able培養とConventional plate（コラーゲンコートプレート）培養を比較

$$\frac{\text{14日間培養した細胞における発現}}{\text{解凍時＝in vivo肝組織}} = \text{14日間の培養の影響による変化}$$

＜Data expression＞
After global normalization, expressed as a Day14/Day0 ratio and compare the effect of Cell-able culture and conventional collagen plate culture.
i.e.,

$$\frac{\text{Expression of 14days culture}}{\text{Expression of liver in vivo}} = \text{Effect of 14 day culture}$$

# Cell-able培養と通常プレート培養における遺伝子発現状態の相違

## Difference in Gene Expression between Cell-able and Conventional Plate

| 主要CYP | Cell-able | Conventional plate |
|---|---|---|
| CYP1A1 | 1.44 | 3.51 |
| CYP1A2 | 0.76 | 0.68 |
| CYP2B6 | 0.59 | 0.36 |
| CYP2C19 | 0.31 | 0.11 |
| CYP2C9 | 0.37 | 0.15 |
| CYP2D6 | 0.26 | 0.18 |
| CYP3A4 | 0.44 | 0.42 |
| CYP3A5 | 0.32 | 0.25 |

| 核内受容体 | | Cell-able | Conventional plate |
|---|---|---|---|
| NR1H3 | LXRa | 0.57 | 0.46 |
| NR1H4 | FXR | 0.76 | 0.99 |
| NR1I2 | PXR | 0.60 | 0.50 |
| NR1I3 | CAR | 0.30 | 0.08 |
| NR1I3 | CAR | 0.34 | 0.12 |
| NR3C1 | GR | 0.91 | 0.84 |
| NR3C1 | GR | 1.26 | 0.87 |
| RXRA | | 1.02 | 1.00 |
| AHR | | 0.48 | 0.41 |
| AHR | | 0.51 | 0.57 |

| トランスポーター | | Cell-able | Conventional plate |
|---|---|---|---|
| ABCB1 | MDR1 | 0.89 | 1.19 |
| ABCB11 | BSEP | 0.56 | 0.26 |
| ABCC2 | MRP2 | 1.03 | 0.61 |
| SLC10A1 | NTCP | 0.40 | 0.10 |
| SLC10A2 | ASBT | 1.12 | 0.67 |
| SLC22A1 | OTC1 | 0.47 | 0.18 |
| SLC22A2 | OTC2 | 2.35 | 0.24 |
| SLC22A4 | OCTN1 | 1.24 | 2.35 |
| SLC22A5 | OCTN2 | 1.26 | 2.07 |
| SLC22A6 | OAT1 | 0.48 | 0.44 |
| SLC22A7 | OAT2 | 0.61 | 0.37 |
| SLCO1A2 | OATP1A2 | 0.17 | 0.24 |
| SLCO1A2 | OATP1A2 | 0.67 | 0.56 |
| SLCO1B1 | OATP1B1 | 0.46 | 0.45 |
| SLCO2A1 | OATP2A1 | 0.29 | 0.26 |
| SLCO2B1 | OATP2B1 | 0.73 | 0.43 |

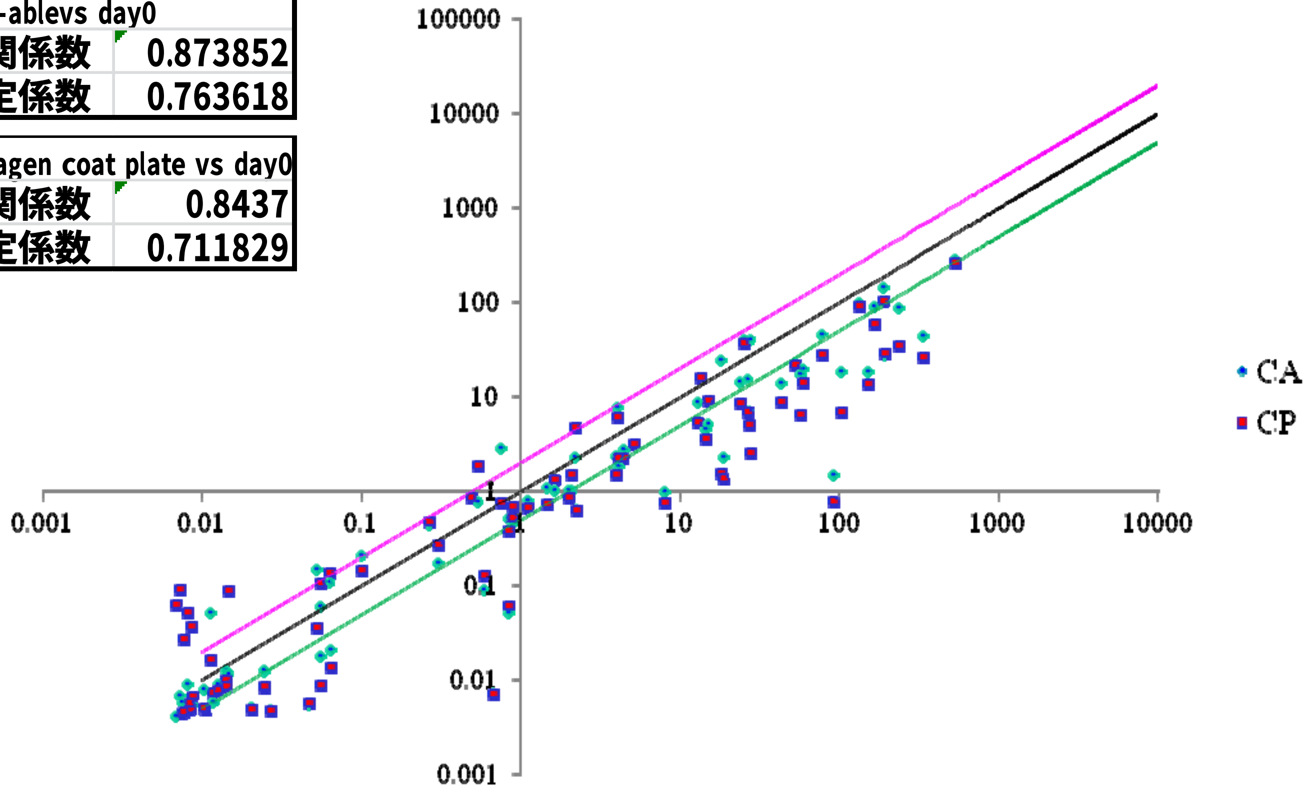
| 肝細胞タンパク | Cell-able | Conventional plate |
|---|---|---|
| Albumin | 0.62 | 0.48 |
| HNF4 alpha | 0.48 | 0.53 |
| HNF4 gamma | 1.29 | 1.33 |
| Tyrosine amino transf | 0.33 | 0.17 |
| Transferrin | 0.68 | 0.25 |

# Cell-able培養と通常プレート培養における遺伝子発現状態の相違
## Difference in Gene Expression between Cell-able and Conventional Plate

### CYPs

| Cell-ablevs day0 | |
|---|---|
| 相関係数 | 0.873852 |
| 決定係数 | 0.763618 |

| Collagen coat plate vs day0 | |
|---|---|
| 相関係数 | 0.8437 |
| 決定係数 | 0.711829 |

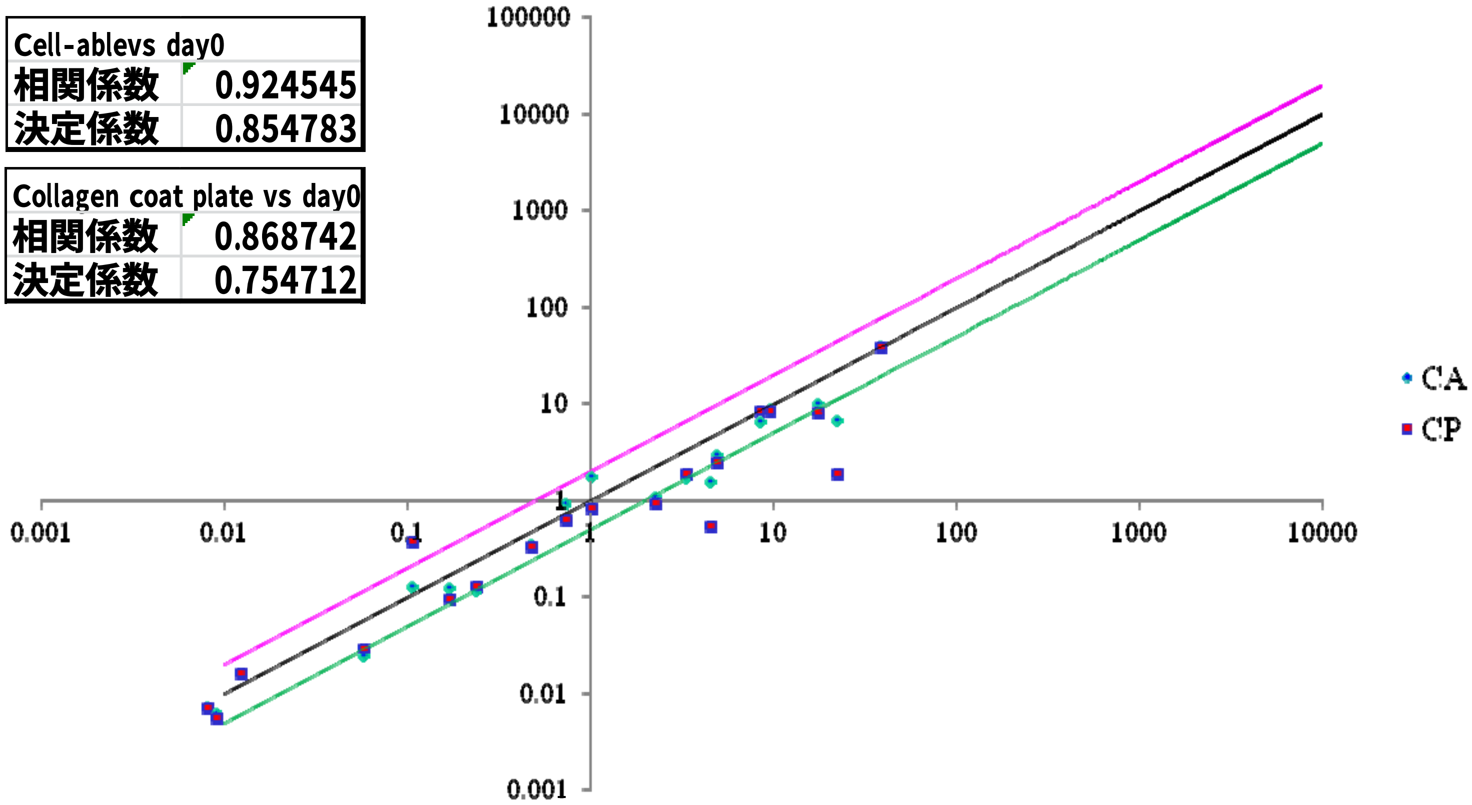


# Cell-able培養と通常プレート培養における遺伝子発現状態の相違
## Difference in Gene Expression between Cell-able and Conventional Plate

### Nuclear Receptors

| Cell-ablevs day0 | |
|---|---|
| 相関係数 | 0.924545 |
| 決定係数 | 0.854783 |

| Collagen coat plate vs day0 | |
|---|---|
| 相関係数 | 0.868742 |
| 決定係数 | 0.754712 |

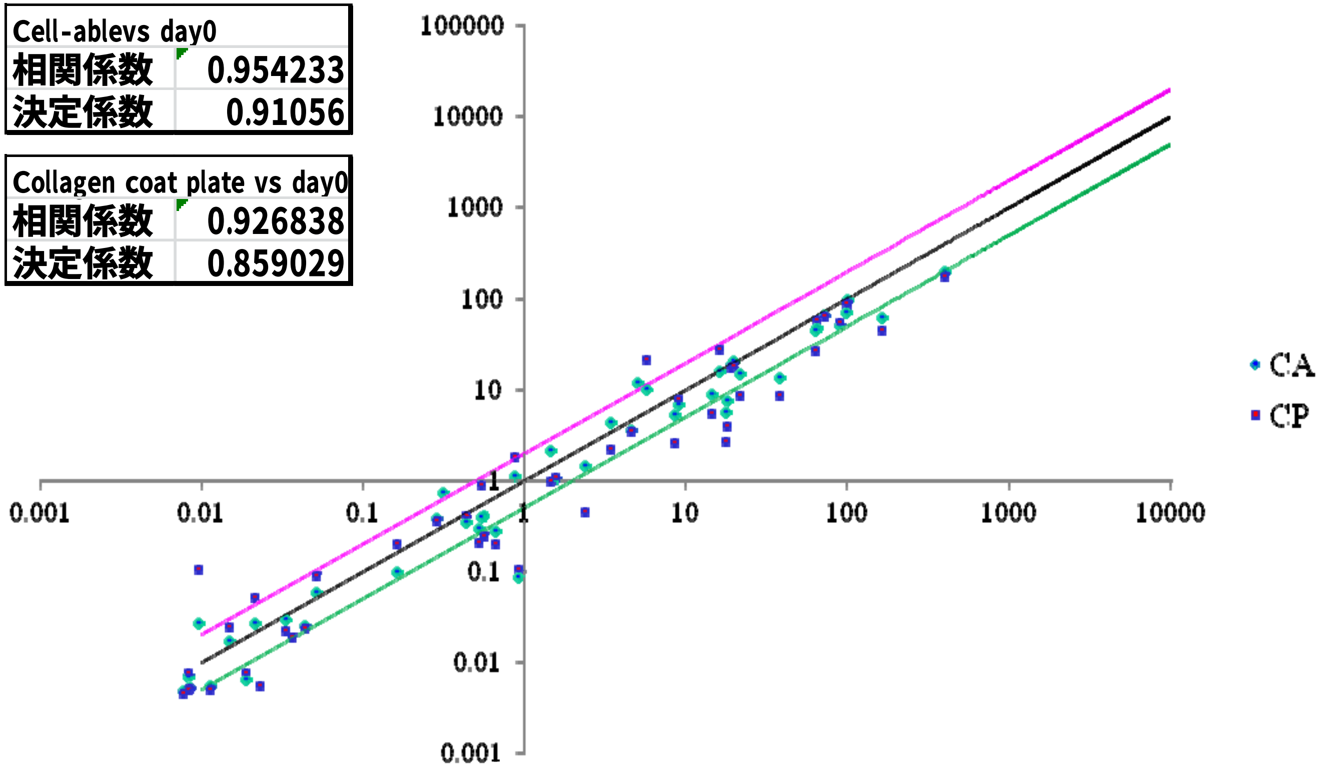


# Cell-able培養と通常プレート培養における遺伝子発現状態の相違
## Difference in Gene Expression between Cell-able and Conventional Plate

## Conjugation Enzymes

| Cell-ablevs day0 | |
|---|---|
| 相関係数 | 0.954233 |
| 決定係数 | 0.91056 |

| Collagen coat plate vs day0 | |
|---|---|
| 相関係数 | 0.926838 |
| 決定係数 | 0.859029 |

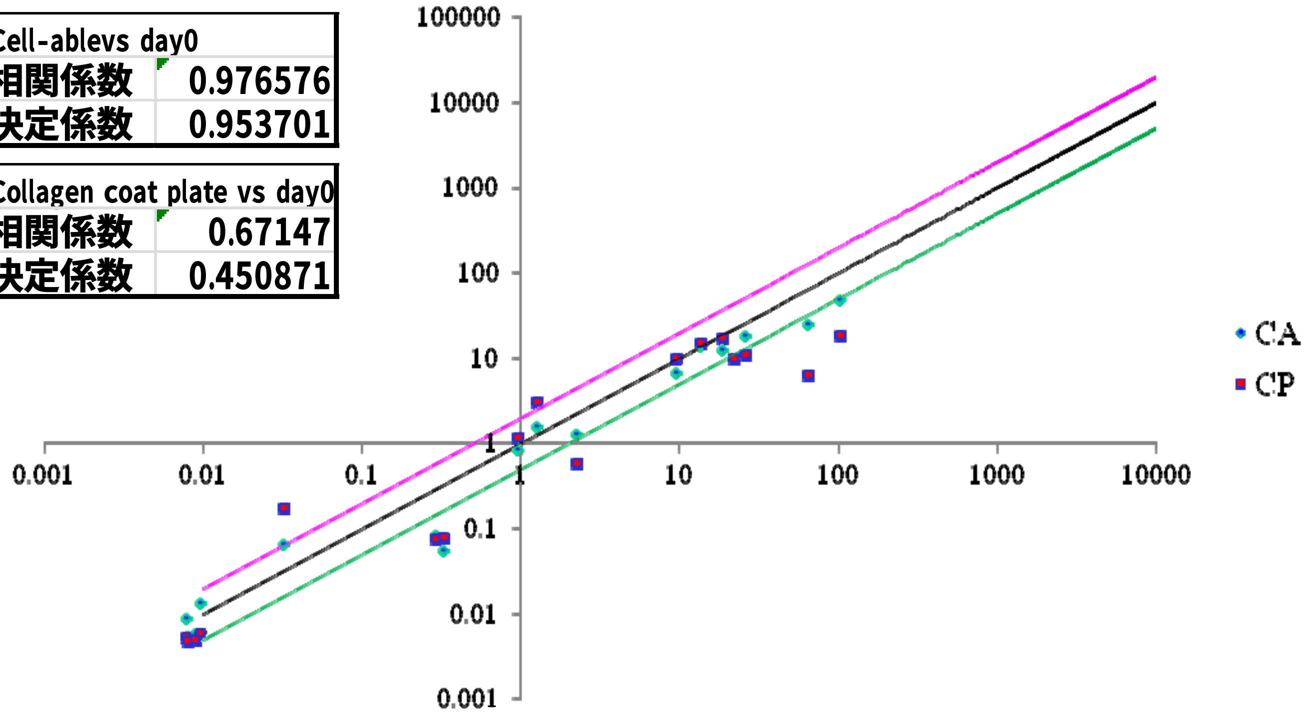


# Cell-able培養と通常プレート培養における遺伝子発現状態の相違
## Difference in Gene Expression between Cell-able and Conventional Plate

### Transporters

| Cell-ablevs day0 | |
|---|---|
| 相関係数 | 0.976576 |
| 決定係数 | 0.953701 |

| Collagen coat plate vs day0 | |
|---|---|
| 相関係数 | 0.67147 |
| 決定係数 | 0.450871 |



# Cell-able培養と通常プレート培養における遺伝子発現状態の相違
## Difference in Gene Expression between Cell-able and Conventional Plate

| 遺伝子発現のDay14対Day0比の相互関係　決定係数の比較<br>R^2 of Day14/Day0 Ratio | | |
|---|---|---|
| | Cell-able | Conventional plate |
| CYPs | 0.764 | 0.712 |
| Nuclear receptors | 0.855 | 0.755 |
| Conjugation enzymes | 0.911 | 0.859 |
| Transporters | 0.953 | 0.451 |
| Liver related genes | 0.806 | 0.733 |

Cell-able培養の方が初期値（Day0）値を維持している．
Cell-able culture maintains in vivo gene expression better than conventional plate culture.

# Cell-able Oncology™

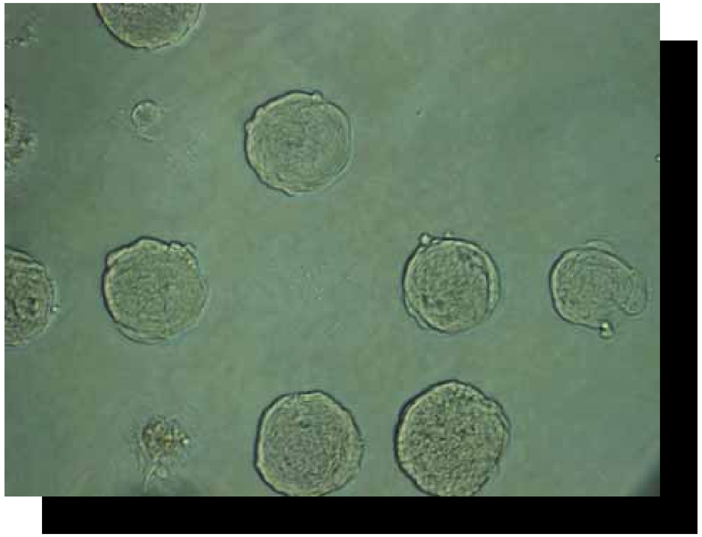
# 初代がん細胞培養

American Association of Cancer Research 2012　発表より

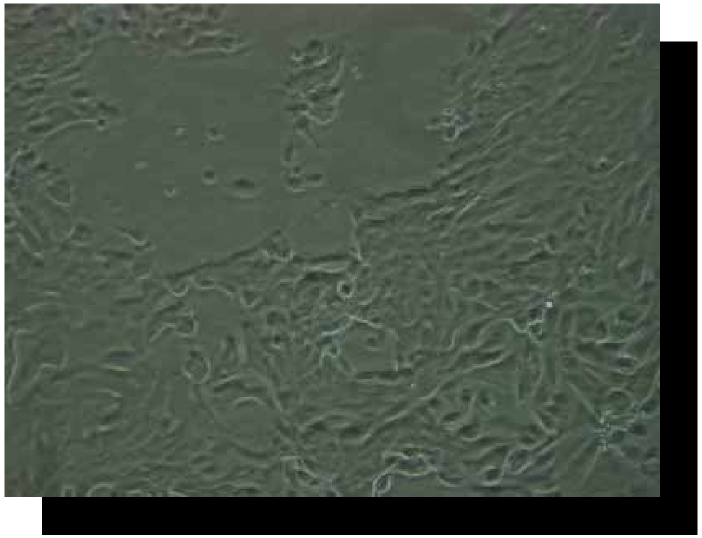
# Cell-able Oncology™
## 患者由来初代卵巣がん細胞の形態　2D培養との比較

摘出卵巣がん
ovarian tumor: endometrioid

Cell-able



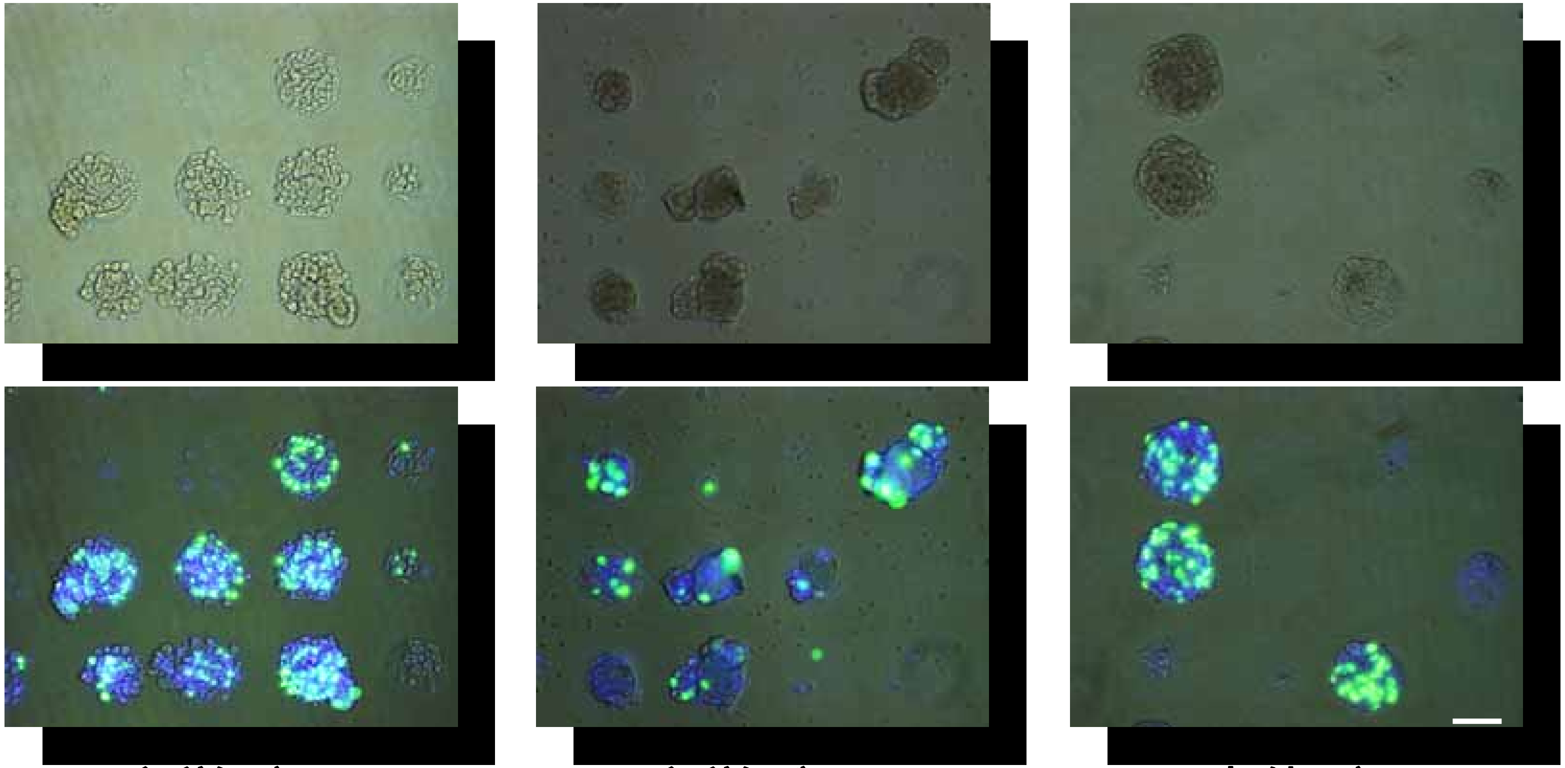
2D plate



AACR 2012　共同発表抜粋

# Cell-able Oncology™

## 卵巣がん・子宮体がんのスフェロイド培養とEdU取込



卵巣がん
(serous, low grade)

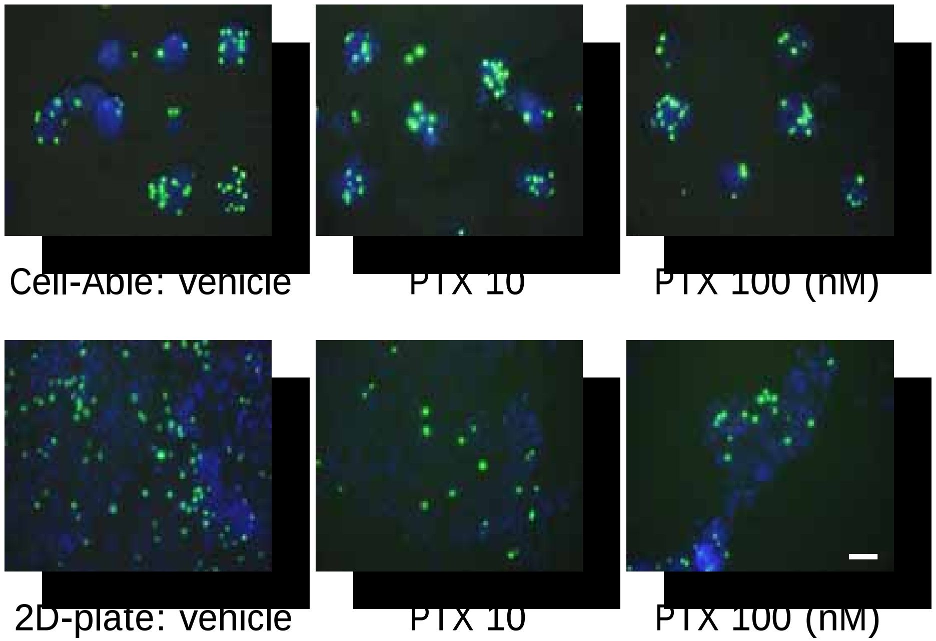
卵巣がん
(clear cell)

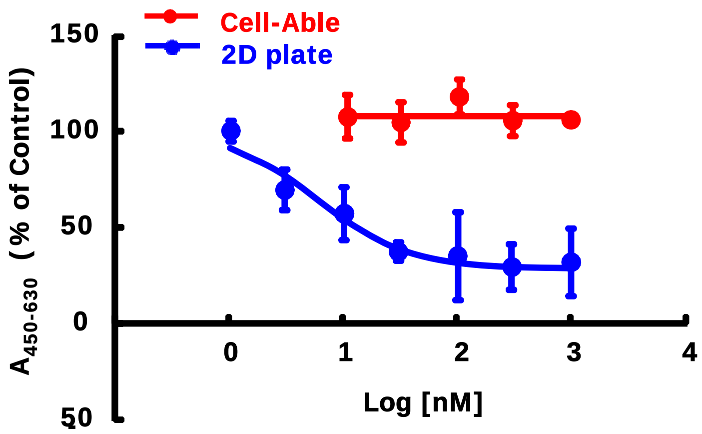
子宮体がん
(grade 2)

二種類のがん細胞がCell-able Oncology™上で増殖
EdU反応時間；3hr

AACR 2012　共同発表抜粋

# Cell-able Oncology™
# 初代子宮体がんの増殖に関する化学療法
# 抗がん薬挙動の比較




| | PTX Cell-Able | PTX 2D plate |
|---|---|---|
| IC50 | >1000 | 16.5 |

Cell-able Oncology™培養の方が臨床上の反応性をよく再現していた．
EdU反応時間；3hr

AACR 2012　共同発表抜粋

# ふたつの技術要素がもたらす培養上の効果
## Cell-able System and its Impact in Cell Culture

✧ 規格化されたスフェロイド　→　初代肝細胞長期機能維持培養、少ないWell間差、in vivo発現の維持、in vivoを再現するがん細胞初代培養

Uniform-size spheroids　→　Long-term functioning hepatocyte primary culture, low inter-well difference, maintenance of in vivo gene expression, tumor cell culture mimicking in vivo

✧ フィーダー細胞の利用　→　初代肝細胞長期機能維持培養、人工肝小葉の構築、低接着性凍結ヒト肝細胞の利用

Use of feeder cells　→　Long-term functioning hepatocyte primary culture, reconstruction of artificial hepatic lobules, use of cryopreserved hepatocytes with low-attaching capability