

2020年 日化協LRI研究報告会

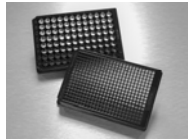
2020年8月21日

# ヒト幹細胞試験による迅速・正確・低コストの 化学物質ハザードAI評価法の開発

京都大学iPS細胞研究所

山根 順子・藤渕 航

# 30物質でのRT-qPCRを用いた予測システム



プロトタイプ  
作成

各種化合物によるIC10決定

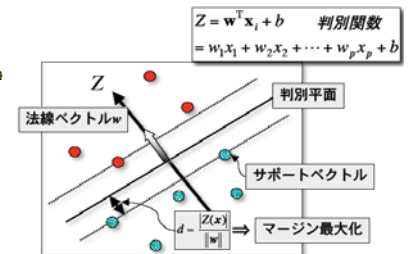
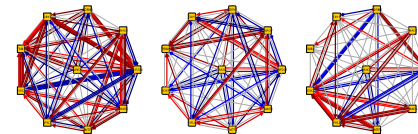
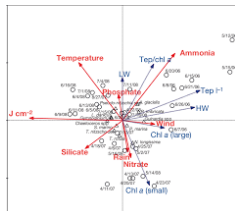
化合物曝露

ライブラリー調整・  
シーケンス

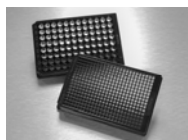
対応分析による  
特徴遺伝子抽出

RT-qPCR実施

予測システムを  
用いた毒性予測



# 30物質でのRT-qPCRを用いた予測システム



プロトタイプ  
作成

各種化合物によるIC10決定

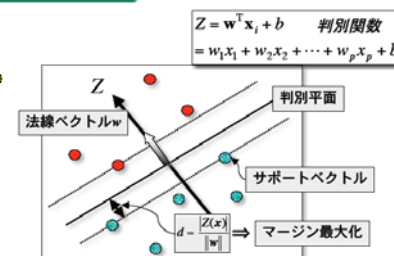
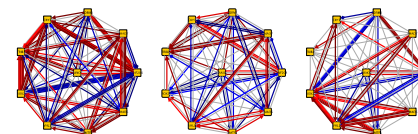
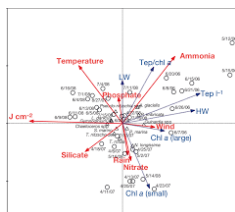
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## 肝毒性物質 10 chemicalsのリスト・曝露濃度

肝毒性物質は日化協と合同で選定

( $\mu$ M)

	DILI rank	Dose 1	Dose 2	Dose 3	Dose 4	Dose 5	Dose 6	Dose 7	Dose 8	Dose 9	Dose 10
Amiodarone	DILI: 8	0.01	0.02	0.05	0.1	0.4	1.2	3.7	11.1	33.3	100
Cyclosporin A	DILI: 7	0.003	0.008	0.02	0.07	0.2	0.6	1.8	5.6	16.7	50
Atorvastatin	DILI: 5	0.001	0.002	0.005	0.01	0.04	0.12	0.37	1.1	3.3	10
Clotrimazole	DILI: 3	0.003	0.008	0.02	0.07	0.2	0.6	1.8	5.6	16.7	50
Ibuprofen	DILI: 3	0.04	1.1	3.2	9.6	28.8	86.4	259	777	2333	7000
Chlorpromazine	DILI: 2	0.02	0.05	0.1	0.4	1.2	3.7	11.1	33.3	100	300
Aspirin	DILI: 0	1	2	5	14	41	123	370	1111	3333	10000
Chlorpheniramine	DILI: 0	0.1	0.2	0.5	1.4	4.1	12.3	37	111	333	1000
Carbon Tetrachloride	Group2B	1	3	8	23	68	205	615	1844	5533	16600
2,4-DNT	Group2B	0.01	0.03	0.09	0.2	0.8	2.4	7.4	22.2	66.6	200

ATP assay: 公比3で10濃度検討 N=4で実施

MTT assay: 公比3で8濃度検討 N=4で実施

最高曝露濃度 (Dose 10) は最大可溶量を適用  
溶媒は全てDMSO

Controlとして0.01% DMSO添加

## MTT/ ATP assayの実施条件

	MTT assay	ATP assay
細胞種	KhES-3細胞（男性株）	KhES-3細胞（男性株）
培地/コーティング剤	StemFit medium/ i-Matrix-511	Essential 8 medium/ VTN-N
化合物曝露時間	48 hrs	48 hrs
Assay reagent kit	Cell Counting Kit-8 (Dojindo)	CellTiter-Glo® Luminescent Cell Viability Assay (Promega)
測定機器	PE Envision 2104 Multilabel Reader1 (PerkinElmer) 吸光度(450nm)測定	PE Envision 2104 Multilabel Reader1 (PerkinElmer) 発光測定
IC10決定 ソフトウェア	R package “drc” from CRAN model. LL4 (4 parameters log-logistic) $f(x) = c + \frac{d - c}{1 + \exp\{b(\log(x) - \log(e))\}}$  R version 3.6.3 (2020-02-29)	R package “drc” from CRAN model. LL4 (4 parameters log-logistic) $f(x) = c + \frac{d - c}{1 + \exp\{b(\log(x) - \log(e))\}}$  R version 3.6.3 (2020-02-29)
IC10計算式	IC10<- 1/b * log ((d-c)/(0.9-c)-1)+log (e) IC10<- exp (IC10)  b<- coefficients (model. LL4) c<- coefficients (model. LL4) d<- coefficients (model. LL4) e<- coefficients (model. LL4)	IC10<- 1/b * log ((d-c)/(0.9-c)-1)+log (e) IC10<- exp (IC10)  b<- coefficients (model. LL4) c<- coefficients (model. LL4) d<- coefficients (model. LL4) e<- coefficients (model. LL4)

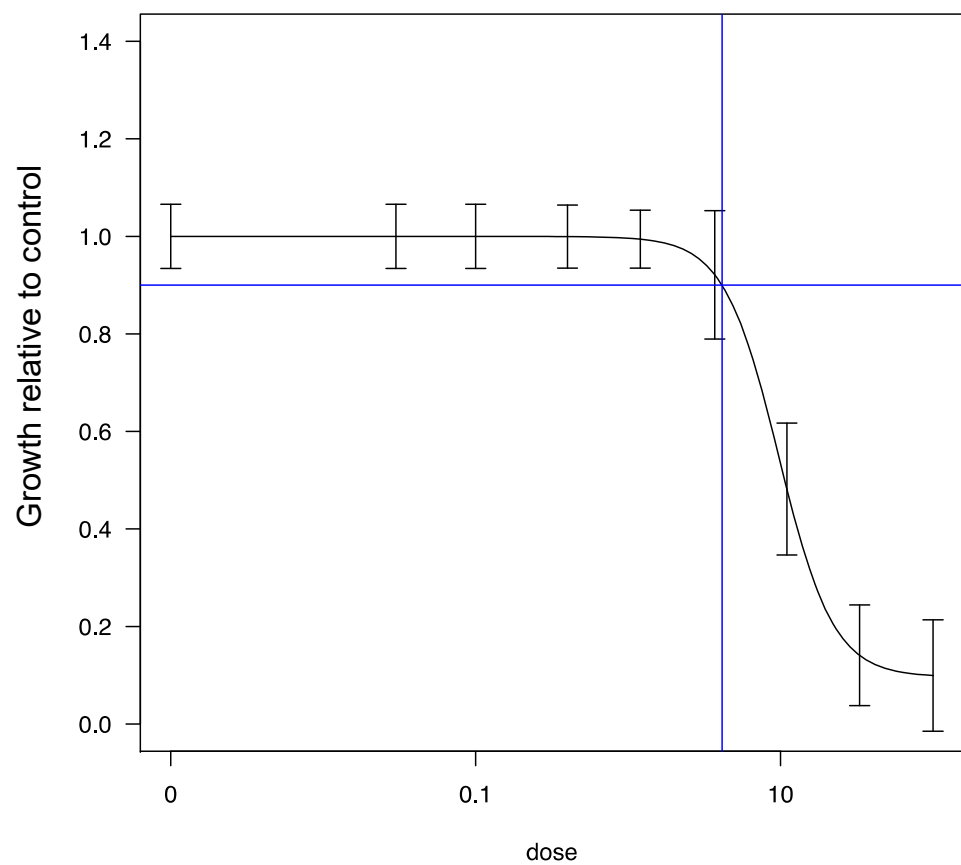
# MTT/ATP assayによるIC10決定

Amiodarone (DILI: 8)

MTT assay

4.1  $\mu$ M

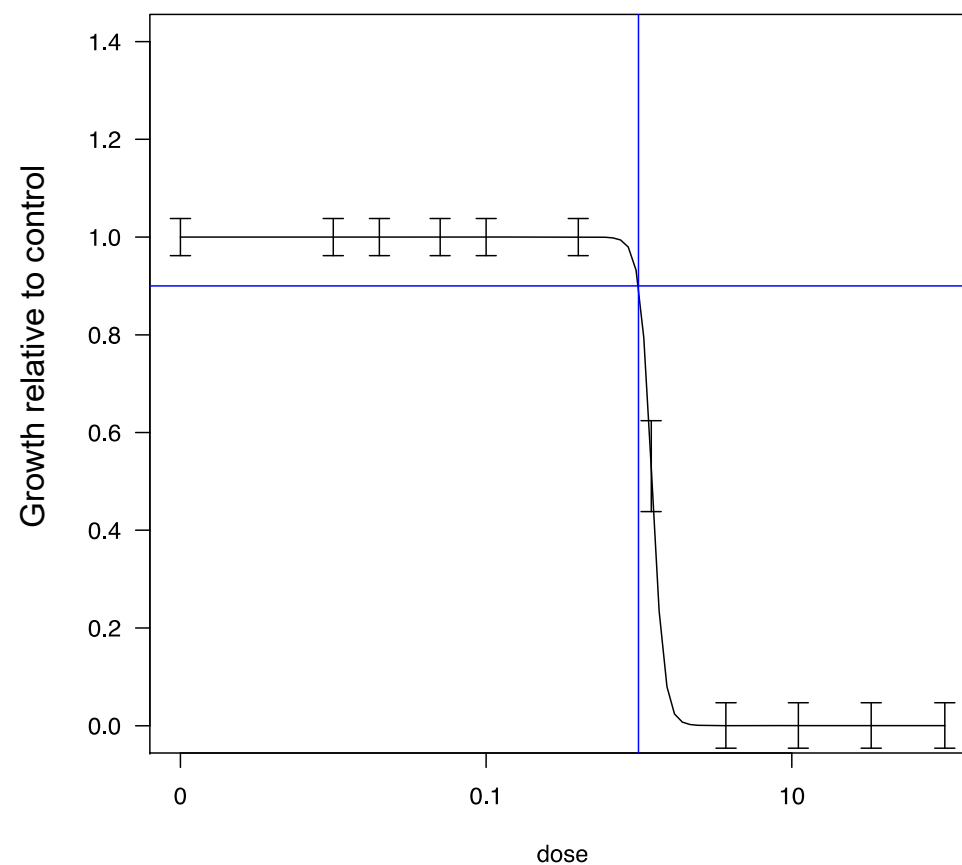
IC10 is : 4.12389601999574



ATP assay

0.99  $\mu$ M

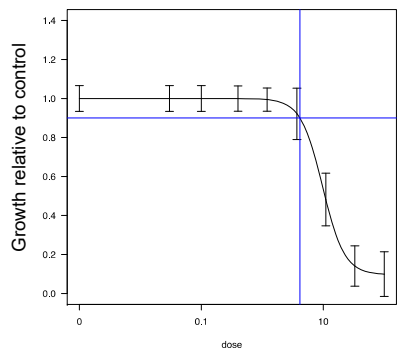
IC10 is : 0.992174981980825



## MTT assay IC10 results

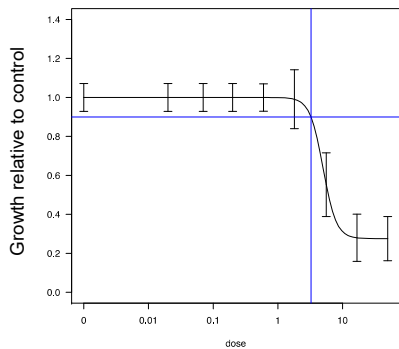
Amiodarone  
IC10: 4.1  $\mu\text{M}$

IC10 is : 4.12389601999574



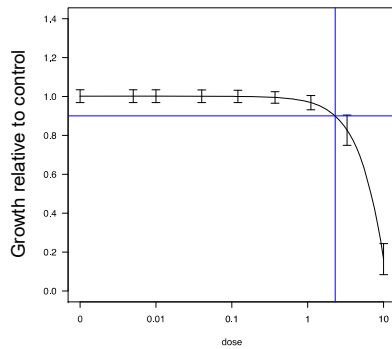
Cyclosporin A  
IC10: 3.2  $\mu\text{M}$

IC10 is : 3.2502764435014



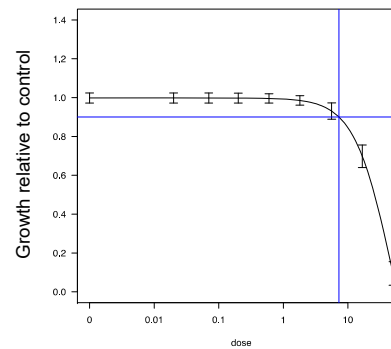
Atorvastatin  
IC10: 2.3  $\mu\text{M}$

IC10 is : 2.29913860893602



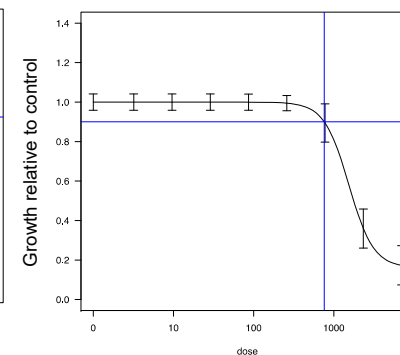
Clotrimazole  
IC10: 7.2  $\mu\text{M}$

IC10 is : 7.26303466992707



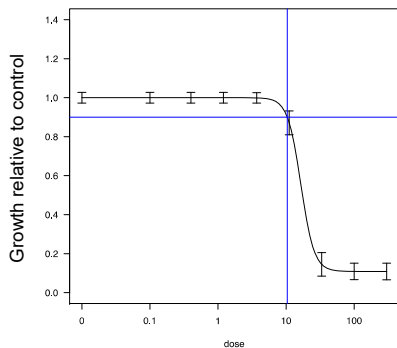
Ibuprofen  
IC10: 759  $\mu\text{M}$

IC10 is : 759.730217800397



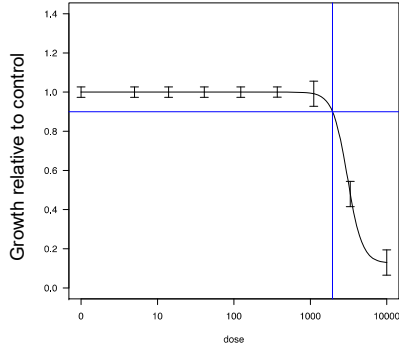
Chlorpromazine  
IC10: 10.3  $\mu\text{M}$

IC10 is : 10.3998092549904



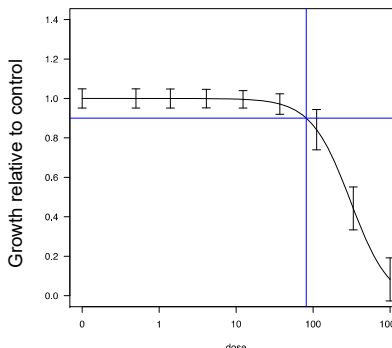
Aspirin  
IC10: 1995  $\mu\text{M}$

IC10 is : 1951.62361468229



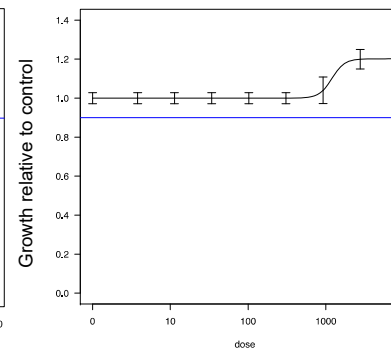
Chlorpheniramine  
IC10: 81  $\mu\text{M}$

IC10 is : 81.5321015309294



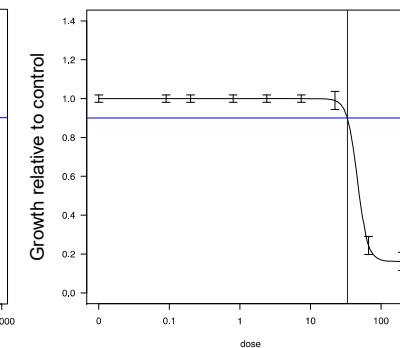
Carbon Tetrachloride  
IC10: NA

IC10 is : NaN



2,4-DNT  
IC10: 33  $\mu\text{M}$

IC10 is : 33.2957867508473

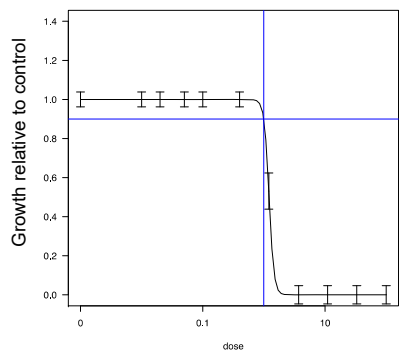


## ATP assay IC10 results

Amiodarone

IC10: 0.99  $\mu\text{M}$

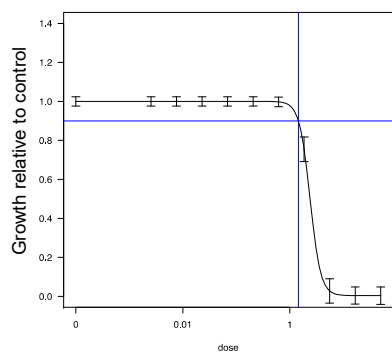
IC10 is : 0.992174981980825



Cyclosporin A

IC10: 1.45  $\mu\text{M}$

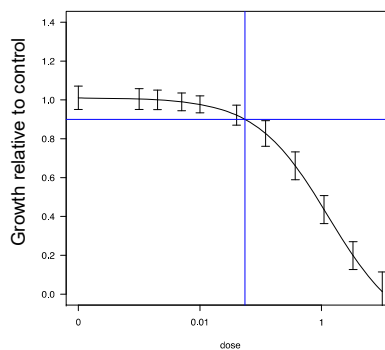
IC10 is : 1.44957213463966



Atorvastatin

IC10: 0.05  $\mu\text{M}$

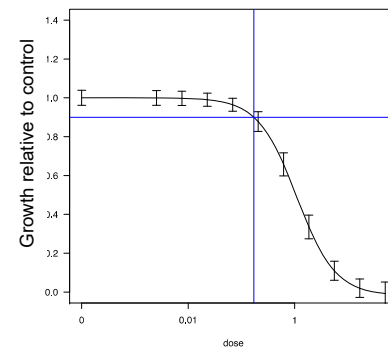
IC10 is : 0.054933013793395



Clotrimazole

IC10: 0.17  $\mu\text{M}$

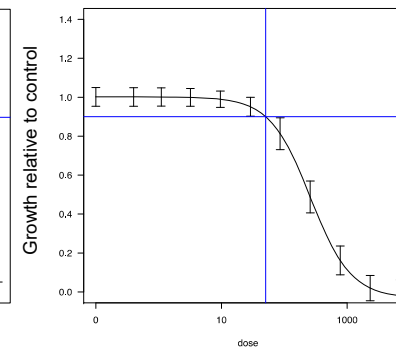
IC10 is : 0.170741800107148



Ibuprofen

IC10: 50  $\mu\text{M}$

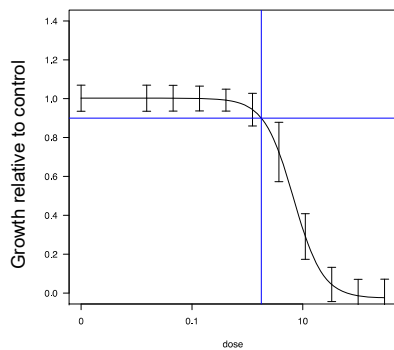
IC10 is : 50.4484790167917



Chlorpromazine

IC10: 1.78  $\mu\text{M}$

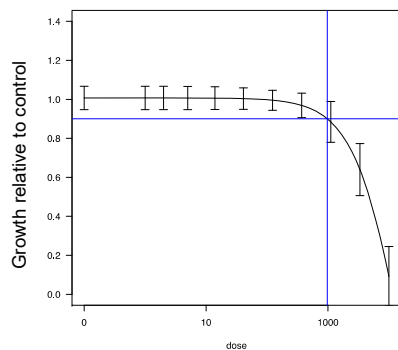
IC10 is : 1.78287125068417



Aspirin

IC10: 974  $\mu\text{M}$

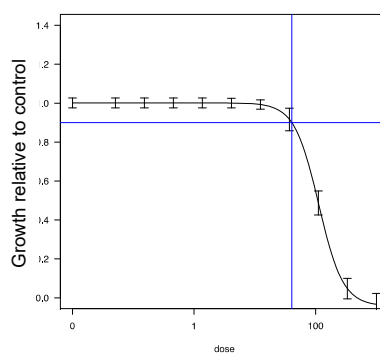
IC10 is : 974.994772805925



Chlorpheniramine

IC10: 40.5  $\mu\text{M}$

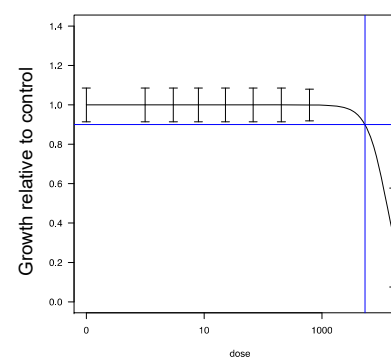
IC10 is : 40.5175905897681



Carbon Tetrachloride

IC10: 5385  $\mu\text{M}$

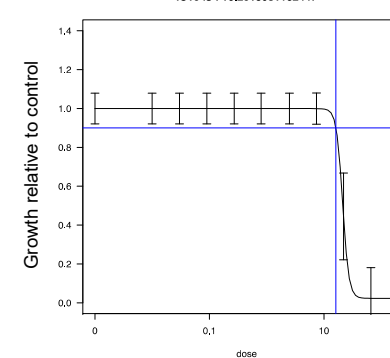
IC10 is : 5385.84032945367



2,4-DNT

IC10: 16  $\mu\text{M}$

IC10 is : 16.2015931162447



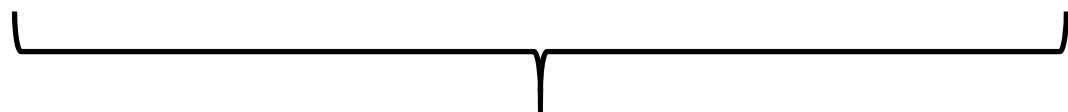


# 予測プロトタイプシステムの候補物質

肝毒性	
Amiodarone	DILI:8
Cyclosporine A	DILI:7
Atorvastatin	DILI:5
Clotrimazole	DILI:3
Ibuprofen	DILI:3
Chlorpromazine	DILI:2
Aspirin	DILI:0
Chlorpheniramine	DILI:0
Carbon Tetrachloride	Group2B
2,4-DNT	Group2B

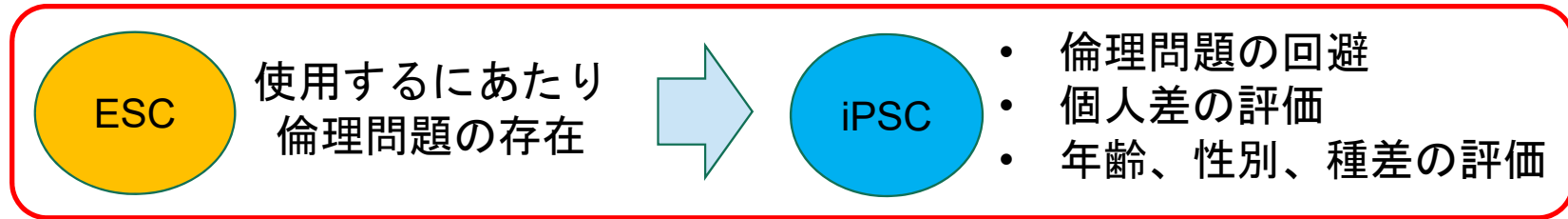
腎毒性
Axitinib
Pamidronate
Gentamicin
Cisplatin
Cyclosporine A

神経毒性
Valproic acid
Phenytoin
Acrylamide
Hexanedione
Cyclopamine



肝毒性物質の対照被験物質として使用

# ヒトESCからiPSCへの移行の取り組み



	ESC IC <sub>10</sub> (nM)	iPC IC <sub>10</sub> (nM)	iPSC/ESC IC <sub>10</sub>
RT1	50	10	0.2
RT2	30	100	26
RT3	5x10 <sup>3</sup>	1x10 <sup>3</sup>	0.2
RT4	15	200	13.3
RT5	1.5x10 <sup>6</sup>	1x10 <sup>6</sup>	0.67
NT1	2x10 <sup>3</sup>	3x10 <sup>4</sup>	15
NT2	2x10 <sup>3</sup>	5x10 <sup>3</sup>	2.5
NT3	2x10 <sup>3</sup>	3x10 <sup>3</sup>	1.5
NT4	1x10 <sup>4</sup>	1.5x10 <sup>5</sup>	15
NT5	2x10 <sup>3</sup>	2x10 <sup>5</sup>	100

RT: 腎毒性物質  
NT: 神経毒性物質

ESC: KhES-3

iPSC: アジア人女性、単球由来

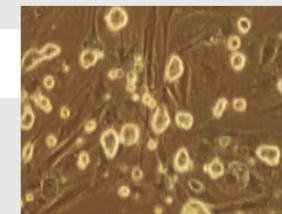
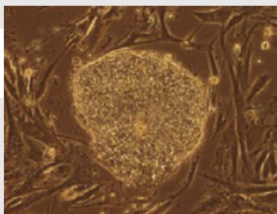
ESCとiPSCでは化合物に対する細胞反応の差が存在



iPSCをESCのような高感受性に近づけられないか？

# ナীব化の手法は完全には確立されていない：改良YAP法を暫定使用

	Primed	Naïve
多能性状態	着床後	着床前
形態	Flat型	Dome型
維持に必要な supplement	bFGF, TGF-beta	LIF, 4or 5 inhibitors/ Activin
維持コンディション	Non-hypoxia	Hypoxia



Takashima et al. を一部改変

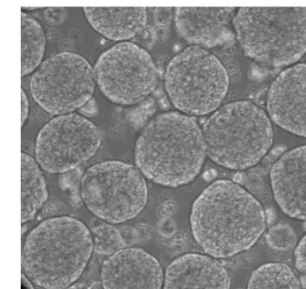
It is known that iPSCs can be cultured in spheroids without differentiation

Takara Bio Europe AB  
Cellartis® DEF-CS™  
500 Xeno-Free  
3D Spheroid Culture  
Medium User Manual

My medium and method is based on 3 methods in

Key components:

- ROCK kinase and E-cadherin activation (ROCK is necessary for E-cad)
- epithelial-to-mesenchymal transition is impeded by E-cadherin, leading to failure to generate germ layers inside spheroids – i.e. differentiation is inhibited
- Soft surface facilitates spheroid formation



## Cell Reports

### YAP:

ERK1/2i (PD0325901 0.5 μM)  
GSK3βi (CHIR99021 3 μM)  
LIF (10 ng/ml)

cAMP, AMPK activator (Forskolin 10 μM)

YAP activator (LPA 10 μM)

In N2B27 medium

## Cell Stem Cell 2014 Theunissen et al.

### SiLA:

ERK1/2i (PD0325901 1 μM)  
GSK3βi (IM-12 1 μM)  
LIF (10 ng/ml)

B-Raf kinase i (SB590885 0.5 μM)

Src i (WH4023 1 μM)

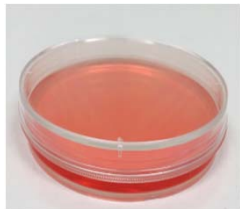
ROCKi (Y-27632 10 μM)

Activin (20 ng/ml)

In N2B27 medium

# ナীব化細胞構築マーカーとしてEOS (Early transposon, Oct-4, Sox2) システムを使用

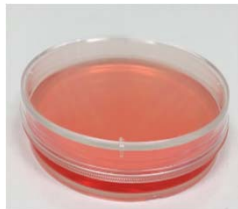
Seed cells in at least 3cm dish



Day 1



Transfection\*

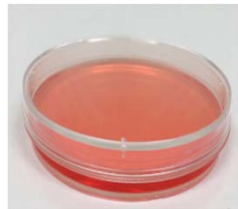


Day 2

\*Extremely important to have high efficiency



Puromycin selection\*\*

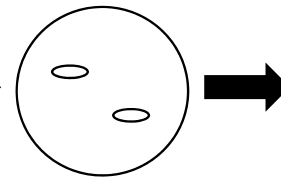


Days 3-6

\*\* Will explain separately



Normally only 2-3 cells remain

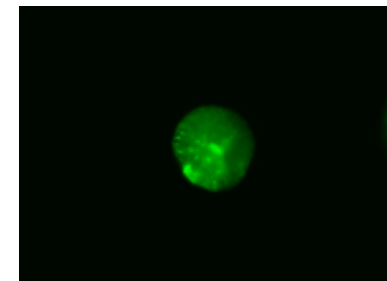


Grow for 7 days

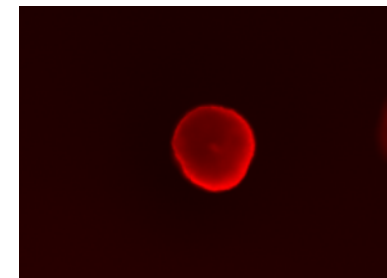
Colony



ナীবマーカーでの発現確認

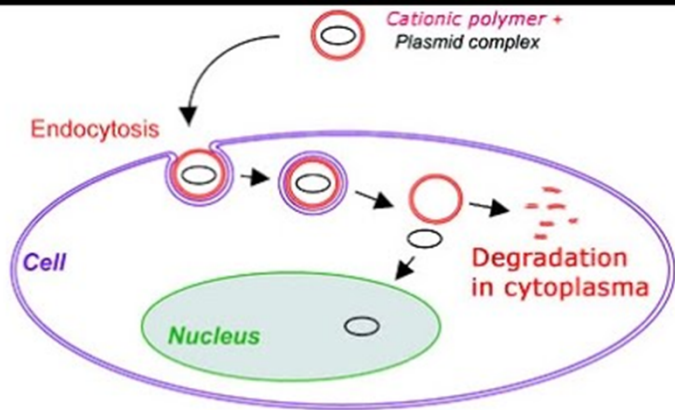


EOS GFP

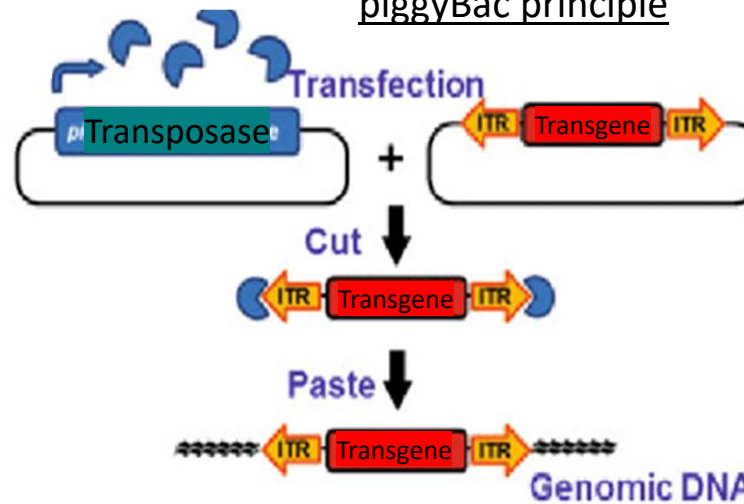


SUSD2

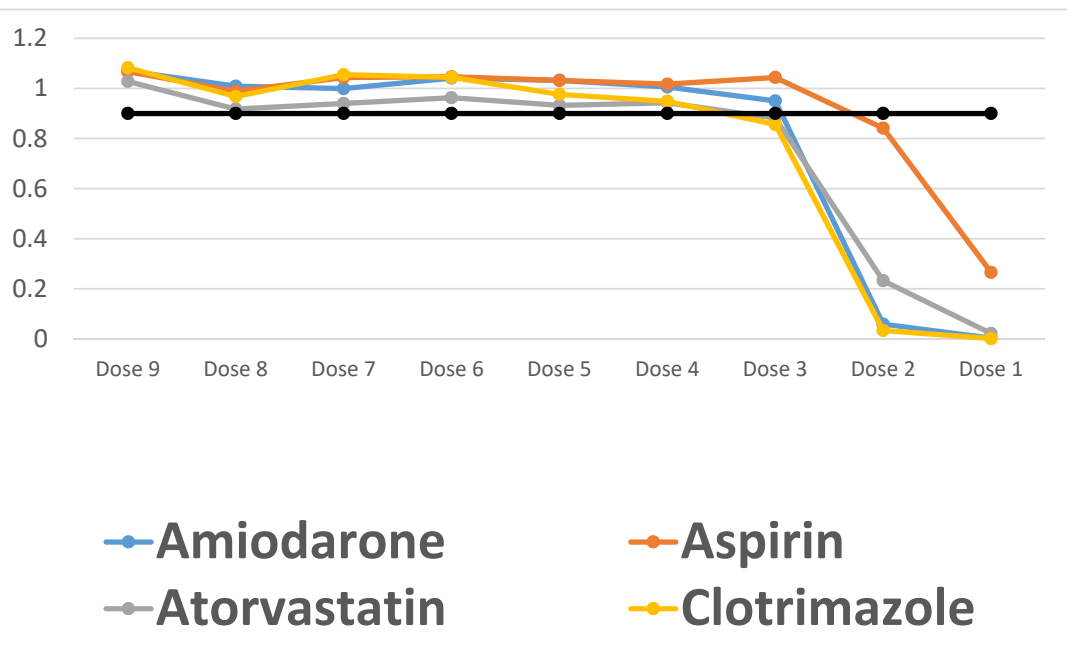
## Transfection principle



## piggyBac principle

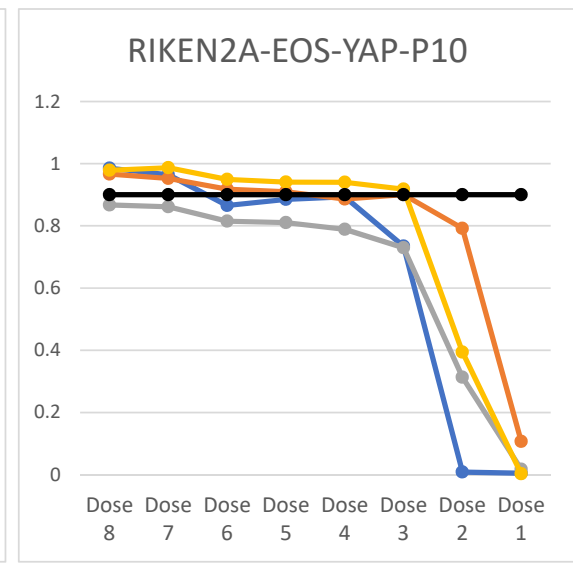
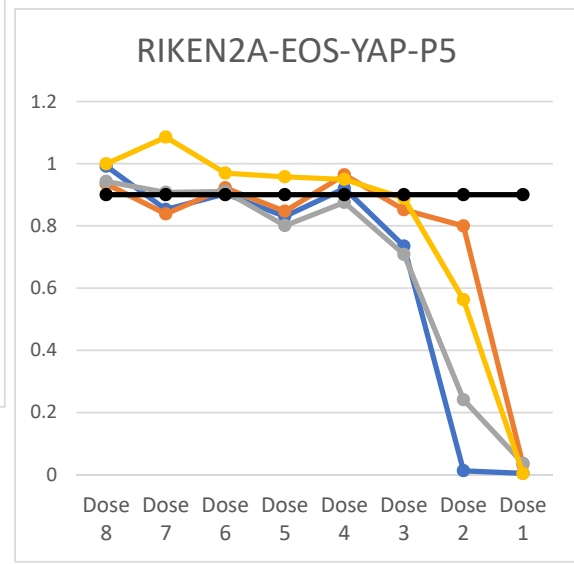
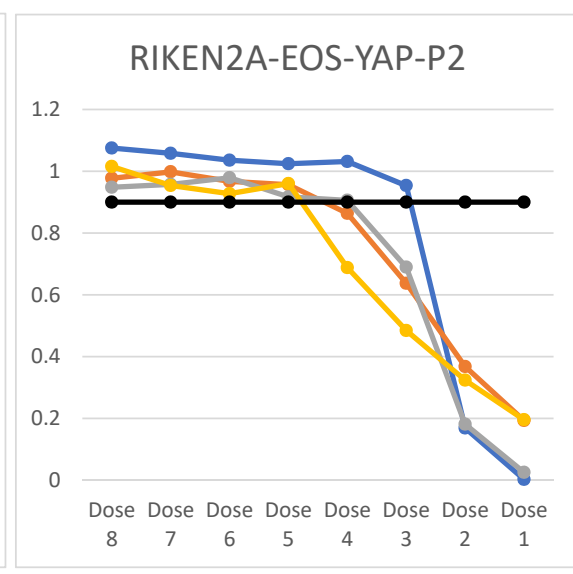
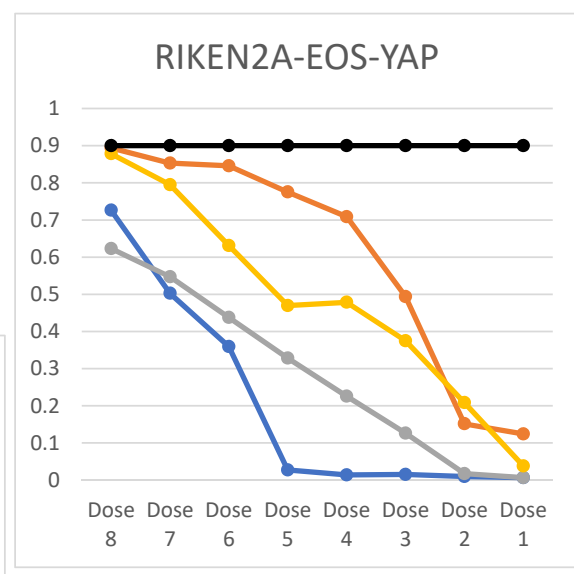


# 細胞のナイーブ化による 感受性の増加



Amiodarone      Aspirin  
Atorvastatin      Clotrimazole

iPS細胞 (RIKEN-2A: 男性株) での  
肝毒性ATPアッセイ

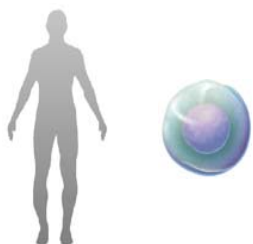


Amiodarone      Aspirin      Atorvastatin      Clotrimazole      IC10

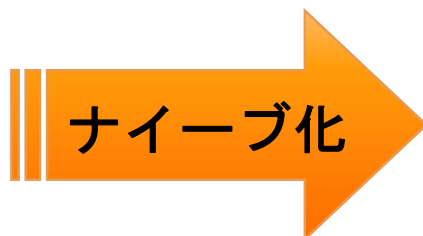
# 将来の展望

肝毒性	
Amiodarone	DILI:8
Cyclosporine A	DILI:7
Atorvastatin	DILI:5
Clotrimazole	DILI:3
Ibuprofen	DILI:3
Chlorpromazine	DILI:2
Aspirin	DILI:0
Chlorpheniramine	DILI:0
Carbon Tetrachloride	Group2B
2,4-DNT	Group2B

マイiPS細胞



リセットiPS細胞

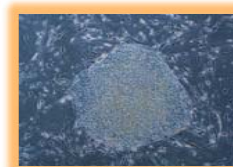
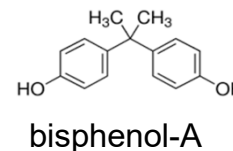


腎毒性	
Axitinib	
Pamidronate	
Gentamicin	
Cisplatin	
Cyclosporine A	

神経毒性	
Valproic acid	
Phenytoin	
Acrylamide	
Hexanedione	
Cyclopamine	



毒性検査を可能に



ヒトES細胞での曝露  
遺伝子発現データベース

プロトタイプ  
作成



予測システム  
作成

- ・肝毒性
  - ・腎毒性
  - ・神経毒性
  - ・心毒性
  - ・非遺伝的発ガン性
  - ・抗ウイルス薬
- 等

毒性試験に使用するための  
標準iPS細胞を作成



ESC (DB) と  
iPSC (試験対象) の  
ブリッジング



hESTから  
hiPSTへの発展