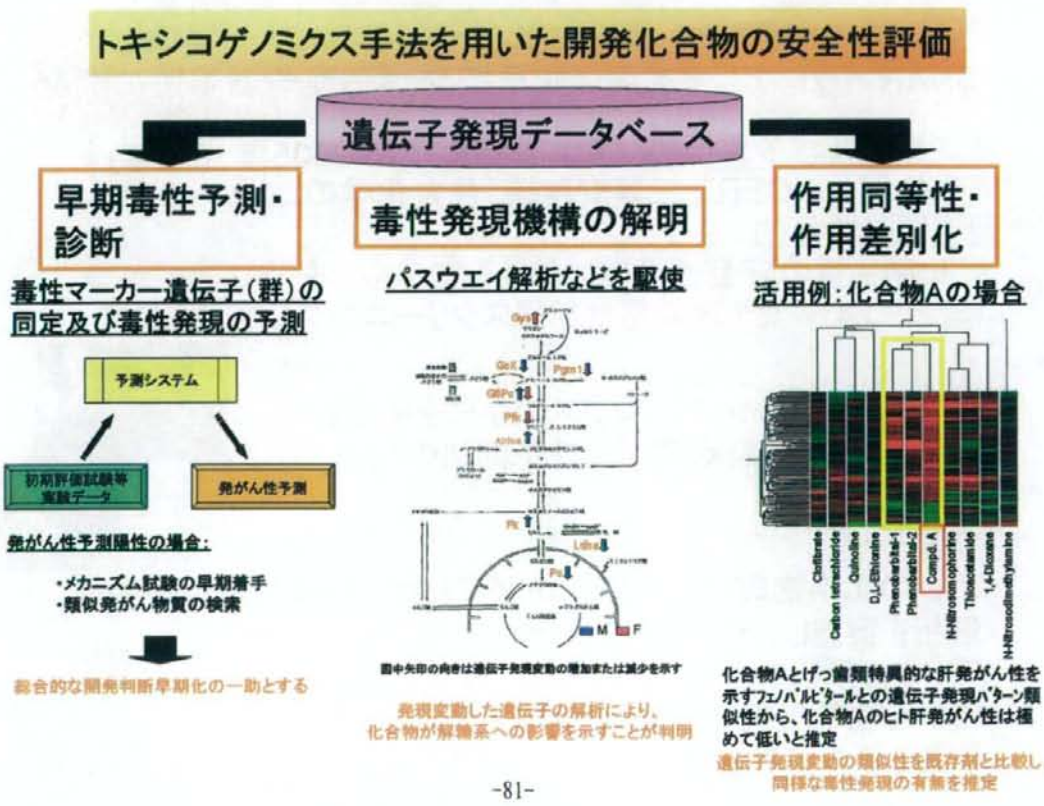
トキシコゲノミクスデータ解析の課題

- In-houseのデータ解析におけるトキシコゲノミクスデータベースおよびバイオマーカーの活用は、薬物安全性評価に際して極めて有効な手法となっている
- しかしTG-GATEsのような外部データベースとの比較解析に際しては、データ互換性が依然として大きな問題となる
- MAQC論文では、個別遺伝子signal, fold change、p値のパラメータのうち、fold changeを使った解析が最もデータベース間の互換性が高いことを報告しており、このような知見を考慮してデータベース間比較解析の互換性を高める努力が必要

12

化学メーカーにおける
遺伝子発現解析技術の活用
と
標準化動向

住友化学株式会社
生物環境科学研究所
住田　佳代

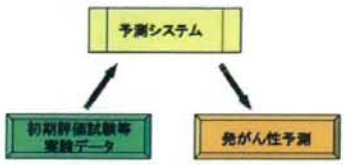


トキシコゲノミクス手法を用いた開発化合物の安全性評価

遺伝子発現データベース

早期毒性予測・診断

毒性マーカー遺伝子(群)の同定及び毒性発現の予測



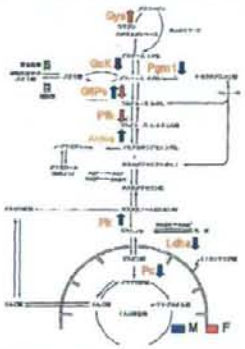
発がん性予測陽性の場合:

- メカニズム試験の早期着手
- 類似発がん物質の検索

総合的な開発判断早期化の一助とする

毒性発現機構の解明

パスウエイ解析などを駆使

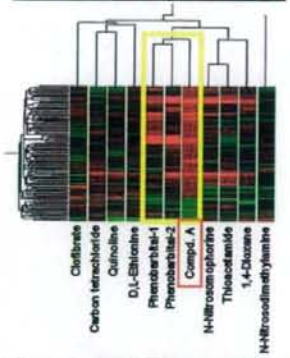


図中矢印の向きは遺伝子発現変動の増加または減少を示す

発現変動した遺伝子の解析により、化合物が解糖系への影響を示すことが判明

作用同等性・作用差別化

活用例:化合物Aの場合



化合物Aとげっ歯類特異的な肝発がん性を示すフェノバルビタールとの遺伝子発現パターン類似性から、化合物Aのヒト肝発がん性は極めて低いと推定

遺伝子発現変動の類似性を既存剤と比較し、同様な毒性発現の有無を推定

NEDO PJ『高精度・簡易有害性(ハザード)評価システム開発』

DNAマイクロアレイを用いた早期発がん性予測手法の開発

研究期間及び予算　約45億円／2001年～2005年　(5カ年)

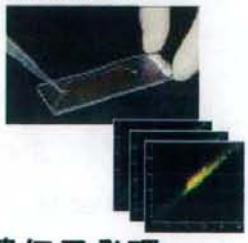
研究体制　CERI、三菱安科研、住友化学のコンソーシアム

研究開発の目的

化学物質の安全性評価における発がん性を"短期間"かつ"低コスト"で予測可能な新規スクリーニング法を開発する

＝

トキシコゲノミクスの手法を応用した新規発がん性予測法の開発



アプローチ

- 85種の化学物質についてラット肝における網羅的遺伝子発現解析を実施し、データを蓄積(⇒データベース構築)
- データの解析(⇒バイオインフォマティクス)
- 発がん性予測式の作成(⇒発がん性予測モデルの構築)
- 予測モデルの性能評価(⇒発がん性予測モデルの検証)

NEDOPJ研究成果

トレーニングデータ(72物質)

予測率:94.4% (擬陽性率:11%、擬陰性率:2%)

外部バリデーション試験

- ✓ 肝発がん及び非発がん物質は高い精度で予測可能(6/6物質)
- ✓ 肝以外の発がん性物質の予測精度は落ちる傾向(0/1物質)
- ✓ 発がん性が曖昧な物質はスコア値が±として算出される傾向

病理診断マーカー探索へ応用

■背景

・グルタチオン*S*-トランスフェラーゼ胎盤型酵素(GST-P):
代表的かつ有用なラット肝前がん病変マーカー

・一部の肝前がん病変(ペルオキシソーム誘導剤で誘発)は抗ラットGST-P抗体を用いて検出できない

■目的

GST-Pでは検出できないラット肝前がん病変に対する新規分子マーカー探索

■方法

病変の誘導 → 病変および隣接正常組織の特異的採取 → totalRNAの単離と遺伝子発現解析 → 遺伝子発現データの詳細な解析と検証実験

トキシコゲノミクスの手法をマーカー候補遺伝子群の抽出に利用

前癌病変においてmRNA量の増加もしくは減少が認められた遺伝子(GST-P陰性)

GenBank 登録番号	遺伝子名	GST-P 陰性巣				GST-P 陽性巣			
		DEN→WY[a]		DEN→CF[b]		DEN のみ		DEN→CF	
		強度[c]	変動率[d]	強度	変動率	強度	変動率	強度	変動率
Up-regulated genes									
M23566	α_2-マクログロブリン	385.7	2.6	3507.5	39.3	ND[e]	—	ND	—
X61654	ad1-antigen	568.0	2.5	1019.5	3.5	ND	—	ND	—
X60767	cdc2 promoter	30.1	2.5	19.4	2.3	18.7	2.0	18.3	2.1
AA860039	EST	22.6	2.1	7.8	7.5	28.1	4.3	16.7	3.5
AI070295	EST	88.2	8.6	58.8	2.3	ND	—	58.0	1.7
Down-regulated genes									
J00738	α-2u グロブリン	3.1	-2.6	175.0	-3.7	1110.8	-1.5	54.9	-3.5
M18363	P-450(M-1)	3.9	-6.5	512.0	-2.6	403.8	-2.3	233.7	-3.0

[a] WY: Wy-14,643. [b] CF: クロフィブレート. [c] 発現強度
[d] 変動率:正常組織に対してn倍の変動 [e] ND: 検出限界以下

新規病理診断マーカー: α_2-マクログロブリン

■結果 ・前がん病変において発現増加が認められた遺伝子

GenBank Acc. No.	遺伝子名	DEN→ Wy-14,643		DEN→Clofibrate	
		シグナル値	発現変動倍率(倍)	シグナル値	発現変動倍率(倍)
M23566	α_2-マクログロブリン	385.7	2.6	3507.5	39.3
X61654	ad1-antigen	568.0	2.5	1019.5	3.5
X60767	cdc2 promoter	30.1	2.5	19.4	2.3
AA860039	EST	22.6	2.1	7.8	7.5
AI070295	EST	88.2	8.6	58.8	2.3

GST-P陰性ラット肝前癌病変に対する新規マーカーとしてα_2-マクログロブリン同定

➡ トキシコゲノミクスの手法を活用して、抗ラットGST-P抗体で検出できないラット肝前がん病変の検出マーカーを見出した。

トキシコゲノミクス手法を用いた開発化合物の安全性評価

遺伝子発現データベース

早期毒性予測・診断

毒性マーカー遺伝子(群)の同定及び毒性発現の予測

予測システム

初期評価試験等実験データ

発がん性予測

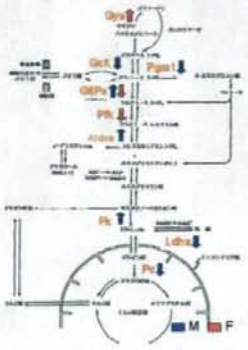
発がん性予測陽性の場合:

・メカニズム試験の早期着手
・類似発がん物質の検索

総合的な開発判断早期化の一助とする

毒性発現機構の解明

パスウエイ解析などを駆使

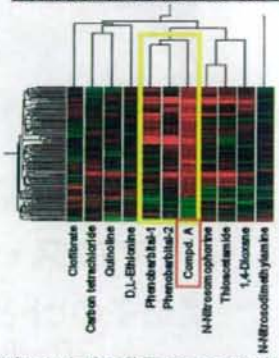


図中矢印の向きは遺伝子発現変動の増加または減少を示す

発現変動した遺伝子の解析により、化合物が解糖系への影響を示すことが判明

作用同等性・作用差別化

活用例:化合物Aの場合



化合物Aとげっ歯類特異的な肝発がん性を示すﾌｪﾉﾊﾞﾙﾋﾞﾀｰﾙとの遺伝子発現ﾊﾟﾀｰﾝ類似性から、化合物Aのヒト肝発がん性は極めて低いと推定

遺伝子発現変動の類似性を既存剤と比較し、同様な毒性発現の有無を推定

生科研でのトキシコゲノミクス研究活用

開発化合物の問題毒性の機構解明

・発現変動遺伝子の意義を解析し、毒性機構を推定

➡ 問題毒性回避へのヒントをfeed back

化合物の作用の同等性(作用類似化合物)

・種々の解析手法を用いて、開発化合物の遺伝子発現プロファイルが既存のどの化合物と類似しているかを検討

➡ 問題毒性に対するプロアクティブな対応

ヒトとの相関性：霊長類の活用

マウス・ラット

遺伝子のヒトとの類似性：
70～80%

霊長類

遺伝子のヒトとの類似性：
90～99%

毒性学的により精緻な評価が可能

マーモセットDNAチップ作製

サイズ：　ラットと同等（～300 g）
　　サルの1/20
繁殖性：　1.5年で性成熟（繁殖可）
　　サルの1/3

飼育性・大量供給性に優れる

遺伝子の取得

外部委託：　網羅的取得
　　➡ 配列決定

社内研究：
- 解析用基本遺伝子
- 毒性関連遺伝子
- 公知情報収集・整理

チップ作製

外部委託

約23,000プローブ搭載

RNA標準化研究班 班会議

平成21年1月22日(木)

目次

1. ﾄｷｼｺｹﾞﾉﾐｸｽ･ｲﾝﾌｫﾏﾃｨｸｽﾌﾟﾛｼﾞｪｸﾄでの活動状況

2. Extended OECD/IPCS Advisory Group on Toxicogenomics (Utrecht, Netherlands) 会議報告

Phase I:

Multi-site analysis
2007 – 2008

Platform: Affymetrix

参加施設

Astellas Pharma Inc.
Daiichi Sankyo Co., Ltd,
Dainippon Sumitomo Pharma Co., Ltd.
Eisai Co., Ltd.
Mitsubishi Tanabe Pharma Corporation
Ono Pharmaceutical Co., Ltd.
Otsuka Pharmaceutical Co., Ltd.
Sanwa Kagaku Kenkyusho Co.,Ltd
Shionogi & Co., Ltd.
Takeda Pharmaceutical Co., Ltd.
Toxicogenomics Project

Alphabetical order

参加施設

Group	Dose	Animal No.	Sample No.
Control			C 1a
	0	1	C 1b
			C 1c
	0	2	C 2
	0	3	C 3
	0	4	C 4
	0	5	C 5
Low			L 1a
	300	1	L 1b
			L 1c
	300	2	L 2
	300	3	L 3
	300	4	L 4
	300	5	L 5
High			H 1a
	1000	1	H 1b
			H 1c
	1000	2	H 2
	1000	3	H 3
	1000	4	H 4
	1000	5	H 5

プロトコール

Animal Study: CRO

Crj: CD(SD) IGS rat
Acetaminophen 0, 300, 1000 mg/kg (n=5)
Sampling: 24h after dosing

⇩

Tissue homogenate: TGP

*Three aliquots were prepared from one of each group in order to assess intra-laboratory variation.

⇩

RNA extraction
GeneChip analysis : Each site
Rat Genome 230 2.0

⇩

Raw data process: Each site
Affymetrix Microarray Suite 5.0 (MAS5.0)

⇩

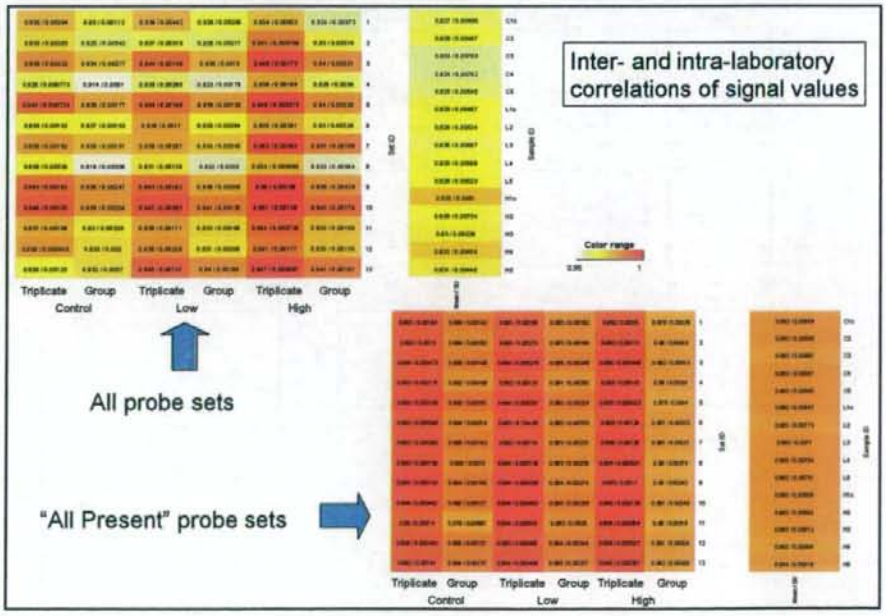
Analysis: TGP & Each site
Normalization : Global mean

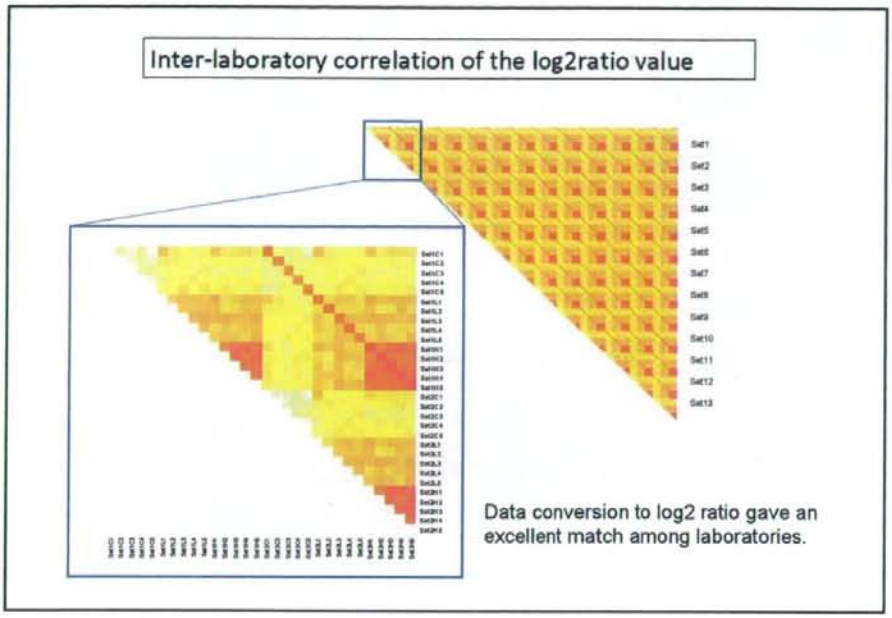
バリデーションで使用されたSOP

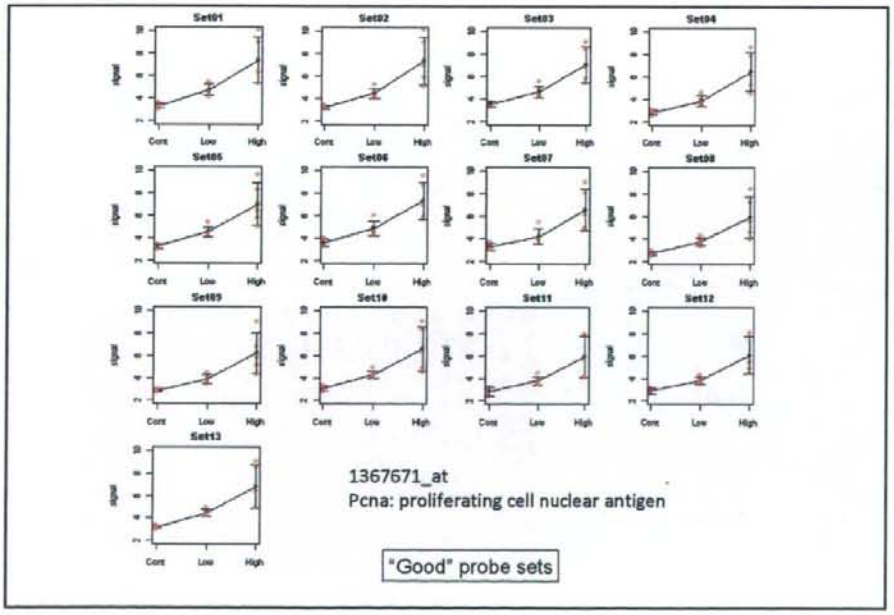
SET ID	SOP Type	wash & stain	RNA QC	4 or 15oC OD measurement	260/280 in QC after fragmentation
1	TGP	self	Gel	15 Multiskanspectrum	+ Gel
2	TGP	self	Gel	15 Multiskanspectrum	+ Gel
3	TGP	self	Bioanalyzer2100	4 NanoDrop	- Bioanalyzer2100
4	TGP	kit	Bioanalyzer2100	15 NanoDrop	- Gel
5	TGP	kit	Bioanalyzer2100	4 NanoDrop	- -
6	Original	self	Bioanalyzer2100	4 Hitachi U-2000	- -
7	TGP	kit	Bioanalyzer2100	4 Beckman DU 640	- -
8	TGP	kit	Bioanalyzer2100	4 Shimadzu UV2450	- -
9	TGP	kit	Bioanalyzer2100	4 NanoDrop	- -
10	TGP	self	Bioanalyzer2100	4 NanoDrop	- Bioanalyzer2100
11	Original	kit	Bioanalyzer2100	15 NanoDrop	- Bioanalyzer2100
12	TGP	self	Bioanalyzer2100	4 NanoDrop	- Bioanalyzer2100
13	Original	kit	Bioanalyzer2100	4 NanoDrop	- Bioanalyzer2100

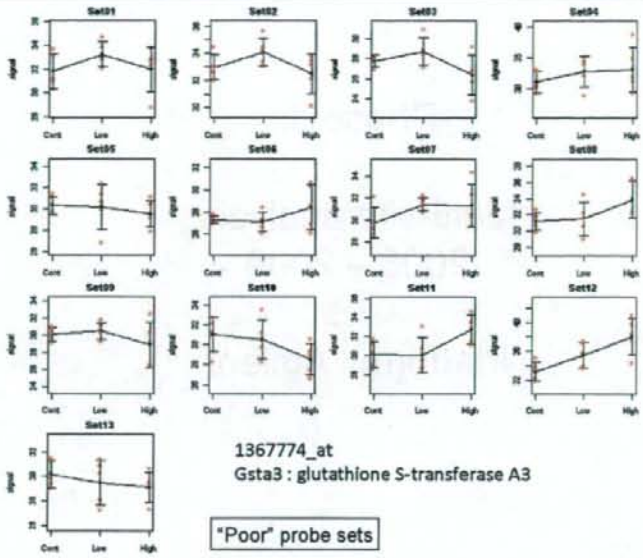
毒性所見

Compound	Acetaminophen		
Species, strain, initial age, sex	Rat [Crl:CD(SD)], 6 week, Male		
Dose (mg/kg)	Vehicle	300	1,000
No. of animals	5	5	5
No. of died animals	0	0	0
Clinical signs	(-)	(-)	(-)
Body weight (% of control)	(-)	(-)	(-)
Body weight gain (g)	5.9	3.8	4.4-8.7
Food consumption (% of control)	(-)	(-)	458
Hematology (% of control)			
Ret	(-)	↘83	↘82
Blood chemistry (% of control)			
K	(-)	(-)	↗115
IP	(-)	(-)	↑124
LDH	(-)	(-)	↗148
Terminal body weight (% of control)	(-)	(-)	(-)
Absolute organ weight (% of control)			
Liver	(-)	(-)	(-)
Spleen	(-)	(-)	↘80
Relative organ weight (% of control)			
Liver	(-)	↗106	(-)
Spleen	(-)	(-)	(-)
Macroscopic findings			
Liver	(-)	(-)	(-)
Spleen	(-)	(-)	(-)
Femur	(-)	(-)	(-)
Lymph node	(-)	(-)	(-)
Histopathology (±: very slight, +: slight, 2+: moderate, 3+: severe)			
Liver			
Change, acidophilic, Hepatocyte, Centrilobular	(-)	(-)	4/5(+)
Cellular infiltration, Centrilobular	(-)	(-)	3/5(+)
Necrosis, Hepatocyte, Centrilobular	(-)	(-)	1/5(+),
Hypertrophy, Hepatocyte, Centrilobular	(-)	(-)	1/5(+)
Spleen	(-)	(-)	(-)
Bone marrow	(-)	(-)	(-)
Lymph node	(-)	(-)	(-)




Inter-laboratory correlation of the log2ratio value
Data conversion to log2 ratio gave an excellent match among laboratories.


Set01
Set02
Set03
Set04
Set05
Set06
Set07
Set08
Set09
Set10
Set11
Set12
Set13
signal
Conc
Low
High
1367671_at
Pcna: proliferating cell nuclear antigen
"Good" probe sets



総括

1. Minor difference in SOP did not affect the gene expression data.
2. Overall intra- and inter-laboratory correlations were good.
3. When using signal values, exclusion of "absent" probe sets gave better correlation.
4. The most excellent correlation was obtained when the data were converted to log2 ratio.
5. The present work would supply essential and basic data for regulatory science in the near future.

Phase II:

Multi-site analysis
2008 – 2009

Platform: Agilent

Tissue samples of TGP validation study

Group	Dose	Animal No.	Sample No.
Control			C 1a
	0	1	C 1b
			C 1c
	0	2	C 2
	0	3	C 3
	0	4	C 4
	0	5	C 5
Low			L 1a
	300	1	L 1b
			L 1c
	300	2	L 2
	300	3	L 3
	300	4	L 4
	300	5	L 5
High			H 1a
	1000	1	H 1b
			H 1c
	1000	2	H 2
	1000	3	H 3
	1000	4	H 4
	1000	5	H 5

Agilent

+

企業

2009年度中に解析
を終了させる予定

Extended OECD/IPCS Advisory Group on Toxicogenomics Meeting

日時: 2008年6月19-20日

場所: Utrecht, The Netherlands

目的

1. To exchange information on progress of the phase 1 of the US Toxcast Program (the central piece of the OECD Molecular Screening Project led by the US) and on other countries and Stakeholders' advances in high throughput/high content screening for prioritization of chemicals.
2. To identify potential regulatory needs and applications of molecular screening.
3. To discuss further partnership of member countries and stakeholders to the Molecular Screening project and agree on the next steps for this project.

議題(1)

DAY 1: Thursday, 19 June 2008		
09h00	1	**Opening (10min)**
		The meeting will be opened by the OECD/IPCS Secretariats. The host country, the Netherlands (Betty Hakkert, RIVM), will deliver welcoming remarks. The meeting will be asked to approve the agenda, and discuss changes in meeting papers and scheduling of the agenda items if necessary.
09h10	2	**Scope and objectives (20min)**
		The Secretariat (Take Fukushima, OECD/ENV/EHS) and the lead country of the OECD Molecular Screening Project, the United States (Bob Kavlock, EPA/ORD/NCCT) will present the scope and objectives of the meeting, as well as the goal to be achieved at the end of the meeting.
09h30	3	**Report from the ICCA LRI Workshop (20min)**
		The International Council of Chemical Associations Long-Range Research Initiative (ICCA-LRI) will hold a workshop entitled "21st Century Approaches to Toxicity Testing, Biomonitoring, and Risk Assessment" in Amsterdam on 16-17 June 2008 and one of breakout sessions will be dedicated to identify lessons learned from using new advances in toxicity testing/molecular screening (e.g., genomics, HTS, HCS) and to discuss how these various approaches will produce information useful to risk assessment. The workshop chair, Richard D. Phillips (ExxonMobil Petroleum and Chemical) will outline the main results of the workshop for background information concerning the state-of-art in technologies. Details of each country or company programs will be revisited at Agenda Item 6.
09h50	4	**Potential Regulatory Needs and Applications of the Molecular Screening**
		The Secretariat (Take Fukushima, OECD/ENV/EHS) will report the outline of the OECD Molecular Screening Project. The United States and other OECD member countries are invited to present view on potential regulatory applications of the Molecular Screening.- -In the United States: Phil Sayre (U.S. EPA/OPPT/RAD) -In Canada: Tim Singer (Health Canada) ** Speakers could be added on a request basis.*
10h30		*Coffee break (20min)*

議題(1)

DAY 1: Thursday, 19 June 2008		
09h00	1	**Opening (10min)**
		The meeting will be opened by the OECD/IPCS Secretariats. The host country, the Netherlands (Betty Hakkert, RIVM), will deliver welcoming remarks. The meeting will be asked to approve the agenda, and discuss changes in meeting papers and scheduling of the agenda items if necessary.
09h10	2	**Scope and objectives (20min)**
		The Secretariat (Take Fukushima, OECD/ENV/EHS) and the lead country of the OECD Molecular Screening Project, the United States (Bob Kavlock, EPA/ORD/NCCT) will present the scope and objectives of the meeting, as well as the goal to be achieved at the end of the meeting.
09h30	3	**Report from the ICCA LRI Workshop (20min)**
		ical Associations Long-Range Research Initiative (ICCA-LRI) will hold a workshop o Toxicity Testing, Biomonitoring, and Risk Assessment" in Amsterdam on 16-17 June will be dedicated to identify lessons learned from using new advances in toxicity nomics, HTS, HCS) and to discuss how these various approaches will produce nt. illips (ExxonMobil Petroleum and Chemical) will outline the main results of the on concerning the state-of-art in technologies. Details of each country or company da.
09h50	4	**Potential Re ds and Ap**
		The Secretariat (Ta shima, O The United States and o OECD mem ular Screening Project. the Molecular Screening.- -In the United States: Phil Sayre (U.S. EPA/OPPT/RAD) -In Canada: Tim Singer (Health Canada) ** Speakers could be added on a request basis.*
10h30		*Coffee break (20min)*

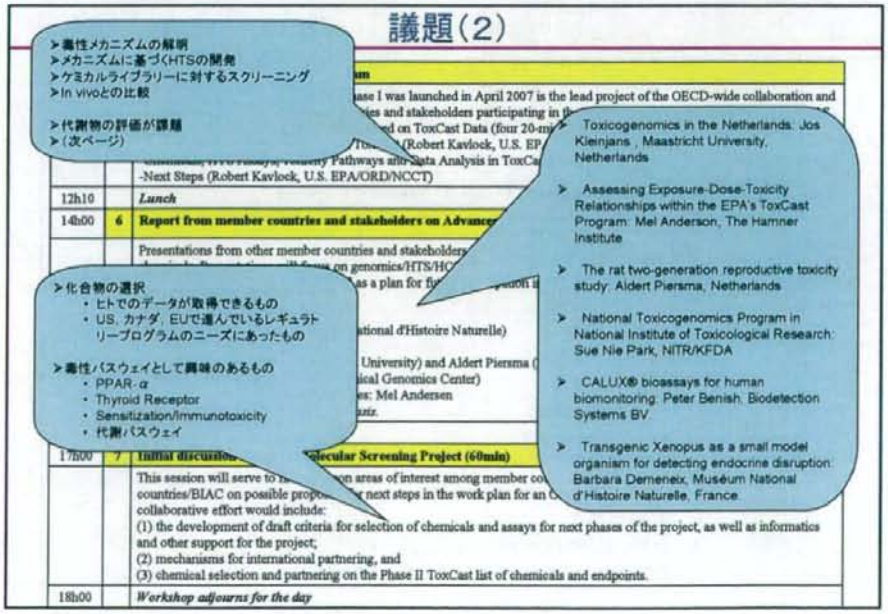
➢EPAが考えるmolecular screeningの利点
- 使用動物、コスト、時間の削減
- パスウェイ、メカニズムの解釈

➢ToxCastの進捗
- Phase I: 14 HPV 化合物
- Phase II: HPV, MPV化合物を予定
- ナノマテリアルについても計画

➢Domestic Substance Listの内容
➢化合物の分類基準等

議題(2)

10h50	5	**Report from the U.S. ToxCast Program**
		The U.S. ToxCast Program of which Phase I was launched in April 2007 is the lead project of the OECD-wide collaboration and would work as the model for other countries and stakeholders participating in the *OECD Molecular Screening Project*. The U.S. EPA will make a series of presentations, based on ToxCast Data (four 20-minute presentations): -Overview of Purpose and Structure of ToxCast (Robert Kavlock, U.S. EPA/ORD/NCCT) -Chemicals, HTS Assays, Toxicity Pathways and Data Analysis in ToxCast (David Dix, U.S. EPA/ORD/NCCT) -Next Steps (Robert Kavlock, U.S. EPA/ORD/NCCT)
12h10		***Lunch***
14h00	6	**Report from member countries and stakeholders on Advances in HTS & HCS for Prioritizing Environmental Chemicals**
		Presentations from other member countries and stakeholders will be made in areas related to prioritization of environmental chemicals. Presentations will focus on genomics/HTS/HCS data generated and other updates on implementation of each Molecular Screening Programs, as well as a plan for future participation in the OECD Molecular Screening and the US ToxCast (seven 20-minute presentations): -Canada: Tim Singer (Health Canada) -France: Barbara Demeneix (Muséum national d'Histoire Naturelle) -Korea: Sue Nie Park (FDA/NITR) -Netherlands: Jos Kleinjans (Maastricht University) and Aldert Piersma (RIVM) -United States: Chris Austin (NIH Chemical Genomics Center) -The Hamner Institute for Health Sciences: Mel Andersen * *Speakers could be added on request basis.*
16h30		***Coffee break (30min)***
17h00	7	**Initial discussion about the Molecular Screening Project (60min)**
		This session will serve to find common areas of interest among member countries. The US will lead a discussion with member countries/BIAC on possible proposals for next steps in the work plan for an OECD-wide project. The proposal for this collaborative effort would include: (1) the development of draft criteria for selection of chemicals and assays for next phases of the project, as well as informatics and other support for the project; (2) mechanisms for international partnering, and (3) chemical selection and partnering on the Phase II ToxCast list of chemicals and endpoints.
18h00		***Workshop adjourns for the day***



ToxCast

Phase	Number of chemicals	Chemical Criteria	Purpose	Number of Assays	Cost per Chemical	Target Date
I	320	Data Rich (pesticides)	Signature Development	>400	$ 20k	FY07-08
IIa	>300	Data Rich Chemicals	Validation	>400	$ 15-20k	FY09
IIb	>100	Known Human Toxicants	Extrapolation	>400	$ 15-20k	FY09
IIc	>300	Expanded Structure and Use Diversity	Extension	>400	$ 15-20k	FY10
III	Thousands	Data Poor	Prediction and Prioritization	???	$ 10-15k	FY11-12

議題(3)

DAY 2: Friday, 20 June 2008		
9h00	8	**Further discussion about the Molecular Screening Project (90min)**
		Continuation of Item 7.
10h30		***Coffee break (30min)***
11h00	9	**Development of Action Items for the Molecular Screening Project (90min)**
		Given discussions at previous agenda items, this session will serve to make recommendations as to which actions should be taken, as well as priorities and time line.
12h30	10	**Conclusion and next steps (30min)**
		Following the discussion, the meeting will be invited to agree on next steps.
13h00	11	**Any other business (30min)**

次のステップ
・サブグループを組織
① Thyroid Signaling
② Cancer Epigenetics
③ PPAR alpha
④ Sensitization/Immunotoxicity
⑤ Databases
⑥ Chemical Nomination
⑦ Developmental and Reproductive Effects

別添4

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Sanosaka T, Namihira M, Asano H, Kohyama J, Aisaki K, Igarashi K, Kanno J, Nakashima K.	Identification of genes that restrict astrocyte differentiation of midgestational neural precursor cells.	Neuroscience	155(3)	780 - 788	2008
Niida A, Smith AD, Imoto S, Tsutsumi S, Aburatani H, Zhang MQ, Akiyama T.	Integrative bioinformatics analysis of transcriptional regulatory programs in breast cancer cells.	BMC Bioinformatics	9	404	2008
Ishiguro A, Toyoshima S & Uyama Y	Current Japanese regulatory situations of pharmacogenomics in drug administration.	Exp Review Clin Pharmacol	1	505 - 514	2008
森和彦、宇山佳明	国際共同治験の基本的考え方について	医薬品研究	39	557 - 575	2008

Neuroscience 155 (2008) 780–788

IDENTIFICATION OF GENES THAT RESTRICT ASTROCYTE DIFFERENTIATION OF MIDGESTATIONAL NEURAL PRECURSOR CELLS

T. SANOSAKA,[a] M. NAMIHIRA,[a] H. ASANO,[a] J. KOHYAMA,[a] K. AISAKI,[b] K. IGARASHI,[b] J. KANNO[b] AND K. NAKASHIMA[a]*

[a]*Laboratory of Molecular Neuroscience, Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5, Takayama, Ikoma, Nara 630-0101, Japan*

[b]*Division of Cellular and Molecular Toxicology, Biological Safety Research Center, National Institutes of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*

Abstract—During development of the mammalian CNS, neurons and glial cells (astrocytes and oligodendrocytes) are generated from common neural precursor cells (NPCs). However, neurogenesis precedes gliogenesis, which normally commences at later stages of fetal telencephalic development. Astrocyte differentiation of mouse NPCs at embryonic day (E) 14.5 (relatively late gestation) is induced by activation of the transcription factor signal transducer and activator of transcription (STAT) 3, whereas at E11.5 (mid-gestation) NPCs do not differentiate into astrocytes even when stimulated by STAT3-activating cytokines such as leukemia inhibitory factor (LIF). This can be explained in part by the fact that astrocyte-specific gene promoters are highly methylated in NPCs at E11.5, but other mechanisms are also likely to play a role. We therefore sought to identify genes involved in the inhibition of astrocyte differentiation of NPCs at midgestation. We first examined gene expression profiles in E11.5 and E14.5 NPCs, using Affymetrix GeneChip analysis, applying the Percellome method to normalize gene expression level. We then conducted *in situ* hybridization analysis for selected genes found to be highly expressed in NPCs at midgestation. Among these genes, we found that *N-myc* and high mobility group AT-hook 2 (*Hmga2*) were highly expressed in the E11.5 but not the E14.5 ventricular zone of mouse brain, where NPCs reside. Transduction of *N-myc* and *Hmga2* by retroviruses into E14.5 NPCs, which normally differentiate into astrocytes in response to LIF, resulted in suppression of astrocyte differentiation. However, sustained expression of *N-myc* and *Hmga2* in E11.5 NPCs failed to maintain the hypermethylated status of an astrocyte-specific gene promoter. Taken together, our data suggest that astrocyte differentiation of NPCs is regulated not only by DNA methylation but also by genes whose expression is controlled spatio-temporally during brain development. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

*Corresponding author. Tel: +81-743-72-5471; fax: +81-743-72-5479.
E-mail address: kin@bs.naist.jp (K. Nakashima).

Abbreviations: bHLH, basic helix–loop–helix; BMP, bone morphogenetic protein; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; DIG, digoxigenin; E, embryonic day; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; GEO, Gene Expression Omnibus; *gfap*, glial fibrillary acidic protein; *Hmga2*, high mobility group AT-hook 2; JAK, janus kinase; LIF, leukemia inhibitory factor; NPC, neural precursor cell; SSC, sodium chloride sodium citrate; STAT, signal transducer and activator of transcription.

0306-4522/08$32.00+0.00 © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2008.06.039

Key words: *N-myc*, *Hmga2*, epigenetics, Percellome method, differentiation.

The mammalian CNS is composed of neurons, astrocytes, and oligodendrocytes. Although these three cell types are derived from common multipotent neural precursor cells (NPCs), their differentiation is spatially and temporally regulated during development (Temple, 2001). Fetal telencephalic NPCs divide symmetrically in early gestation to increase their own numbers, and then undergo neurogenesis through mostly asymmetric divisions. Toward the end of the neurogenic phase, NPCs acquire multipotentiality to generate astrocytes and oligodendrocytes as well as neurons. It has recently become apparent that NPC fate determination is controlled by both extracellular cues, including cytokine signaling, and intracellular programs such as epigenetic gene regulation (Edlund and Jessell, 1999; Takizawa et al., 2001; Hsieh and Gage, 2004).

Interleukin (IL) -6 family cytokines such as cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) activate the janus kinase (JAK) –signal transducer and activator of transcription (STAT) signaling pathway and are known to induce astrocyte differentiation of NPCs (Bonni et al., 1997; Rajan and McKay, 1998). Gene knockouts of LIF (Bugga et al., 1998), LIF receptor β (Koblar et al., 1998), the common receptor component gp130 (Nakashima et al., 1999a) and STAT3 (He et al., 2005) all result in impaired astrocyte differentiation *in vivo*, emphasizing the contribution of JAK-STAT signaling to astrogliogenesis in the developing CNS. Bone morphogenetic proteins (BMPs) are another group of astrocyte-inducing cytokines. They synergistically induce astrocytic differentiation of NPCs via formation of a complex between STATs and BMP-activated transcription factor Smads, bridged by the transcriptional coactivators p300/CBP (Nakashima et al., 1999b).

In addition to these extracellular factors, intracellular programs and factors also play critical roles to regulate astrocytic differentiation of NPCs. We have previously shown that a CpG dinucleotide within a STAT3-binding element (TTC*CG*AGAA) in the astrocytic marker glial fibrillary acidic protein (*gfap*) gene promoter is highly methylated in NPCs at midgestation (embryonic day (E)11.5), when the cells differentiate only into neurons but not into astrocytes. Since STAT3 does not bind to the methylated cognate sequence, NPCs at midgestation do not express *gfap* even when stimulated by STAT3-activating cytokines such as LIF. As gestation proceeds, the STAT3-binding

site becomes gradually demethylated in NPCs, enabling them to express *gfap* in response to LIF stimulation (Takizawa et al., 2001). Thus, we have proposed that DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation during brain development. However, the important question of how this astrocyte-specific gene promoter becomes demethylated in NPCs remains unanswered.

Neurogenic basic helix–loop–helix (bHLH) transcription factors have been also shown to regulate astrocyte differentiation during early neural development. Mice carrying mutations in *mash1* and *math3* (Tomita et al., 2000), or, to a lesser extent, *mash1* and *ngn2* (Nieto et al., 2001) exhibit decreased neurogenesis and premature astrogliogenesis. Conversely, overexpression of neurogenic bHLH factors, either *in vivo* during the gliogenic period (Cai et al., 2000) or in cultured NPCs exposed to CNTF (Sun et al., 2001), promotes neurogenesis at the expense of astrogliogenesis. A possible mechanism underlying the repressive effect on astrogliogenesis is that Ngn1 binds to p300/CBP and sequesters them away from STAT3, thereby preventing STAT3 from activating astrocytic gene expression (Sun et al., 2001). Such a mechanism may ensure the restriction of astrocyte differentiation in NPCs that would otherwise differentiate into neurons under the influence of high-level neurogenic bHLH factor expression during the neurogenic period.

Although these studies have provided us with an integrated insight into the mechanism of neurogenic-to-gliogenic switching in NPCs, they do not preclude the involvement of other, as yet unknown, factors. To identify such factors, we first in this study examined gene expression profiles of mid- and late-gestational NPCs by Affymetrix GeneChip analysis, which is widely used to obtain a complete picture of developmental stage-specific gene expression (Abramova et al., 2005; Ajioka et al., 2006). We then performed *in situ* hybridization experiments to investigate the spatio-temporal expression pattern of genes that were found to be highly expressed in midgestational NPCs. Two genes, *N-myc* and high mobility group AT-hook 2 (*Hmga2*), were highly expressed in the ventricular zone of E11.5 but not of E14.5 mouse brain. Transduction of *N-myc* and *Hmga2* into E14.5 NPCs resulted in suppression of astrocyte differentiation, even in the presence of LIF. However, the prolonged expression of these genes in E11.5 NPCs failed to preserve the hypermethylated status of the astrocyte-specific *gfap* promoter. These results suggest that the inhibition of astrocyte differentiation in midgestational NPCs is regulated not only by DNA methylation of astrocyte-specific gene promoters but also by transcription-regulating factors whose expression is controlled spatiotemporally during brain development.

EXPERIMENTAL PROCEDURES

NPC culture

Timed-pregnant ICR mice were used to prepare NPCs. The protocols described below were carried out according to the animal experimentation guidelines of Nara Institute of Science and Technology that comply with National Institutes of Health Guide for Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. NPCs were prepared from telencephalons of E11.5 and E14.5 mice and cultured as described previously (Nakashima et al., 1999b). Briefly, the telencephalons were triturated in Hanks' balanced salt solution by mild pipetting with a 1-ml pipet tip (Gilson, Middleton, WI, USA). Dissociated cells were cultured in N2-supplemented Dulbecco's Modified Eagle's Medium with F12 (GIBCO, Grand Island, NY, USA) containing 10 ng/ml basic FGF (R&D Systems, Minneapolis, MN, USA) (N2/DMEM/F12/bFGF) on culture dishes (Nunc, Naperville, IL, USA) or chamber slides (Nunc) which had been precoated with poly-L-ornithine (Sigma, St. Louis, MO, USA) and fibronectin (Sigma).

Immunocytochemistry

E11.5 and E14.5 NPCs cultured on coated chamber slides were washed with PBS, fixed in 4% paraformaldehyde in PBS, and stained with the following primary antibodies: rabbit anti-SOX2 (1:1000, Chemicon, Temecula, CA, USA), mouse anti-βIII-tubulin (1:500, Sigma), rabbit anti-GFAP (1:2000, Dako, High Wycombe, UK). The following secondary antibodies were used: Alexa488-conjugated goat anti-rabbit IgG (1:500, Molecular Probes, Eugene, OR, USA), Cy3-conjugated goat anti-mouse IgG (1:500, Chemicon). Nuclei were stained using bisbenzimide H33258 fluorochrome trihydrochloride (Nacalai Tesque, Kyoto, Japan). All experiments were independently replicated at least three times.

Sample preparation and GeneChip analysis

These procedures were conducted according to the Percellome method (Kanno et al., 2006) to normalize mRNA expression values to sample cell numbers by adding external spike mRNAs to the sample in proportion to the genomic DNA concentration and utilizing the spike RNA quantity data as a dose-response standard curve for each sample. Cells cultured on coated dishes were washed with PBS, lysed in 500 ml of RLT buffer (Qiagen K.K., Tokyo, Japan) and transferred to a 1.5-ml tube. Two separate 10-μl aliquots were treated with DNAse-free RNase A (Nippon Gene, Tokyo, Japan) for 30 min at 37 °C, followed by proteinase K (Roche Diagnostics, Mannheim, Germany) for 3 h at 55 °C, and then transferred to a 96-well black plate. PicoGreen fluorescent dye (Molecular Probes) was added to each well, and then incubated for 2 min at 30 °C. The DNA concentration was measured using a 96-well fluorescence plate reader with excitation at 485 nm and emission at 538 nm. Lamda phage DNA (PicoGreen kit, Molecular Probes) was used as standard. The appropriate amount of spike RNA cocktail was added to the sample homogenates in proportion to their DNA concentration. Five independent *Bacillus subtilis* poly-A RNAs were included in the grade-dosed spike cocktail. Total RNAs were purified using an RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. First-strand cDNAs were synthesized by incubating 5 μg of total RNA with 200 U SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 100 pmol T7-$(dT)_{24}$ primer [5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3′]. After second-strand synthesis, the double-stranded cDNAs were purified using a GeneChip Sample Cleanup Module (Affymetrix, Washington, DC, USA), according to the manufacturer's instructions, and labeled by *in vitro* transcription using a BioArray HighYield RNA transcript labeling kit (Enzo Life Sciences, Farmingdale, NY, USA). The labeled cRNA was then purified using a GeneChip Sample Cleanup Module (Affymetrix) and treated with fragmentation buffer at 94 °C for 35 min. For hybridization to a GeneChip Mouse Genome 430 2.0 Array (Affymetrix), 15 μg of fragmented cRNA probe was incubated with 50 pM control oligonucleotide B2, 1× eukaryotic hybridization control (1.5 pM BioB, 5 pM BioC, 25 pM BioD and 100 pM Cre), 0.1 mg/ml herring sperm