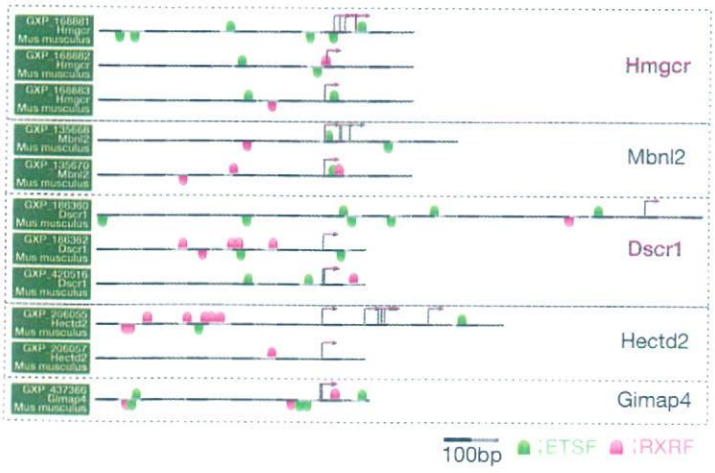

図6：TEF非依存遺伝子の *in silico* プロモーター解析

TEF非依存遺伝子約20のうち，TCDF優位の5遺伝子を絞り込み，Genomatix Software GmbHの提供する *in silico* プロモーター解析の結果を示す．5つの遺伝子に共通して，ETSファミリーとRXRファミリーの転写因子の結合配列を認めた．Hmgcr；3-hydroxy-3-methylglutaryl-coenzyme A reductase，Mbnl2；muscleblind-like 2，Dscr1；Down syndrome critical region homolog 1 (human)，Hectd2；HECT domain containing 2，Gimap4；GTPase，IMAP family member 4．

ことになる．この際の毒性も概してTEFに従うことが知られており，TCDDの1に対してTCDFの10が同等の影響を及ぼす．しかし，リガンド分子個有の作用には受容体毒性学上，興味があるところであり，培養細胞に対する影響を検討した際にこの値が逆転する場合があることを見いだしたことから，TCDF特有の作用がある可能性をマウス肝において遺伝子発現レベルで検討することとした．

TCDDとTCDFについて，以下のような同一プロトコールを用いての実験を行った．12週齢雄C57BL/6マウスの1群3匹，20群を用意し，0，1，3，10，および30 μg/kgの用量で単回強制経口投与後，2，4，8，および24時間後に肝を採取し，Affymetrix GeneChip，MOE430 2.0によりPercellome遺伝子発現データを得た．2時間目の反応を見やすくするために，仮想0時間に2時間溶媒対照群の値を流用し，用量軸5点，時間軸5点から成る5×5の三次元Surfaceを作製した．さらに，TCDD＝1，TCDF＝0.1というTEF値に従った反応を示す遺伝子を抽出するために，TCDDの0，1，3 μg/kg群から成る3×5のSurfaceとTCDFの0，10，30 μg/kg群から成る3×5のSurfaceを用意した．そして，この3×5のSurface同士について，上述のtmfアルゴリズムにより類似度を計算し，類似性の十分に高い遺伝子のリストを得た．次にコピー数が同等であるか，反応が投与依存的変動として生物学的蓋然性があるかを3×5および5×5のSurfaceにより確認し，TEFに忠実に従うTEF依存遺伝子（図1，2）を約140，従わないTEF非依存遺伝子を約20得た．TEFに従うと判定された遺伝子群を，Ingenuity Pathway Analysis (Ingenuity Systems, Inc.) により既知情報と照合するとAhRの下流の第1相代謝酵素やNrf2下流の第2相代謝酵素を中心に，AhRを中心としたPathwayの構成要員であることが示され（図3），上述したTEFについて現在想定されている分子背景に合致するものであった．従わない遺伝子についても，5×5のSurface同士を比較し，TCDDとTCDFで反応のパターンが異なるもの，および類似していてもTEF値の10倍差を説明できないもの，すなわち，TCDFが同等あるいはより強い反応を示すものを抽出した（図4）．TEF非依存遺伝子群は既知情報との照合で予想どおりAhRを含まないPathwayを描き出した（図5）．*In silico* プロモーター解析ソフトウェア (Genomatix Software GmbH) に甘い条件で遺伝子リストを投入した結果，すべてに共通するものとして多数のエレメント，例えば，E2F，EKL，ETS，HES，NR2，RXR，SP1，TBPなどのファミリーが見いだされたが，AhR結合配列は抽出されなかった．さらに，非依存遺伝子のうちTCDF優位の5遺伝子を絞り込みパスウェイ解析を行った結果，TNFを中心とし，ESR1やABCA1を含むネットワークが描かれ，*in silico* プロモーター解析では5遺伝子に共通するものとしてETSファミリーとRXRファミリーの結合部位が選択された（図6）．ETSはERK/MAPKシグナル系の下流に位置し，その1つであるETS2の強制発現系の実験などからp53系を介する胸腺系のアポトーシス，あるいはダウン症候群との関連性などが指摘される．これらの既知情報ベースの解析結果は限られた共通の公開情報源を基にしているため，概して同じリストに収束する．しかし，得られたリストのうち，この検索に投入しなかった遺伝子（図5中の灰色）について，再度Surfaceを吟味すると選定基準ぎりぎりで排除されていた遺伝子が見つかる．ここでは，図5中のTgfb1 (transforming growth factor beta 1)，Hspa1a (heat shock protein 1A)，およびFdft1 (farnesyl diphosphate farnesyl transferase 1) が該当する．このような既知情報と実際のデータとの往復が，データ解釈の向上と今後の検証実験の計画立案に役立つものと見込まれる．

おわりに

このTCDDとTCDFの実験結果の比較によるダイオキシン類化合物の生体影響に関わる分子メカニズム解析はいまだ途上にあり，追加としてAhRノックアウトマウスを用いた投与実験やChIP（クロマチン免疫沈降）解析などによる確認作業が考えられる．ここでは，Percellome Projectの投与実験の組み合わせと，それらに対するPercellome法の利点を生かした網羅的な解析が，環境化学物質をはじめとする外来性化学

物質（Xenobiotics）の生体影響に関する分子生物学的メカニズム解明研究のユニークな糸口を提供する手段としても利用可能であることを示すことができたと考える．誌面の都合上，他に譲るが，ヒトに対する催奇形性があり使用禁止となっていたが，癌や難治性炎症性疾患の治療薬として再登場したサリドマイドについて，成獣雄マウスの肺に及ぼす影響と経胎盤的にマウス胎仔に及ぼす影響とをPercellome解析により対比すると，間葉系成分に対する共通の抑制シグナルの存在が示唆される事例を見いだした．異なったプロトコールで異なった組織に対して行われた実験の間でも，このように共通のメカニズムを抽出しうる可能性を見ており，今後の複合的展開に大きな期待を抱いているところである．今後，本法の利点を生かした解析をさらに進めるとともに，データ・解析ツールの公開Webサイトの充実，および，実験のみならずデータ解析・データマイニングについての共同研究を含めた展開を加速させていきたい．

謝辞 本Percellome Projectの遂行にあたっては，当毒性部の全メンバー，特に松田菜恵，辻 昌貴，森田紘一，今井あや子，安東朋子，安部麻紀，森山紀子，近藤優子，青柳千百合，相原妃佐子，渡辺 忍の各氏の卓越した働きに深謝する．三次元Surface可視化およびそれを用いた解析ツール群のアルゴリズム開発は共筆者の相崎健一主任研究官による．データベース関連，MADIC実装などのIT開発はNTTコムウェア，日本NCR（日本テラデータ）との共同研究に負うところ大である．本研究は厚生労働科学研究費補助金H13-生活-012，H13-生活-013，H14-トキシコ-001，H15-化学-002，H18-化学-一般-001などによる．

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Special Review

Percellome Projectによる毒性トランスクリプトミクスの新しい試み

Percellome Project as a New Approach to Toxicology Transcriptomics

菅野 純　北嶋 聡　相﨑健一　五十嵐勝秀　中津則之　高木篤也　小川幸男　児玉幸夫

Jun Kanno, Satoshi Kitajima, Ken-ichi Aisaki, Katsuhide Igarashi, Noriyuki Nakatsu, Atsuya Takagi, Yukio Ogawa, Yukio Kodama

身の回りの物質の毒性（有害性）を予測し，その被害を未然に防ぐのが毒性学の役割である．この精度向上を目指したトキシコゲノミクス研究を実施する際に，マイクロアレイなどから細胞１個当たりのmRNAコピー数を得るPercellome法を開発した．90化合物のマウス肝初期応答データを採取し終え，新たな対象（反復投与，胎児毒性，吸入毒性，多臓器連携）を加えたPercellome Projectを展開している．

key words

トキシコゲノミクス，分子毒性学，遺伝子発現カスケード，標準化，Percellome法，３次元多層(Millefeuille）データ

菅野 純　国立医薬品食品衛生研究所 安全性生物試験研究センター 毒性部　E-mail：kanno@nihs.go.jp

1985年東京医科歯科大学大学院医学研究科博士課程修了．人体病理学，実験病理学専攻．国立医薬品食品衛生研究所毒性部室長を経て，2002年より同部長．内分泌かく乱関連などの分子毒性学研究，トキシコゲノミクスプロジェクトなどを厚生労働所掌業務との有機的連携のもとに推進．

北嶋 聡，相﨑健一，五十嵐勝秀，中津則之，高木篤也，小川幸男，児玉幸夫　国立医薬品食品衛生研究所 安全性生物試験研究センター毒性部

はじめに

医薬品，食品，化粧品，生活関連用品など，身の回りの物質が我々の身体に取り込まれた際に生じる可能性のある毒性（有害性）を予測し，それらの使用に際しての被害を未然に防ぐのが毒性学の役割である[注1]（図1）．具体的には，人々の安全を確保するために使用法（用途）や使用量（残留量）を制限したり，場合によっては禁止したりするための科学的根拠を提供するが，その際，人の身代わりとして実験動物を用いる場合が多い．このような毒性学の精度向上の一環として，従来からの毒性研究（毒性症候学，毒性病理学，など）に加えてのトキシコゲノミクス（Toxicogenomics）研究が進められている．

トキシコゲノミクスでは，物質が生体に及ぼす影響をトランスクリプトームとして観測・解析する．その際，①分子毒性学を構築し種差や個体差の問題，複合暴露の問題などを解決するためには，遺伝子発現カスケードの全容解明を目指す必要がある，②形態学的に変化が現れた段階のトランスクリプトームは，遺伝子発現カスケードの最終段階に過ぎない，③形態変化の現れないごく初期段階を含む遺伝子発現カスケードを描出するためにはまとまった量のデータの蓄積が必須である，との観点から，筆者らは，マイクロアレイや定量PCRから細胞１個当たりのmRNAコピー数を得るPercellome手法と，そのデータ解析のための３次元多層（Millefeuille）システムを開発・実用化した．遺伝子発現量が共通の尺度，すなわち“コピー数／細胞”で表現されることから，検体間，実験間，マイクロアレイのバージョン間，異なったプラットホーム間，などのデータ比較が直接的に行えるようになり，数年かけて蓄積したデータの有機的活用が可能となった．現在，90種類の化学物質によるマウス肝の初期応答データを採取し終えたところである．新たな対象（反復投与，胎児毒性，吸入毒性，多臓器連携）を加えたPercellome Projectの概要を紹介する．

注1　環境への配慮も含まれる．

Ⅰ．Percellome法：細胞１個当たりのmRNA絶対量を得る方法

原理は単純である．サンプルの細胞数を計測し，外部標準mRNA（スパイクRNA）を細胞１個当たり決まった分子数だけそのサンプルに添加し，そしてRNA抽出，測定に移る．サンプルのRNAの測定値を，スパイクRNAの値を基準に，細胞1個当たりのコピー数に換算する．実際には細胞数を直接計測するのが困難なことが多いため，その代替指標として細胞核内のゲノムDNA量を用いる[1), 2)]．定量性・直線性の検証にはLBM標準サンプル（肝[L]と脳[B]を100：0，75：25，50：50，25：75および0：100に混合した5サンプルから成るセット）を用いる．なお，スパイクRNAは，5種類の枯草菌遺伝子のmRNAを濃度公比３で混合したカクテル（dose-graded spike cocktail；GSC）として用意した．高精度を要求されるDNA定量法は手作業プロトコールおよび自動ロボット（PerkinElmer JANUS）のプロトコールを準備

図1. 毒性学の対象

毒性学は，身の回りの物質が引き起こす障害を予測し，その発生を未然に防ぐことを目的としている．トキシコゲノミクス（毒性ゲノミクス）は，最先端の網羅的遺伝子発現解析技術を用いて，従来の毒性学の予測の精度を著しく向上，迅速化させることで，国民の健康安全の確保にさらに貢献することを目指している．

中である．カクテルとも共同研究ベースで供給可能である（連絡先：kanno@nihs.go.jp）．また，ERCC（The External RNA Control Consortium）と連絡をとるとともに，国際的標準化への関与を深めるため平成18年度厚労科研費「医薬品などの有効性・安全性評価に資する遺伝子発現解析の国際的標準化に関わる研究（H18-特別-指定-023）」を立ち上げた．現在，この他にシックハウス症候群を考慮した低用量域での吸入毒性トキシコゲノミクス，1匹のマウスから多臓器を採取しそれらの連携状況をトランスクリプトームから解析する多臓器トキシコゲノミクスを開始し，特徴的な遺伝子について組織内の発現分布を*in situ*ハイブリダイゼーションで確認する作業を並行している．また，下記の3次元データをweb公開するサーバを整備し，一部の化合物から3次元多層（Millefeuille）データを順次閲覧可能とした（http://toxicomics. nihs.go.jp/db/）．

Ⅱ．3次元多層（Millefeuille）データシステム：生物系研究者に優しいデータ可視化と解析

医薬品を含む毒性既知の90化合物について単回経口投与後のトランスクリプトームデータを取得して，初期応答遺伝子カスケードを解析するための基盤データベースを構築した．現在，第二段階として反復暴露データ集積を開始した．データは，用量軸，時間軸，および遺伝子発現軸から成る3次元表示により，遺伝子発現の用量および時間に依存した変化を1枚の曲面として表すことで可視的に変化を判別しやすいように配慮した（図2）．これにより，コンピュータが選び出した遺伝子クラスターの中身を確認する際，特に，mRNAの合成分解のスピードなどの知見から生物学的にありえないパターン（用量軸の方向にも時間軸の方向にもジグザグな変化など）を排除する際に威力を発揮している．

1つの実験から排出されるGeneChip約50枚のデータを一括処理する能力を持ったPercellome自動換算・データ品質管理（QC）に関わるソフトウエアに加えて，3次元多層（Millefeuille）データに最適化した，発現パターン類似性による候補遺伝子検索，およびそれを発展させた教師無しクラスタリング[3]を中心とした解析システム（MF System，MFシリーズ，開発：相﨑健一）を独自に実用化し，開発継続中である（図3）．これらにより，データQCはその日のうちに，基本的な発現情報検索から全遺伝子の教師無しクラスタリングまでを3日間で完遂できるものとなっている．

この基本解析を用いて，発現パターンによって分類された候補遺伝子リストが多数生成される．一部の幸運な例ではただちに新規と思われる毒性関連反応を見いだすことができた．またそうでない場合のための1つの補強手段とし

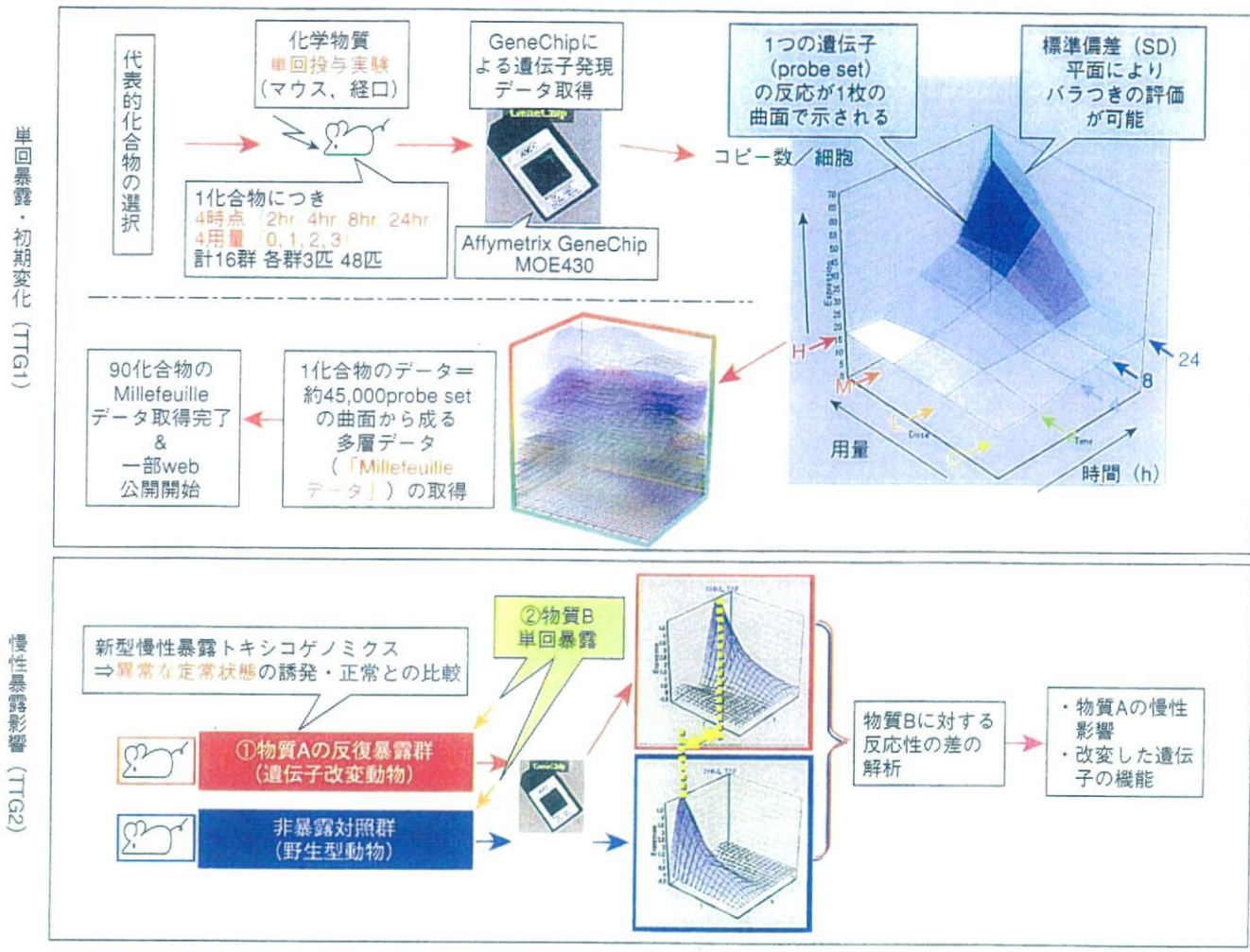

図2. Percellome法と3次元表示による多層（Millefeuille）データシステムを用いたプロジェクトの根幹部分の概要
単回投与による遺伝子発現初期変化データを90化合物について取得（上段）．現在，反復投与の影響を検討中（下段）．H；高用量（high），M；中用量（medium），L；低用量（low），C；コントロール（control）．

て，Gene Ontologyなどの既存知識を利用して候補遺伝子リストの理解を支援するソフトウエア（MF GoPlot）を用意した．このツールは一種の化合物クラスタリングとしても利用することができる．

さらに候補遺伝子リストを基に複数化合物間比較を行い，複数条件下においても同期して発現する遺伝子群を自動抽出するシステムも開発済みである．本システムで得られた同期遺伝子群はシグナルカスケードの構成単位である可能性があり，データベース化しつつ，その解析を進めている（5TB規模のデータベース部分および，大量計算アルゴリズム実装は（株）NTTコムウェアおよび（株）日本NCR/Teradataとの共同開発による）．

Ⅲ．Percellome手法のリアルタイムPCRを含む他のプラットホームへの適用

Percellome手法は，GSCの受け入れ条件を整えることにより，様々なプラットホームに適用可能である．その1つとして最も定量性が高いとされるリアルタイムPCR（ABI PRISM 7900 HT・96ウェルプレート）への適用例を示す．現行のRT-PCR絶対定量法では，遺伝子ごとに検量線が必要であり，多数のサンプルについて多数の遺伝子を検討するには不向きである．Percellome RT-PCRでは，マイクロアレイと同様の原理を用いる．すなわち，サンプル破砕液に，その細胞数に比例する量のスパイクカクテル（GSC）を添加し，それらのCt値をPCRプレートごとの検量線とすることにより，測定したい遺伝子のCt値を細胞1個当たりのmRNAコピー数に換算する．これにより，GAPDHやActinなどのハウスキーピング遺伝子が変動してしまう際の問題，例えば，少数の遺伝子を検討する際にGlobal normalization法を適用し難い問題などが解決される．共通サンプルを測定しデータを比較することにより，Affymetrix GeneChipのPercellome結果と9割程度の整合性が確認され，

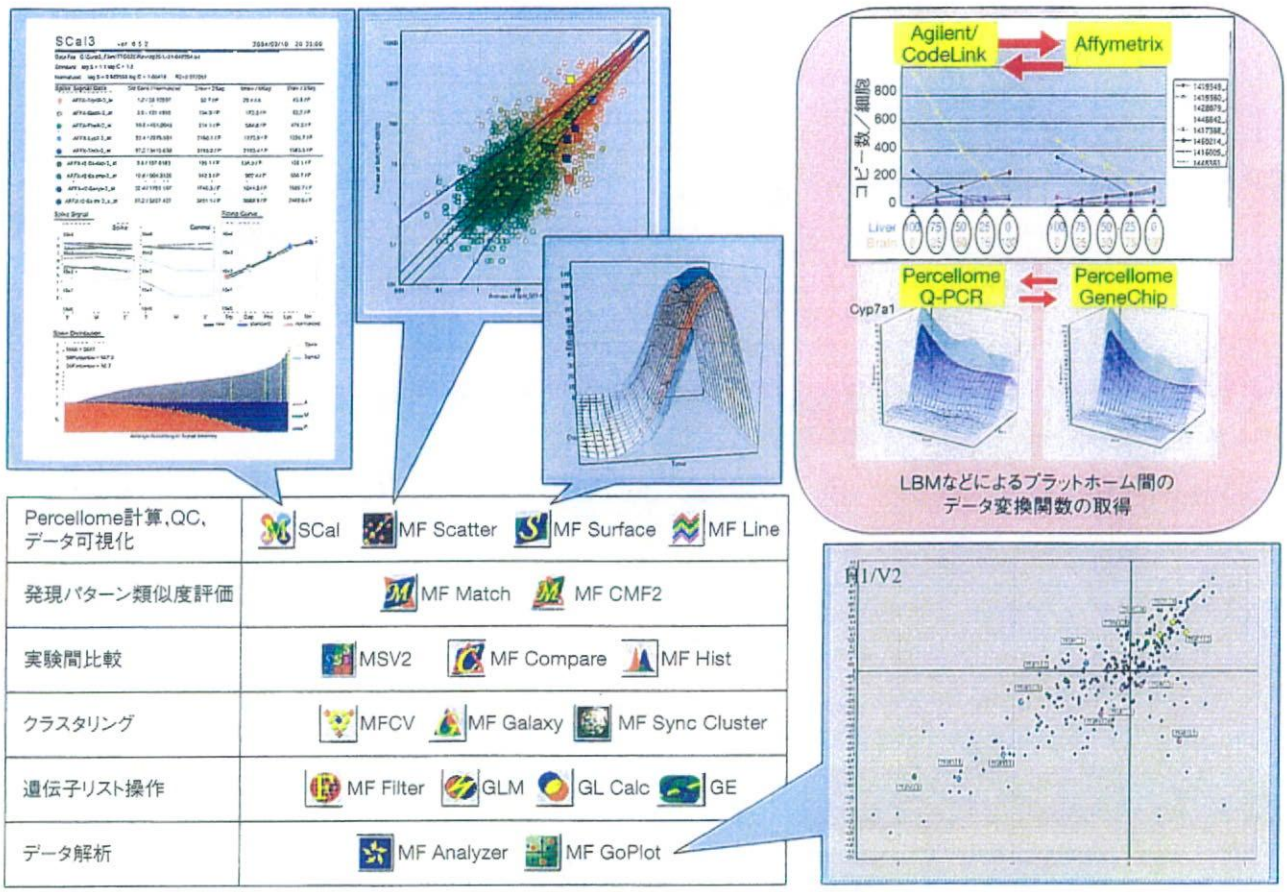

図3. 3次元多層（Millefeuille）データの解析などに用いる独自開発プログラム群

品質管理とともにPrecellome計算を自動的に実施するSCal，Plotソフトウエア，3次元曲面の描画ソフト（MF Surface），など．右上はプラットホーム間のデータ変換情報の得方を示す．LBMを用いる方法（上段）と，実際の実験サンプルを用いる方法（下段）がある．いずれも，一度，両方のプラットホームでそれらのサンプルを測定する必要がある．

GeneChipとPercellome RT-PCRとの間でのコピー数の換算式がいくつかの遺伝子について得られている．この他に，Agilent社製の単色マイクロアレイとCodeLinkアレイにGSCを測定可能なカスタムアレイを用意し終え，LBMサンプルのデータなどをもとに，これらとの間の換算式も得つつある（図3右上）．

Percellome法は，Affymetrixの新しいエキソンアレイの定量性・直線性の検討にも適応可能である．Affymetrix社のHuman Exon 1.0 ST Arrayと従来型の発現アレイHuman Genome U133 plus 2について，性質の異なるヒト癌細胞株2株から調製したLBM様標準サンプル（100：0，75：25，50：50，25：75および0：100混合5サンプル）による比較を行い，両アレイ間の相関性の高いprobe setを多数検出することができた．また，既知のエキソンに対して設計されたprobe setでは発現が見られ，イントロンに対して設計されたprobe setでは発現が見られない，あるいは，既知のsplicing variantに対応したprobe setの発現が検出された，などの基本性能が確認された．しかし，Percellome法を適用して未知のsplicing variantの検出力を向上させるためには，現状では各エキソン間の定量性に問題があることが示唆された．定量値を算出する補正アルゴリズムの開発など，何らかの対策が必要であることが考えられ，現在，Affymetrix社に確認を行っている．

Ⅳ．核内受容体原性毒性のPercellomeトキシコゲノミクス解析

受容体原性毒性とは，化学物質が受容体（リガンド依存的転写因子を含む）に選択的に結合してシグナルをかく乱し，その結果生じる有害性を指す．代表例としてはダイオキシンが挙げられる．AhR（Arylhydrocarbon receptor）ノックアウトマウスでは，ダイオキシンを大量に投与しても毒性がほとんど観察されない．すなわち，野生型マウスがダイオキシンで死ぬメカニズムには，AhRが必須であり，AhRからの異常なシグナルがマウスを死に至らせていることに

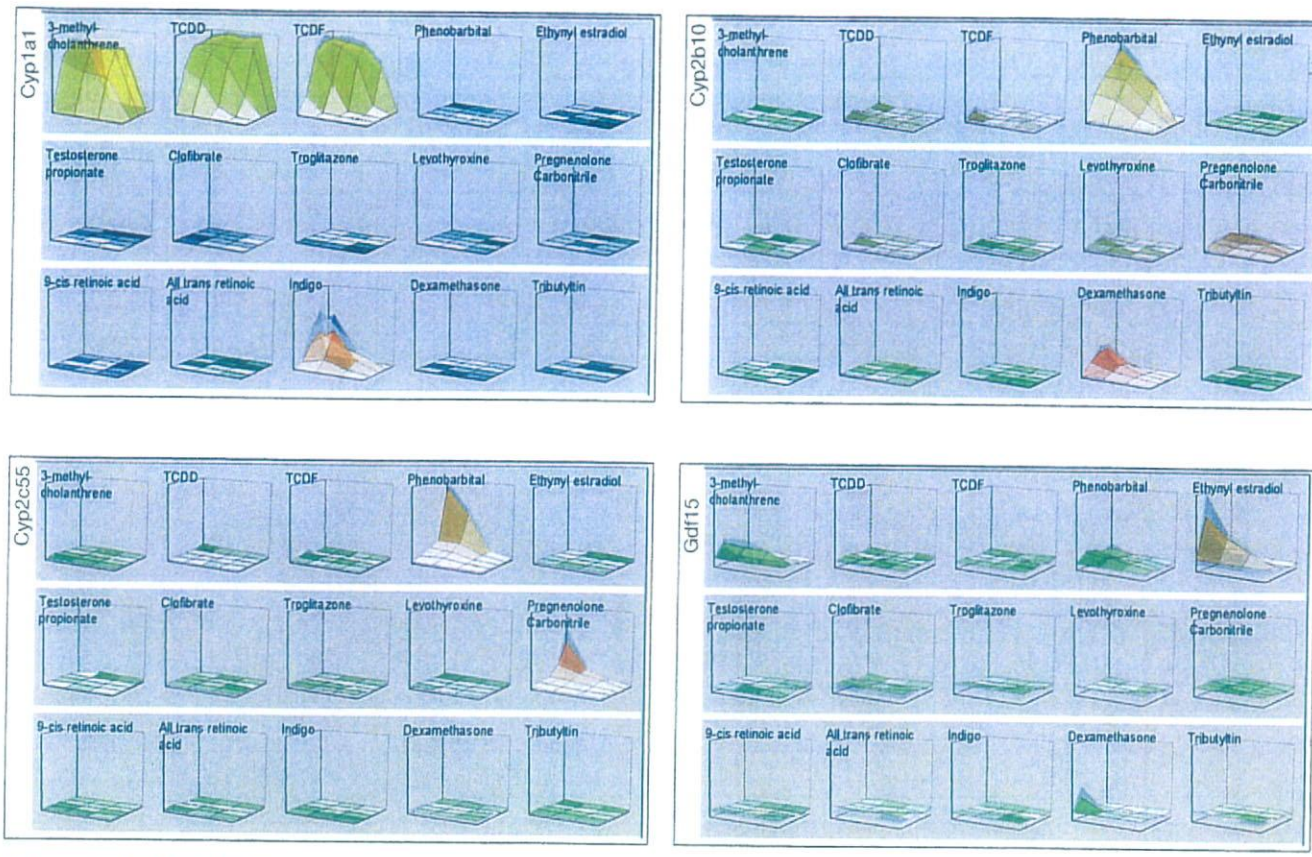

図4. 化合物間の発現比較

15種類の核内受容体リガンド化合物（各3次元グラフ内に表示）によるCyp1a1（左上），Cyp2c55（左下），Cyp2b10（右上）および，Gdf15（右下）の遺伝子発現を3次元表示したもの．各軸は，図2のとおり．縦軸のスケールは遺伝子ごとに共通．リガンドに選択的な遺伝子の発現が確認される．

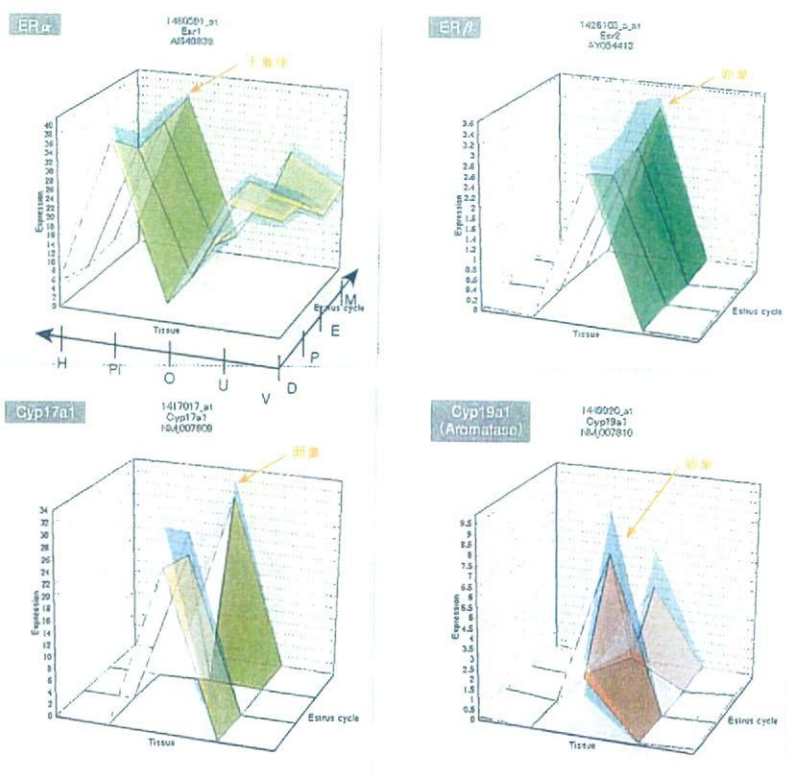

図5. 臓器間の発現比較

マウスの性周期（Diestrus，Proestrus，Estrus，Metestrusの4日間で1周期）ごとの視床下部（H），下垂体（Pi），卵巣（O），子宮（U）および膣（V）における，ER α，ER β，Cyp17a1（steroid-17 α-hydroxylase），およびCyp19a1（Aromatase）の遺伝子発現変動を3次元表示したもの．後二者の酵素は卵巣において周期性を持って発現している．

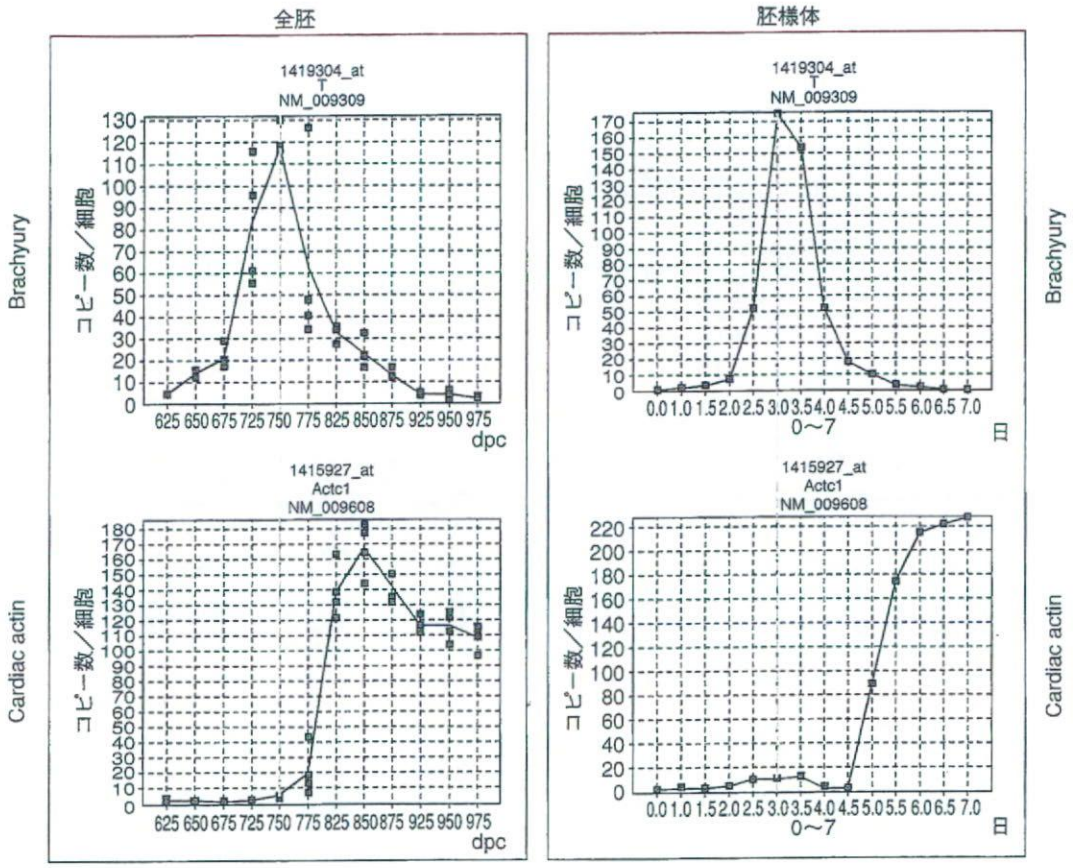

図6. マウス胎児（全胚）と胚様体の発現比較

マウス全胚の胎生6.25日〜9.75日までの遺伝子発現と，胚様体の1日〜7日目までの遺伝子変動の網羅的データベースから，初期中胚葉分化マーカーであるBrachyury遺伝子と，Cardiac actin遺伝子の経時変化を示す．

なる．エストロジェン活性化学物質による有害影響（内分泌かく乱化学物質問題）も同様にER（estrogen receptor）を介する受容体原性毒性と考えられ，胎生期にERを発現する組織が，低用量シグナルかく乱影響の重要標的であると考えられている．

ここでは，受容体原性毒性研究の基盤として，Percellome手法を適用して，①核内受容体作動性物質によるマウス雄肝臓の遺伝子発現変動，②性周期に伴うマウス雌生殖器遺伝子発現変動，③生後の発達過程におけるマウス雌生殖器遺伝子発現変動，の3種類のデータベースを構築した．例えば，①では10種類の核内受容体に作用する典型物質について，単回経口投与後，2，4，8，24時間目の変動を解析し，Ethynyl-estradiolがGDF15，TCDDがCyp1a1，9-cis Retinoic AcidがCyp26a1，DexamethasoneがCyp2b10，ClofibrateがCyp4a14，PCNがCyp2c55など，各々の受容体に特徴的な遺伝子発現を誘導するところがとらえられている（図4）．②の性周期データベースは視床下部，下垂体，卵巣，子宮，膣を対象としており，性周期との関連が網羅的にとらえられている（図5）．これらのデータベースは，今後，各種の候補物質が引き起こす変化を詳細に解析する際の基準として利用される．

V．発生トキシコゲノミクスへの応用

発生毒性学は，個体発生過程におけるダイナミックな遺伝子発現調節の分子機構を把握することにより，さらに正確なものに補強されると考える．現在，C57BL/6マウス胚の器官形成期初期にあたる胎生6.5〜9.5日（プラグ確認日：0.5日）の，①全胚の遺伝子発現変動解析，②遺伝子欠失マウス全胚との比較，および③標的が明らかな既知発生毒性物質投与による本データベースの具体的な適用，を実施している．①についてはすでに0.25日間隔（Time point 計12点）の遺伝子発現データベースを得て，②遺伝子欠失胚のデータといくつかの注目すべき遺伝子についてはwhole mount ISHを用いた発現の検証を加えた．これと並行して，ES細胞からhanging drop法で得た胚様体の0.5日間隔の遺伝子発現データとの比較を実施している．個体発生に関与

する遺伝子群の多くは経時的に激しく変化しており，既知発生毒性物質投与実験については標的遺伝子シグナルカスケードを解析中である（図6）．

おわりに

ノーザンブロットでは実験サンプルにだけバンドが見られ，対照サンプルには遺伝子発現がないという結果を得ても，細胞1個当たりで定量してみると，対照が10コピーに対して実験サンプルが20コピーである場合がある．“無”が“有”になったのではなく，“10”が“20”になったのである．

さて，筆者らの属する毒性学でも，医学の分野でも，疾患概念や毒性概念が整理され，患者や実験動物を診断する際には，まず，そのどれに当てはまるかを検討する．すなわち，どの“典型”に近い症例であるかを検討することから始まることが多い．

しかし，最近の医学・生物学には多因子疾患・多因子形質発現制御の概念が導入され，今から何年かの後には，“21世紀初頭までは，患者の遺伝子多型を調べずして治療を行っていた時代”として，“血液型を調べずに輸血していた時代”と並び称されるようになる可能性がある．このような多因子概念が定着すると，その多くは，“有（100%）”“無（0%）”の組み合わせではなく，“70%”“50%”“90%”といった半端な数の組み合わせであることが考えられる．すなわち，今までの離散値的な“典型”例を基準とするアプローチから，連続値的な病態“スペクトラム”を直接扱うアプローチに変革していく可能性が考えられる．その際の網羅的データの解析とその蓄積の必要性を考えるとき，遺伝子発現データの定量化・標準化という問題は，今まで以上に重みを増すと考えられる．生命現象の網羅的解析にはトランスクリプトームだけでは不十分であることは自明であるが，この定量性を確保することは，これから実現されるであろう網羅的プロテオミクスなどの基盤としても重要ではないかと考える．

マイクロアレイなどから得られるトランスクリプトーム情報が，今後の医薬品審査や化学物質の安全性評価の際に必須なものとなる時代がすぐそこまで来ていることを念頭に，筆者らはPercellome法をさらに展開し，Percellome Projectデータベースを可能な限り高精度に保ちつつ毒性学的な内容を充実させるべく最大限の活動を継続して行く所存であるが，この技術，あるいは研究内容が毒性学以外の研究分野にもお役に立つことができれば幸甚である．

謝辞　本システムの開発とプロジェクトの遂行に当たっては，当毒性部の全メンバー，特に松田菜恵，辻 昌貴，森田紘一，今井あや子，安東朋子，安部麻紀，森山紀子，近藤優子，青柳千百合，相原妃佐子，渡辺 忍の各氏の卓越した働きに深謝する．本研究は厚生労働科学研究費補助金H13-生活-012，H13-生活-013，H14-トキシコ-001，H15-化学-002，H18-化学-一般-001などによる．

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Effects of Environmental Antiandrogenic Chemicals on Expression of Androgen-Responsive Genes in Rat Prostate Lobes

Tomoharu Suzuki,[a] Nariaki Fujimoto,[b] Shigeyuki Kitamura,[c] and Shigeru Ohta*,[a]

[a]*Graduate School of Biomedical Sciences, Hiroshima University,* [b]*Research Institute for Radiation Biology and Medicine, Hiroshima University, 1–2–3, Kasumi, Minami-ku, Hiroshima 734–8551, Japan, and* [c]*Nihon Pharmaceutical University, 10281, Komuro, Inamachi, Kitaadachi-gun, Saitama 362–0806, Japan*

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Rat prostate, which is usually used in the Hershberger assay for evaluating the antiandrogenic activity of environmental chemicals *in vivo*, has a complex structure consisting 4 lobes, *i.e.*, the ventral prostate (VP), lateral prostate (LP), dorsal prostate (DP) and anterior prostate (AP). The VP is considered to have no counterpart in primates, while the LP and DP are histologically similar to human prostate. However, the Hershberger assay focuses on the VP, not the other lobes. Moreover, there are few other methods for assessment of antiandrogenic activity *in vivo*. We therefore investigated androgen-responsive genes in the DP, as well as VP, following treatment with environmental chemicals reported to be androgen antagonists. Male castrated F344 rats were treated with testosterone (0.5 $mg \cdot kg^{-1} \cdot day^{-1}$) alone or together with flutamide (6 $mg \cdot kg^{-1} \cdot day^{-1}$) as a reference antiandrogen or fenthion (25 $mg \cdot kg^{-1} \cdot day^{-1}$) or fenitrothion (25 $mg \cdot kg^{-1} \cdot day^{-1}$) or 2,4,4′-trihydroxybenzophenone (2,4,4′-triOH-BP) (300 $mg \cdot kg^{-1} \cdot day^{-1}$) for 7 days. Testosterone significantly increased the expression of kallikrein S3, cystatin-related protein-1 (CRP-1) and prostatein C3 mRNAs in the VP, and prostate secretory protein of 94 amino acids (PSP94) mRNA, but not stem cell growth factor (SCGF) mRNA, in the DP. Coadministration of flutamide blocked the testosterone-induced increases of all three mRNAs in the VP, but not that of PSP94 mRNA in the DP. Coadministration of fenitrothion significantly reduced the testosterone-induced increase of kallikrein S3 mRNA, while fenthion significantly increased the testosterone-induced increase of PSP94 mRNA. 2,4,4′-TriOH-BP significantly increased the testosterone-induced increases of CRP-1 and prostatein C3 mRNAs. These results indicate that the effects of environmental chemicals on the prostate are very complex. The Hershberger assay alone appears to be inadequate for risk assessment, and it may be useful to employ androgen-responsive genes as additional markers.

Key words —— antiandrogenic activity, androgen-responsive genes, rat prostate lobes, Hershberger assay, quantitative reverse transcriptase polymerase chain reaction

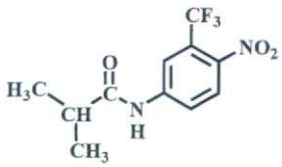
INTRODUCTION

Many environmental xenobiotics exert hormonal effects at the cellular and organism levels. These compounds are able to mimic the biological activity of sex hormones and thyroid hormone, and are called endocrine-disrupting chemicals. Initially, estrogenic chemicals such as alkylphenols and bisphenol A were discovered,[1,2] while more recently, several environmental pollutants were discovered to be androgen antagonists.[3,4]

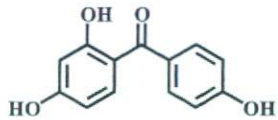
*To whom correspondence should be addressed: Graduate School of Biomedical Sciences, Hiroshima University, 1–2–3, Kasumi, Minami-ku, Hiroshima 734–8551, Japan. Tel.: +81-82-257-5325; Fax: +81-82-257-5329; E-mail: sohta@hiroshima-u.ac.jp

The Hershberger assay has been used to detect chemicals with androgen receptor (AR)-mediated activity *in vivo*.[5–7] The advantages of this assay are that is straightforward, quick and relatively specific to androgenic/antiandrogenic compounds. The endpoint of this assay involves weighing the accessory sex organs of castrated male rats treated with an AR agonist and test compounds.[8,9] However, the Hershberger assay is usually focused on the rat ventral prostate (VP), not other lobes. The rat prostate has a complex structure, consisting of a VP, lateral prostate (LP), dorsal prostate (DP) and anterior prostate (AP). The rodent VP is considered to have no counterpart in primates, while the LP and DP are histologically similar to the human prostate.[10]

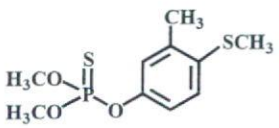
We recently reported the lobe-specific expres-



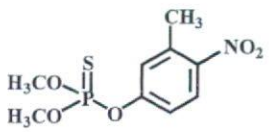
Flutamide



2,4,4'-Trihydroxybenzophenone



Fenthion



Fenitrothion

Fig. 1. Structures of Benzophenone, Fenthion and Fenitrothion

sion and lobe-specific response to androgen of several androgen-responsive genes.[11] In the VP, kallikrein S3, cystatin-related protein-1 (CRP-1) and prostatein C3 were highly responsive to androgen treatment. On the other hand, in the LP and DP, prostate secretory protein of 94 amino acids (PSP94), and stem cell growth factor (SCGF) were responsive. In the present study, we used three antiandrogenic chemicals, fenthion, fenitrothion and 2,4,4′-trihydroxybenzophenone (2,4,4′-triOH-BP), as well as the reference antiandrogen flutamide (Fig. 1), and quantitatively analyzed the changes of expression of the above genes in the DP and VP after administration of these chemicals to castrated rats using the same schedule as in the Hershberger assay.[4,12,13] Based on the results, we discuss whether androgen-responsive genes might be suitable markers for assessment of the antiandrogenic activity of environmental chemicals.

MATERIALS AND METHODS

Chemicals —— Testosterone propionate, fenthion and fenitrothion were purchased from Wako Junyaku KK, Osaka, Japan, flutamide from Sigma (St. Louis, MO, U.S.A.) and 2,4,4′-triOH-BP from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan.

Animals —— Animal experiments were conducted according to "A Guide for the Care and Use of Laboratory Animals of Hiroshima University." Male F344 rats were purchased at 4 weeks of age from Charles River Japan Co. (Kanagawa, Japan) and maintained with free access to basal diet and tap water. All animals were surgically castrated at 5 weeks old. At the age of 7 weeks, they were divided into 6 groups each consisting of 6 animals. The rats were treated once a day for 7 days with subcutaneous doses of 0.3 ml of vehicle (dimethyl sulfoxide), testosterone propionate ($0.5\,mg \cdot kg^{-1} \cdot day^{-1}$), testosterone propionate plus flutamide ($6\,mg \cdot kg^{-1} \cdot day^{-1}$), testosterone propionate plus fenitrothion ($25\,mg \cdot kg^{-1} \cdot day^{-1}$), testosterone propionate plus fenthion ($25\,mg \cdot kg^{-1} \cdot day^{-1}$) or testosterone propionate plus 2,4,4′-triOH-BP ($300\,mg \cdot kg^{-1} \cdot day^{-1}$). Animals were sacrificed under anesthesia and the prostate and seminal vesicles were removed, immediately frozen in liquid nitrogen, and stored at −80 °C.

Quantification of mRNAs by Real-time RT-PCR —— RNA preparation was carried out with a Total RNA Isolation kit (Promega Co., Madison, WI, U.S.A.). Total RNA (2 μg) was reverse-transcribed with 200 U of MMLV-RT (Invitrogen Corp., Carlsbad, CA, U.S.A.) and 2.5 pmol of oligo-dT primer (Invitrogen) in 25 μl of buffer containing 1 mM dNTP, 100 mM Tris–HCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$, 60 mM dithiothreitol and 5 U/μl RNasin with incubation at 37 °C for 60 min. A real-time PCR method with a QuantiTect Sybr Green PCR kit (Qiagen, Valencia, CA, U.S.A.) and an ABI Prism 7700 (PerkinElmer Life Sciences, Boston, MA, U.S.A.) was employed for quantitative measurement, following the supplied protocol.[14] Specific primer sets with a Tm of about 59 °C were designed for each mRNA (Table 1). The PCR conditions were 15 min of initial activation followed by 45 cycles of 20 sec at 94 °C, and 30 sec at 58 °C and 40 sec at 72 °C. Prior to quantitative analysis, PCR products were prepared separately and purified by gel-electrophoresis. Extracted fragments were used as standards for quantification. The DNA sequences were confirmed with a capillary DNA sequencer, ABI 310 (PerkinElmer Life Sciences). All mRNA contents were normalized with reference to β-actin mRNA.

Statistical Analysis —— Statistical comparisons were made using ANOVA followed by Scheffe's test.

RESULTS

Effects of Test Chemicals on mRNA Expression of Androgen-responsive Genes

In order to evaluate the effects of several environmental chemicals on the expression of androgen-

Table 1. Primers for Quantitative PCR of Rat Genes

Gene	GenBank Acc#	5′-Primer	3′-Primer
kallikrein S3	M11566	5′-AATTCCCAACCCTGGCAAGT-3′	5′-CGCTGAGCAAAGGGTTCATC-3′
CRP-1	S57980	5′-TGCTCCTACTGGCCATCTTTG-3′	5′-TGTCAGCACTGTGCGTGTTG-3′
prostatein C3	M71245	5′-CAGTGGTTCTGGCTGCAGTATT-3′	5′-CTAGAAAACACTGCTTGAATTGCTTC-3′
PSP94	U65486	5′-GATCACCTGCTGCACCAAAAC-3′	5′-TTCCTGGGTTCGTCCGTTC-3′
SCGF	XM_218611	5′-AGAGGAAACCACCACAACACCT-3′	5′-GTCCAAAACATGCAGACGGAT-3′
β-actin	X03765	5′-CTGTCCCTGTATGCCTCTGGTC-3′	5′-TGAGGTAGTCCGTCAGGTCCC-3′

Table 2. mRNA Levels Expressed by Reportedly Androgen-sensitive Genes in Castrated Rats in the Experimental Treatment Groups

Treatment group	VP			DP	
	Kallikrein S3 mRNA	CRP-1 mRNA	Prostatein C3 mRNA	PSP94 mRNA	SCGF mRNA
Vehicle Control	0.003 ± 0.0002**	0.0004 ± 0.0002**	0.02 ± 0.005**	1.3 ± 0.32**	0.018 ± 0.003
T	7.3 ± 1.1	46 ± 9.0	84 ± 4.1	11 ± 2.0	0.028 ± 0.010
T+Flu	0.060 ± 0.016**	0.30 ± 0.010**	6.4 ± 1.7**	8.7 ± 1.2	0.022 ± 0.002
T+MPP	5.5 ± 0.70	40 ± 4.8	86 ± 10	19 ± 2.2*	0.045 ± 0.011
T+MEP	4.0 ± 0.66*	23 ± 3.1	84 ± 14	15 ± 2.1	0.028 ± 0.003
T+2,4,4′-triOH-BP	7.4 ± 1.2	93 ± 10*	112 ± 9.0*	6.5 ± 1.1	0.099 ± 0.020*

Castrated male F344 rats were treated with T (0.5 mg·kg^{-1}·day^{-1}) and/or MPP (25 mg·kg^{-1}·day^{-1}), MEP (25 mg·kg^{-1}·day^{-1}), BP (300 mg·kg^{-1}·day^{-1}), Flu (6 mg·kg^{-1}·day^{-1}) for a week. Values are mean ± S.E.M. (n = 6), $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* T. Abbreviations: T, testosterone propionate; Flu, flutamide; MPP, fenthion; MEP, fenitrothion; 2,4,4′-triOH-BP, 2,4,4′-trihydroxybenzophenone.

responsive genes, we carried out quantitative analysis of mRNA expression of three genes in the VP and two in the DP. All of these genes have been reported to be androgen-responsive.[11]

In the VP, expression levels of the kallikrein S3, CRP-1 and prostatein C3 genes in castrated rats were all significantly increased by administration of testosterone (Table 2), while coadministration of flutamide essentially abrogated the effect of testosterone. Coadministration of fenthion had little effect on the action of testosterone, while coadministration of fenitrothion significantly decreased the testosterone-induced increase of kallikrein S3 mRNA. Coadministration of 2,4,4′-triOH-BP significantly enhanced the testosterone-induced increases of CRP-1 and prostatein C3 mRNAs.

In the DP, testosterone increased the expression of PSP94 mRNA, but had no effect on SCGF mRNA, while coadministration of flutamide did not significantly alter the effect of testosterone. Coadministration of fenthion further increased the testosterone-induced expression of PSP94 mRNA, while coadministration of fenitrothion had no effect. Coadministration of 2,4,4′-triOH-BP with testosterone resulted in a significant increase of SCGF mRNA compared with the testosterone-alone group.

DISCUSSION

The Hershberger assay is widely used to study the androgenic and antiandrogenic activity of environmental chemicals. Usually rat prostate is used for this assay. Rat prostate consists of four separate lobes, and although the LP and DP are considered to be homologous to the peripheral zone of human prostate and the AP is similar to the central zone, the VP has no homologous region in human prostate.[15] However, Hershberger assays generally focus on the VP because of its high sensitivity to androgen ablation and to testosterone supplementation after castration. Moreover, there are few alternatives to the Hershberger assay to assess androgenic/antiandrogenic activity *in vivo*. In this study, we assessed the antiandrogenic activities of some known environmental antiandrogens using androgen-responsive genes expressed in the VP and DP as markers. Fenthion and fenitrothion are organophosphorus insecticides; both have been reported to have antiandrogenic activity *in vivo* in the Hershberger assay.[4,12] 2,4,4′-TriOH-BP, a derivative of benzophenone-3 used in sunscreen for humans, is also an antiandrogen.[13] Kallikrein S3, CRP-1 and prostatein C3 are secreted proteins ex-

pressed abundantly in the VP and regulated by androgen.[16–18] We reported that expression of the mRNAs encoding these proteins was increased 10- to 1000-fold in the VP within 24 hr after testosterone treatment in castrated rats.[11] In this study, all the mRNA levels were confirmed to be greatly increased by testosterone and this increase was blocked by co-treatment with flutamide (Table 2). Although fenthion and fenitrothion have been reported to be antiandrogens *in vivo*, we found that they had no effect on the testosterone-induced increases of gene expression in the VP, except for a modest, but significant, decrease of the testosterone-induced increase of kallikrein S3 mRNA by fenitrothion. The reason for this may be the effect of metabolism *in vivo*. Flutamide is converted to hydroxyflutamide, with an increase of about 50-fold in antagonistic activity, while fenthion is inactivated.[12,19] On the other hand, coadministration of 2,4,4′-triOH-BP enhanced the testosterone-induced increase in the expression of CRP-1 and prostatein C3 in the VP (Table 2).

PSP94 is one of the secreted proteins abundantly expressed in DP.[20] Expression of PSP94 was reportedly increased about 2-fold in the DP in castrated rats after testosterone treatment for 24 hr.[11] In this study, testosterone treatment increased PSP94 mRNA, but flutamide did not block this increase. Fenthion significantly enhanced the testosterone-induced increase of PSP94 mRNA, but fenitrothion and 2,4,4′-triOH-BP were ineffective. SCGF is one of the growth factors expressed in rat prostate, and is expressed highly in the DP. It is tightly regulated by androgen in the DP, being up-regulated about 5-fold within 1 hr after testosterone treatment.[11] In this study, however testosterone did not significantly increase the mRNA level of SCGF, while coadministration of 2,4,4′-triOH-BP resulted in a significant increase compared with testosterone alone (Table 2). The reason for this may be the estrogenicity of 2,4,4′-triOH-BP, which acts as an estrogen agonist in MCF-7 human breast cancer cells and ovariectomized rats.[13,21] In the rat, estrogen receptor β (ERβ) is expressed in the prostate, and has a role in prostate growth.[22,23] Its presence may influence the antiandrogenic activity of environmental chemicals. There are differences in response to fenthion, fenitrothion and flutamide between three genes in the VP and two genes in the DP. The reason for this may be the difference in response to testosterone; expression of three genes in the VP greatly increased, on the other hand two genes in the DP did not show great change.

In conclusion, the effects of environmental chemicals on the prostate are very complex, and the Hershberger assay alone appears to be inadequate to understand them. Androgen-responsive genes especially three genes in the VP may be good markers for assessment of androgenic/antiandrogenic activity of environmental chemicals.

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In vivo function of the 5′ flanking region of mouse estrogen receptor β gene

Nariaki Fujimoto [a,*], Osamu Nakajima [b], Shigeyuki Kitamura [c], Shigeru Ohta [c]

[a] *Department of Developmental Biology, Research Institute for Radiation Biology and Medicine (RIRBM), Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan*
[b] *Research Laboratory for Molecular Genetics, Yamagata University, Yamagata 990-9585, Japan*
[c] *Department of Xenobiotic Metabolism and Molecular Toxicology, Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan*

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Abstract

The estrogen receptor (ER) subtypes α and β differentially distributed in tissues, and ERβ is present preferentially in epididymis, testis, prostate, ovary and lung. Although transcription promoter activity has been found in the 5′ flanking (5′f) region of the ERβ gene, it is not known whether the proximal 5′f region is responsible for the tissue-specific distribution. In the present study, we examined the *in vivo* promoter activity of this region in transgenic mice with the lacZ reporter. About 2.2 kbp of the proximal 5′f region of ERβ was cloned and inserted into reporter plasmids. This 5′f region of mouse ERβ, which displayed a substantial promoter activity *in vitro*, was very similar to that in rats, but showed limited homology with the human gene. Three independent lines of mice containing ERβ-5′f-lacZ were obtained. Quantitative measurement of mRNA showed that lacZ was expressed only in the testis, in which sertoli cells as well as a part of the spermatogonia were confirmed to be lacZ-positive, in accordance with the known localization of ERβ expression in the testis. The present study suggests that a 2.2 kbp of the 5′f region of the ERβ gene is able to direct testis-specific expression, but is not itself sufficient to determine the expression in other organs.

Keywords: Estrogen receptor beta; Promoter; Transgenic mouse; *In vivo* analysis

1. Introduction

Estrogen-dependent biological function is mediated by two subtypes of estrogen receptor (ER), α and β [1]. ERα mediates classical estrogen responses, such as uterine growth and mammary gland development. In addition, ERα function is essential for sperm fertilization in the testis [2,3]. ERβ, on the other hand, is necessary to maintain a normal ovulation frequency in the ovary and plays anti-proliferative roles in the prostate and mammary glands. While significant amounts of ERα are present in a variety of sites, including bone, liver and skeletal muscle, as well as reproductive tissues, ERβ distribution seems more limited, with significant expression only in thymus, testis, prostate and ovary. Previous studies have indicated that transcription of the ERα gene is determined by multiple untranslated first exons and promoters [4–6]. Six untranslated first exons have been identified in the human case and four in the rat and the mouse [7–9]. The transcripts are generated by differential promoter usage and differ in the 5′ untranslated exon 1 because of alternative splicing events. In addition, intronic promoter activity was recently found to be involved in the transcription of variant ERα [10]. In the case of ERβ, two isoforms of human ERβ mRNA containing different untranslated 5′-ends have been reported, suggesting the existence of two distinct promoter structures for the gene [11,12]. On the other hand, our previous study and others have demonstrated that rat and mouse ERβ mRNA has a single end structure [13,14]. About 0.7 kbp of the 5′ flanking (5′f) region of ERβ was determined in both

* Corresponding author. Tel.: +81 82 257 5820; fax: +81 82 256 7107.
E-mail address: nfjm@hiroshima-u.ac.jp (N. Fujimoto).

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species and found to be highly homologous. *In vitro* reporter gene analysis demonstrated that the region determines basic as well as cell-specific transcriptional activity in both species.

Although *in vitro* studies have suggested that the 5′f region of ERβ gene may be involved in tissue-specific distribution of ERβ, the question of whether it is sufficient for *in vivo* transcriptional regulation has yet to be addressed. In the present study, therefore, promoter activity of the 5′f region of ERβ gene was examined *in vivo*. The 2.2 kbp region from the transcription starting site was cloned and connected into a lacZ reporter gene to create transgenic mice. The reporter expression was determined by quantitative RT-PCR, as well as lacZ staining, in individual tissues.

2. Materials and methods

2.1. Animals

Animal experiments were conducted according to the guidelines of the Guide for the Care and Use of Laboratory Animals of Hiroshima University and The Animal Welfare Regulations of Yamagata University School of Medicine. BDF1 mice were obtained from CLEA Japan Inc. (Tokyo, Japan) and maintained under constant conditions with free access to basal diet and tap water. Animals were sacrificed under anesthesia and tissues were dissected out, and immediately fixed in RNA Later solution (Ambion, Inc., Austin, TX, USA) for RNA extraction. Portions of the tissues were fixed in 2% formaldehyde/0.2% glutalaldehyde fixation solution, incubated in 20% sucrose solution and embedded in OTC compound (Miles Inc., Elkhart, Indiana, USA) for frozen sectioning.

2.2. Cell culture

The rat prostate cell line, DT3, was maintained in DME medium (Sigma Chemical Co., St. Louis, MO, USA) containing penicillin and streptomycin with 5% FBS (Gibco/Invitrogen Corp., Carlsbad, CA, USA). For hormone treatment, cells were maintained for a week in phenol red-free medium (Sigma Chemicals) containing the same antibiotics along with dextran-charcoal treated sera.

2.3. Genomic DNA library screening

To isolate a genomic 5′f region of ERβ exon 1, a 129SVJ library in λFIX II (Stratagene, La Jolla, CA, USA) was screened with a 809-bp cDNA probe containing the previously reported exon 1 and 5′f region of mERβ (Genbank accession AB034985). Two clones were purified and sequenced to the transcriptional starting site with an ABI model 310 sequencer (PerkinElmer Life Sciences, Boston, MA, USA).

2.4. Construction of luciferase reporter plasmids and transient transfection

Truncated fragments of the 5′f region of mouse ERβ were prepared by accurate PCR with LA-Taq (Takara Bio Inc., Ohtsu, Japan) between −2145, −1343, −692, −149, −60 and +193 (relative to the transcriptional starting site) from the cloned fragment. Each was cloned into PCR2.1-TOPO vector (Invitrogen) and then SacI/XhoI fragments were inserted into the same restriction enzyme site of the pGL_3-basic luciferase reporter plasmid (Promega Co., Madison, WI, USA). phRL-CMV (Promega) was employed as an internal control. DT3 cells were plated at 2×10^4 per well in 48-well plates and transiently transfected with 300 ng of a reporter and 5 ng of phRL-CMV with TransFast transfection reagent (Promega). The weight ratio of TransFast reagent to DNA was 1:1. After 24 h incubation, cells were harvested with 30 μl of cell lysis buffer (Promega) and the firefly and renilla luciferase activities were determined with a Dual Luciferase Assay Kit (Promega) by measuring luminescence with a Wallac Micro-Beta Scintillation Counter (PerkinElmer Life Sciences). Firefly luciferase reporter activity was normalized to renilla luciferase activity from phRL-CMV.

2.5. Construction of lacZ reporter transgene and generation of transgenic mice

A pUC18-based reporter plasmid, placF, containing a nuclear localization signal (nls), lacZ, and mouse protamine polyA was a gift from Richard Palmiter at the University of Washington, Seattle, WA, USA [15]. A 2.3-kb fragment of the 5′f region of mouse ERβ (−2145 to +193, relative to the transcriptional starting site) was cut out from the pCR2.1-TOPO vector and inserted into placF. KpnI/HindIII digestion gave a 6.2 kb fragment of ERβ 5′f connected to nls-lacZ-poly(A). The construct was microinjected into the pronuclei of single-cell fertilized mouse embryos to generate transgenic mice, as previously described [16,17]. To detect the exogenous lacZ gene, genomic DNA was extracted from the tail tissues of pups, and PCR was performed with one primer specific for the ERβ 5′f region and another primer specific for lacZ.

2.6. Quantification of mRNA by real-time RT-PCR

Details were as described previously [18]. Total RNA was isolated from tissue and reverse-transcribed. A QuantiTect Sybr Green PCR kit (Qiagen) and an ABI Prism 7700 (Perkin-Elmer Co.) were employed for quantification of cDNA according to the supplied protocol. The following specific forward and reverse PCR primers with a Tm of about 59 °C were designed: 5′-CGATCTTCCTGAGGCCGATAC and 5′-TGTGAGCGAGTAACAACCCGT for lacZ (+231 to +381, 151 bp) and 5′-TCTGGACACCTCTCTCCTTT and 5′-CAACCGCTCCCGCCAAGCTT for mouse ERβ (+112 to +263, 152 bp) and 5′-CTGTCCCTGTATGCCTCTGGTC and 5′-TGAGGTAGTCCGTCAGGTCCC for mouse β-actin

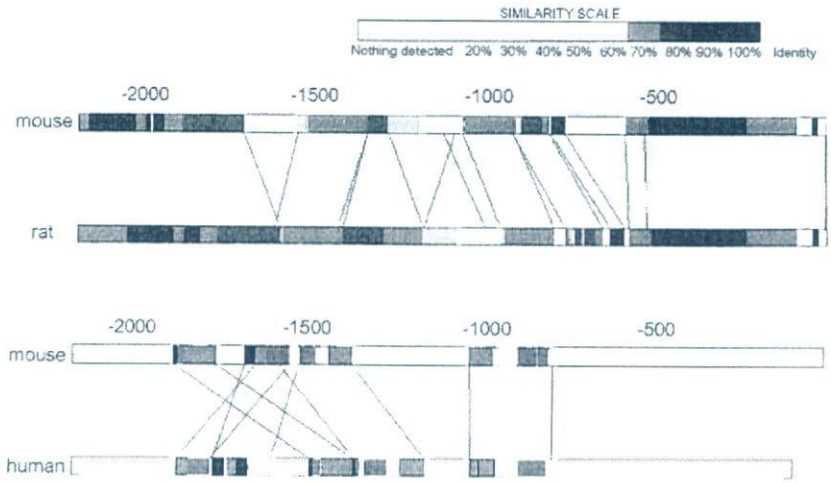

Fig. 1. Sequence comparison of the 5′f regions of mouse, rat and human ERβ. The sequences of 2.2 kbp of the 5′f region of mouse and rat ERβ (DQ273590 and DQ273589) and the corresponding human region (AF191544) were compared by using LFasta (Pôle Bioinformatique Lyonnais).

(+340 to +490, 151 bp). Measured mRNA contents were normalized with reference to β-actin mRNA.

2.7. Quantification of the transgene copy number

The gene copy number of lacZ was also determined with the real-time PCR based method described by Kindich et al. [19]. The genomic DNAs from tails were subject to real-time PCR analysis. The same primer set above was used for the determination of lacZ gene. Androgen receptor gene, a single copy gene in male animals, was employed as a reference. The PCR primers were 5′-CACCATGCAACTTCTTCAGCA (504–524) and 5′-TGAATTGCCCCCTAGGTAACT (+565 to +730 in exon 1, 191 bp).

2.8. Histochemical analysis of lacZ expression in mouse tissues

Sections (40 μm thick) from frozen tissues embedded in OCT compound were mounted onto microscope slides. They were fixed in 0.2% glutalaldehyde in PBS and incubated in PBS containing 1 mg/ml X-gal, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ and 2 mM $MgCl_2$. The reaction was stopped by washing with PBS and the sections were counterstained with 1% eosin, dehydrated in alcohol, treated with xylene, and mounted in Eukitt (O. Kindler GmbH, Freiburg, Germany).

2.9. Statistical analysis

Statistical comparisons were made using Student's *t*-test.

3. Results

3.1. Genomic structure of the 5′ flanking region of mouse ERβ

Two lamda phage DNAs containing a 5′f region of mouse ERβ were obtained from the 129SVJ mouse genomic library. A 2827 bp region from the transcription starting site was sequenced (GenBank accession DQ273590) and compared with the 5′f regions of rat ERβ (DQ273589) and human ERβ (AF191544) by use of the Local Alignment Tool, LFasta, at Pôle Bioinformatique Lyonnais (http://www.pbil.univ-lyon1.fr/pbil.html). High homology is evident between mouse and rat (70–90% similarity throughout the region), but there is only a limited sequence homology of the rodent and human ERβ promoter regions (Fig. 1). Motif analysis (http://www.motif.genome.jp/) showed the 5′f region to contain the consensus promoter motifs AML-1a, SRY, GATA-3 and cdxA (Fig. 2A).

3.2. In vitro promoter activity of mERβ-5′f

The results of examination of the promoter activity of the cloned 5′f region and some of the deletion fragments of mERβ are summarized in Fig. 2B. The luciferase activity of ERβ-5′f (2145/+193)-luc was similar to those of the truncated reporters −1343/+193, −692/+193 and −149/+193, while no activity was seen with −60/+193.

3.3. Generation of mERβ-5′f-lacZ-transgenic mice and lacZ mRNA levels in different tissues at 10 weeks of age

After microinjection and embryo implantation, three lines of transgenic mice (lines 420, 481 and 561) were successfully established. No neonatal or adult deaths were observed in mERβ-5′f-lacZ mice. Quantitative PCR of lacZ in genomic DNA showed that the numbers of transgene copies were 2, 5–6, and 4 per genome in lines 420, 481 and 561, respectively. Table 1 shows the lacZ and ERβ mRNA levels in several tissues of each line of F1 mice at 10 weeks of age. While high ERβ mRNA levels were evident in the epididymis, lung, ventral prostate, testis and ovary, significant lacZ mRNA expression was noted only in testis, although the levels differed among the transgenic mouse lines. In lines 481 and

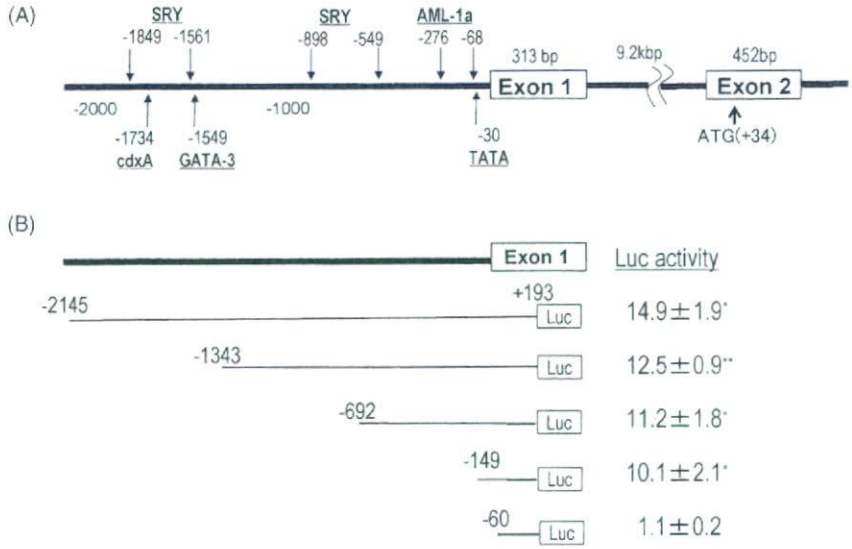

Fig. 2. (A) Structure of the 5′f region of mouse ERβ. Arrows indicate putative *cis*-acting motifs (only consensus motifs are displayed). Numbers indicate positions relative to the transcription starting site (exon 1). (B) Successive 5′-deletion analysis of mouse ERβ promoter activity. Luciferase reporters containing fragments of 5′ flanking regions of ERβ were transiently transfected into an ERβ-positive rat prostate cancer cell line DT3. Values are relative to that with the null-luc-reporter (mean ± S.E.M., $n = 3$).

Table 1
LacZ and ERβ mRNA in mERβ-5′f-lacZ-TG mice

	Ovary	Epididymis	Lung	Ventral prostate	Testis	Liver	Thym	Kidney	Spleen
LacZ									
Line 420	–	0.2	0.5	0.0	238	0.2	n.d.	n.d.	n.d.
Line 481	0.25	35.5	4.9	4.3	1329	5.9	2.1	3	3.5
Line 561	0.16	28.5	15.5	1.4	890	3.8	0.9	0.4	0.4
ERβ									
wt	20.0	9.2	3.2	1.9	0.4	0.0	0.1	0.0	0.0

Values are mRNA levels divided by β-actin mRNA levels (mol/mol β-actin). Eleven-week-old F1 mERβ-5f-lacZ-transgenic mice were sacrificed. Total RNA was isolated from tissues and lacZ mRNA levels were measured by real-time PCR. The numbers of transgene copies were 2, 5–6, and 4 per genome in lines 420, 481 and 561, respectively.

561, lacZ mRNA expression in the epididymis was higher than that in clearly negative tissues.

3.4. *LacZ straining in mERβ-5′f-lacZ-transgenic mice*

LacZ activity was detected in *in situ* frozen tissue sections of epididymis, lung, ventral prostate, testis, ovary and liver in 10-week-old F1 transgenic mice. Positive staining was observed only in the testis (Fig. 3). Localization of staining in the outer line of seminiferous tubules, as well as the triangular shape of the stained cells, suggested that the stained cells were sertoli cells. In addition, a part of the spermatogonia may be lacZ-positive. The partial staining of Leydig cells is probably artifact, since it was also observed in the wild-type testis.

4. Discussion

The 5′f region of the ERβ gene has been cloned and proved to have promoter activity in mice as well as humans in cell culture studies [14,12]. A region containing 692 bp from the transcription start site of mouse ERβ was sequenced and found to contain several putative *cis*-acting elements. Successive 5′-deletion analysis with luciferase reporters suggested that the promoter for basal transcriptional activity was located near the transcriptional start site, while the other elements may contribute to modulating the transcription. Our cloning and *in vitro* promoter analysis of the 5′f region of rat ERβ gave very similar results [13]. In the present study, the region of the mouse ERβ gene extending to −2139 bp was further cloned and luciferase reporter assay was carried out in a prostate cell line, DT3. The results were in line with those of previous reports, since the reporters containing 2.1, 1.3, 0.7 and 0.15 kbp of the 5′f region of mERβ showed similar transcriptional activity, while a −60 bp reporter showed no activity. Comparison of the 5′f regions revealed a high degree of similarity between the mouse and rat sequences, while the human sequence shows only partial homology with those of rodents and contains different *cis*-acting elements (AML-1a, Nkx-2.5 and AP-1). Recently, a second isoform of human ERβ mRNA containing different untranslated 5′-ends was

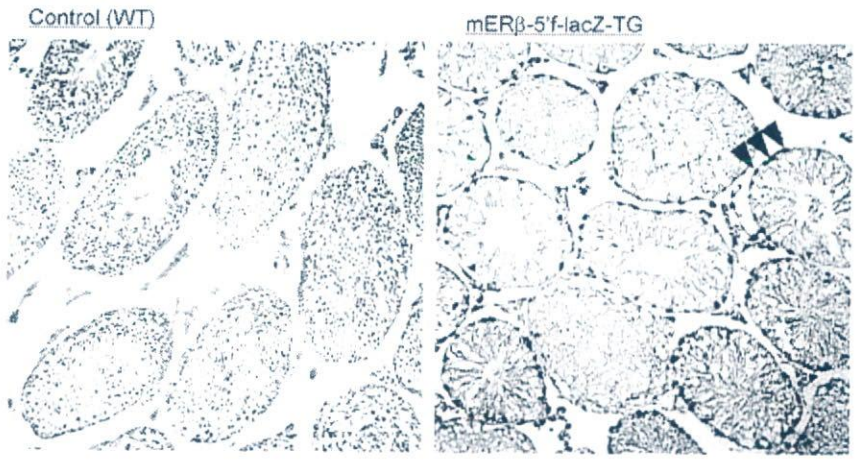

Fig. 3. LacZ staining in testis in WT and ERβ-5′f-TG mice. Sertoli cells as well as a part of the spermatogonia are lacZ-positive (arrowheads). Staining of Leydig cells is an artifact (also present in control).

reported to be specifically expressed in ejaculated spermatozoa and peripheral leukocytes, indicating the existence of two distinct promoter structures for the human ERβ gene [11].

Significant amounts of ERα are present in a variety of sites including pituitary, bone, liver and skeletal muscle, as well as reproductive tissues, such as ovary, uterus and mammary gland, while ERβ expression is restricted mainly to lung, thymus, testis, epididymis, prostate and ovary [1,20,21]. In addition, the tissue distribution of ERβ seems differ among species [22,23]. In humans, higher levels of ERβ mRNA were detected in the thymus, testis and ovary, while the prostate is the major ERβ-positive tissue in rats. On the other hand, in mice, ERβ mRNA is expressed in the epididymis, testis, prostate, ovary and lung. Previous *in vitro* studies have showed that the 5′f region of ERβ is responsible for cell-specific expression, i.e., higher luciferase reporter activities were noted in ERβ-positive cell lines than in ERβ-negative lines [13,14,12]. However, *in vivo* functional analysis remains necessary to determine whether the identified promoter regions control the tissue-specific ERβ expression.

In the present study, we successfully generated transgenic mice containing a mERβ-5′f-lacZ reporter. Three lines were obtained and showed similar tissue distribution patterns of lacZ mRNA expression, which suggested that the expression results from the transgene itself, not from specific insertion sites of the transgene. Data clearly demonstrated a testis-specific expression of lacZ, though a low level of lacZ expression may be present in the epididymis. The differences of mRNA levels among the lines seem to depend on the number of copies of the transgene. In testis, expression of lacZ was localized in sertoli cells, as well as a part of the spermatogonia, in agreement with previous findings based on immunostaining of ERβ expression in the mouse testis [24]. Sertoli cell-specific expression of ERβ has been confirmed by immunostaining, as well by *in situ* hybridization, in rat testis [25,26]. In addition, a recent investigation suggested that 17β-estradiol and 5α-androstane-3β (3β-diol), a possible physiological ERβ ligand converted from 5α-dihydrotestosterone in the tissue, may modulate sertoli cell function through ERβ [27]. Although expression of ERβ in sertoli cells of the testis is evident in rats as well as mice, it remains controversial whether it is expressed in human testis [28].

ERα expression is regulated by multiple untranslated first exons and promoters. At least four promoters have been postulated for the mouse ERα gene [5,8]. Two proximal promoters, called A and B, are responsible for the expression in most ERα-positive organs, including mammary gland, uterus and ovary, while the other two distal promoters seem to determine liver- and testis-specific expression. An *in vivo* functional analysis of the 5′f region of mouse ERα gene using transgenic mice demonstrated that a proximal 5′f region (promoter A) is sufficient for expression in a variety of organs, including mammary gland and uterus [29]. In contrast, our results indicate that the promoter activity of the proximal 5′f region of mERβ gene is able to define only the expression in the testis. Quantitative measurements of lacZ revealed virtually no expression in other ERβ-positive organs, such as lung, ovary or prostate, though a very low level of lacZ mRNA was detected in the epididymis. Other enhancer elements may control other aspects of tissue-specific expression. Intronic enhancers would be one possibility, since they have been reported to be essential for some types of tissue-specific gene expression [30,31].

Complex promoter organization involving multiple promoters seems to be a common feature among the nuclear hormone receptor gene family [8]. It is noteworthy that, despite a number of reports suggesting promoter activity in the upstream region of the nuclear hormone receptors based on *in vitro* assay, *in vivo* function of these promoters has not been proven yet, except for a proximal promoter of mouse ERα. The present study of ERβ promoter, which indicates that the 5′f region alone possesses very limited promoter activity for tissue-specific expression, provides further insight into promoter function in the nuclear receptor family.

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