めではないかと思われた．

カルメジンのノックアウトマウスが報告された後，先述のADAM2ノックアウトマウスが報告されたが，なんとこの二つの遺伝子のノックアウトは同じフェノタイプを示していた．このことを強引に結びつけて考えると，カルメジンがADAM2をシャペロンしている可能性が浮かび上がる．そこで精巣の抽出液をカルメジン抗体で免疫沈降したものをSDS電気泳動しADAM2抗体を用いてウエスタンブロットしてみると，確かにADAM2がカルメジンと共沈していた．精巣内にはカルメジンと相同性を有するユビキタスな分子シャペロンであるカルネキシンも発現している．カルネキシンを免疫沈降すると多くのタンパク質が共沈するが，その中にADAM2は含まれておらず，明らかにカルメジンはADAM2と特異的に結合していることが確認された．さらに検討すると，通常ADAM1とADAM2はヘテロダイマーを形成しているので抽出液をADAM2で免疫沈降したときにはADAM1も同時に落ちてくる．ところが，カルメジンをノックアウトするとADAM2で免疫沈降してもADAM1が落ちてこなくなることが分かった．これはカルメジンがADAM2の折り畳みに関与しており，その折り畳みがなければADM1/ADAM2のヘテロダイマー（ファーティリン）の形成がうまくいかないことを示すものである．これでカルメジンとADAM2の両ノックアウトマウスが同じフェノタイプを有する理由が説明できた．

ところが驚いたことに，その後発表されたADAM1aやADAM3のノックアウトマウスがまたしてもカルメジンと同じフェノタイプを示したのである．ADAMファミリーはお互いに連携した発現様式をとっているようで，ADAM1aノックアウトマウスの場合にはADAM2の発現に影響はないが，ADAM3は精子からなくなるらしい[27]．また，ADAM3をノックアウトしたときにはADAM2の値はそれほど減らない[28]ので，これまでのノックアウトマウスの結果を総合すると，ADAM3が透明帯への結合に関与している最下流の因子ということになる．

しかし残念ながら，ADAM3をヒトゲノム上でコンピュータ検索しても偽遺伝子と思われるものしか検出されない．ヒト精子にはADAM3が存在しないとするとADAM3を最終的な透明帯接着因子とするメカニズムはマウス特有のものであることになる．さらに別の可能性として，ADAM1bと並んで精巣内に存在するADAM1aをノックアウトしたときにADAM3が精子から消えた[27]ように，ADAM2やADAM3が消えたときに実は別に存在する真の透明帯結合因子が精子から消失したことが考えられる．その場合にはヒトやマウスに共通の透明帯結合メカニズムが存在してもおかしくはなくなる．

さて，それではADAM3以外に透明帯結合因子の候補はないのであろうか？ ADAM3以外にもsp56やzonadhesin，などが結合因子として報告されているがその信憑性はノックアウトマウス作製の結果を待たねばならないと思われる．少なくともsp56はカルメジンノックアウトマウスの精子上に残っていることが示されている[29]．また，話は少しずれるがカルメジン，ADAM1a，ADAM2，ACEのノックアウトマウスの精子はみな透明帯に結合できないということ以外に精子が輸卵管内に登っていけないというフェノタイプも共有している[26,27,30,31]．透明帯への結合不全と輸卵管への移行不全がなぜパラレルに起こるのかは大きな謎であると同時に何か重要なことを示唆しているに違いない．

2）細胞膜の融合

透明帯への結合や通過は受精の前段階であり，受精現象の中核部分は配偶子の細胞膜が融合する過程であろう．細胞膜は細胞の境界を決める構造であるが，水平方向に流動性を持っていたり，二重層の表側と裏側で脂質組成を局在化させる機構が存在したり，コレステロールに富んだラフトと呼ばれる構造があるほか，細胞が分裂する際には膜成分の増殖も起こるなど，かなり動的な存在である．細胞が分裂する時にはミオシンなどからできた収縮環ができて，細胞膜にくびれが入り，最終的には中央体とよばれる微細な構造でつながった形になる．しかし，真に細胞が二つに分離するためには，この部分が引きちぎられなければならない．そのときには細胞膜に破れ目ができるはずであるが，おそらく膜の持つ自然な修復機能によって，この穴はふさがる．

細胞膜に人工的に穴をあける操作（たとえば未受精卵の細胞質内にピペットで精子を注入するICSI操作）をしても膜は自動的に修復する．しかし，細胞膜の復元能力は常に一定ではなく，マウスの卵子はヒトの卵子に比べて修復されにくいためICSI操作によって膜を破ると卵子はよく死んでしまう．マウスの場合，大きな穴があくために修復しにくいのか，修復能が劣っているのかははっきりしない．また，同じマウスでも受精卵になると未受精卵のときよりも細胞膜は丈夫になり，凍結保存などの操作にもよく耐えるようになる．精子の細胞膜も射精されたときと，受精能を獲得した後では，かなり違うことが認められている．同じ細胞膜でありながら，状況によって多様な性質を取ることができるのは細胞膜の裏打ち構造や膜を構成する脂質の組成やラフトなどの構造などさまざまな因子の働きによっている．受精とは未受精卵の細胞膜と，受精能を獲得した後に先体反応を起こした精子の細胞膜が融合する過程である．受精が成立するための基本的な状況として，まずお互いの膜が融合に適した環境に適合していることが必要と思われる．その上で何らかの因子が働き融合が引き起

表 1 さまざまな膜融合の例

ひとつの細胞で完結する膜融合
細胞分裂
エキソソーム[50]
ウイルスの出芽
神経顆粒からの伝達物質の分泌（SNARE，クラスリン）
★精子の先体反応[51]（SNARE）
ファゴサイトーシス（SNARE，クラスリン）
ミトコンドリアの融合（mitofusin）[52]
同種細胞の融合
筋芽細胞から筋肉
胎盤絨毛からシンシチオトロフォブラスト
破骨細胞の形成[32]（DC-STAMP）
異種細胞（生物）の融合
★受精（精子-卵子）[37,45]（CD9，Izumo）
ウイルスの感染

こされるのであろう．

実は，受精以外にも生体には膜融合をともなう重要な過程がたくさん存在している（表1）．肝臓や白血球の中には多核の細胞が存在するが，これは核分裂後に細胞質が分裂しないためにできあがる．ところが同様に多核化した細胞である筋肉の場合は成り立ちが肝臓や多核白血球とは異なり，筋原細胞がお互いに融合することによってできあがっている．胎盤絨毛細胞（trophoblast）は自身で融合を起こしシンシチオトロフォブラストを形成し，子宮壁に接触・結合のあと胎盤形成に関わることが知られている．しかしこれらの細胞における融合因子は明らかにされていない．

また，破骨細胞は単球/マクロファージ系に属する細胞であるが，時には100核を越える大型の多核細胞を形成する．この多核化も細胞融合によってもたらされることが知られていたが，最近，この融合を媒介する因子はDC-STAMPと呼ばれるタンパク質であることが明らかにされた[32]．しかしながら，この因子は間接的な働きをするものであり，直接の融合因子が分かっているわけではない[33]．

このほか，細胞内小器官であるミトコンドリアは他の細胞オルガネラの膜と融合することはないが，ミトコンドリア同士は融合したり分裂したりすることが知られる．その融合にはmitofusin1や2が関与することが知られている[34]．膜の融合のメカニズムがもっともよく調べられているのは小胞体におけるタンパク質輸送やエキソサイトーシスである．クラスリンにより被覆された小胞体が出芽するとその根元でダイナミン（GTPase）が膜を融合させて小胞をくびりとる．できあがった小胞はターゲットに向かいそこで小胞上のSNARE（vSNARE）とターゲット上のSNARE（tSNARE）が対になり膜同士を融合に導くと報告されている．受精に先立って精子は先体部分の膜が融合する先体反応を起こす必要があるが先体部分はその中に入っているたくさんの酵素を放出する一種のエキソサイトーシスと考えられる．とするとSNAREの関与があってもいいと思われるが，それを裏付けるようなデータも報告されている（図4）[35]．

ウイルスに限らず，病原性の細菌などもいろいろなメカニズムを駆使して細胞膜を通過し，細胞質内に入り込む仕組みを持っている．ある種のウイルスが標的細胞に融合する過程では小胞体や精子の先体反応で使われるSNAREの仕組みを真似ているらしく，ウイルスの上に単独でvSNAREとtSNAREの複合体からなるような構造を持っており，融合の時にはあたかもSNAREと見まがうばかりのメカニズムで融合を起こしているらしい（図5）．また感染病原体の場合は，細胞内で増殖後，細胞膜をすり

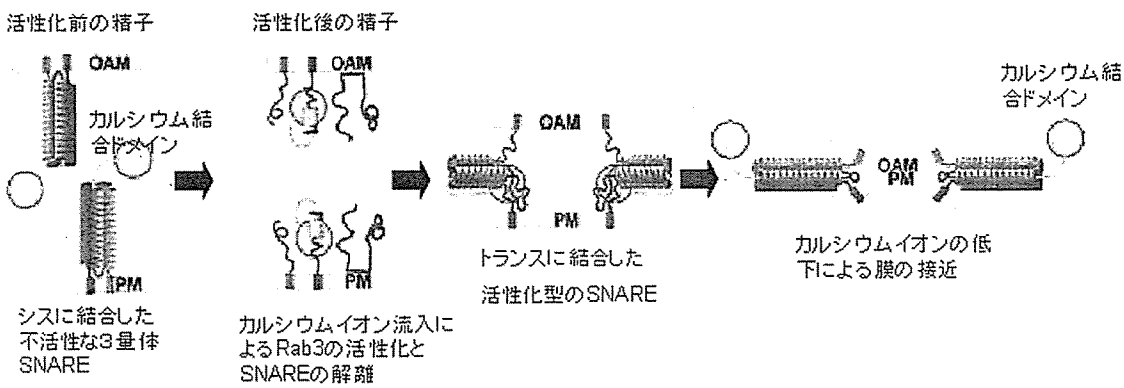


図 4 精子の先体部分の膜（細胞膜と先体外膜）の融合のメカニズム[35]
先体反応時におけるSNAREの関与に関する仮説を示す．精子の細胞膜（PM）と外先体膜（OAM）にはともにRab3，NSF，aSNAPがneurotoxinに抵抗性を示すシス結合したヘテロ3両体を形成しているが，先体内部にカルシウムが入ってくるとRab3が活性化され3量体が解離する．その後トランスに結合する3量体が形成され，カルシウム濃度が再び減少することによりPMとOAMが引き寄せられて先体反応が起こる．

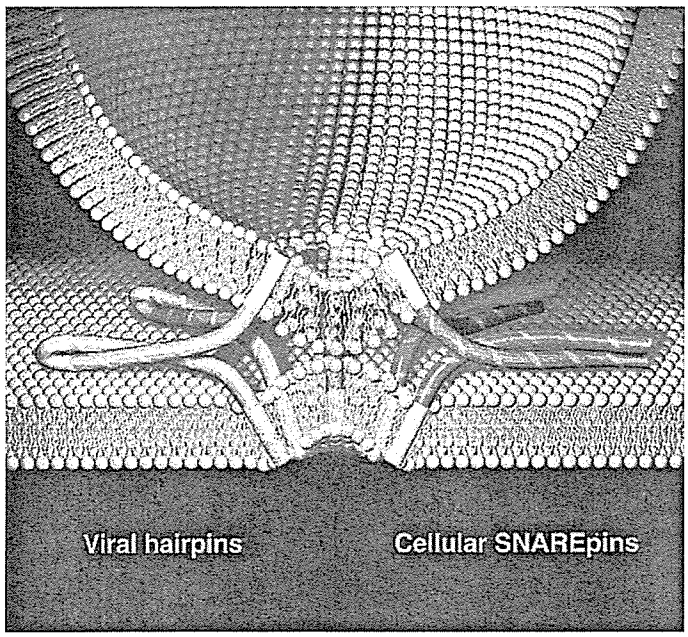


図5 SNAREによく似た構造を持つウイルスの融合タンパク[53]
左はウイルスのSNARE様一本鎖たんぱく質，右は細胞のもつSNARE構造

抜けて外に出なければ，再び他の細胞へと感染を広げることができない．というわけですべての膜融合を伴う反応は病原体にまで範囲を広げると非常に多くの仕組みが存在しているのが現状である．

さまざまな仕組みで膜融合が起こるにしても，そこになにか共通の機構は存在しないのであろうか？ 光学顕微鏡で観察できるような巨大人工脂質膜小胞（リポソーム）にインフルエンザウイルスの膜融合誘導タンパク質ヘムアグルチニンを含ませて，膜融合の過程を再現した実験によると，脂質二重膜が単純に近接しただけでは融合が起こらず，ペプチドが膜を激しく褶曲（折り畳み）して，初めて融合が起こると報告されている[36]．

図6に示すように膜が折れ曲がらないと融合が起こらない．このことを一般的なものとして考えると，膜を形成する脂質層の不安定化が融合が起こるための必須条件になるのかもしれない．それではいったい，受精現象ではどのような膜の変化が起こっているのであろうか？

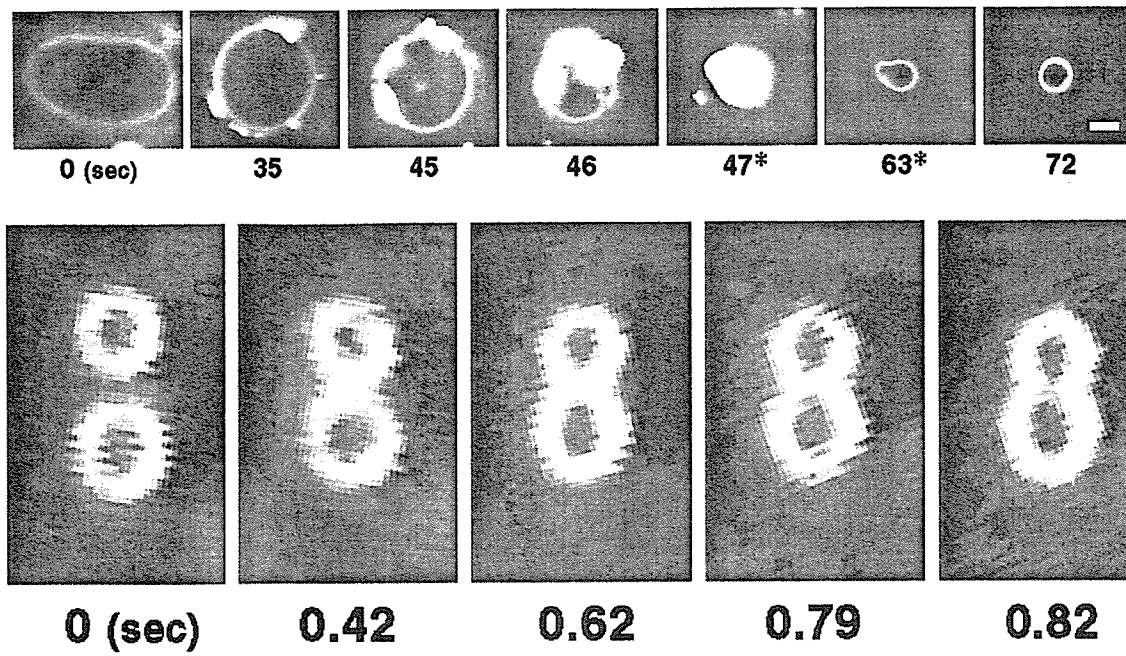
3）精子と卵子の融合

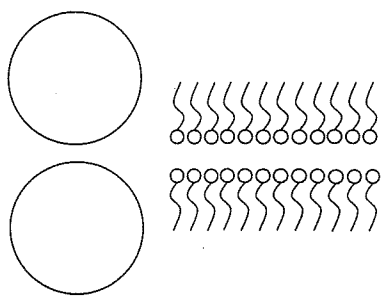
精子-卵子相互作用に関して旧来提唱されてきたメカニズムに対して遺伝子ノックアウトマウスの作製実験はどちらかといえば破壊的な役割を果たしてきたが，建設的な側面ももちろん存在する．いかなる遺伝子をノックアウトしてもその研究を行うためには必ずできあがったマウスは交配して増やさなければならない．というわけで，いまや受精の研究者は座っているだけで，他の研究者がノックアウトした遺伝子が精子や卵子の受精能に関わっているのかどうかを知ることができる．受精研究者にとっては極楽浄土のような世界が展開されようとしている．たとえば，CD9ノックアウトの場合であるが，この遺伝子はそもそも多くの細胞で発現しており，欠損させると免疫担当細胞に影響が出るのではないかという予想のもとにノックアウトマウスが作製されたが，不妊という全く予期されていなかった部分に劇的な影響が現れた．すなわちCD9を欠失させたマウスの雄は正常な受精能を持つが，雌は不妊であった[37]．

なぜ，不妊になるのかを体外受精で調べたところ，卵子の透明帯内に多くの精子が蓄積しているが，これらの精子は卵子と融合できないことがわかった．卵子は普通，最初にやってきた精子と融合すると，多精子受精を防ぐため透明帯反応[38]と呼ばれる反応を起こし，それ以上の精子の侵入を阻止するが，どの精子も卵子と融合できなかったために，精子が次々と侵入し続け，図7に示すような結果になったものと考えられた．

遺伝子ノックアウトにより，卵子が精子と融合するためにはCD9が必須であることが判明し，精子-卵子の融合のメカニズムにかかわる重要因子が受精を専門としない研究者により発見され，融合の分子生物学的研究の扉がはじめて開かれることになったわけである．CD9は四つの膜貫通領域を持つタンパク質で，テトラスパニンと呼ばれるファミリーに属しておりインテグリンなどと結合することが知られていた．卵子上ではインテグリン$\alpha 6$，$\beta 1$が存在しており，インテグリンの合成ペプチドなどを加えると融合阻害が起こることから，インテグリンが精子との融合に必要であるという論文が報告された[39]．この手法は旧来の受精の図式を描くための手法と同じであった．だからとい

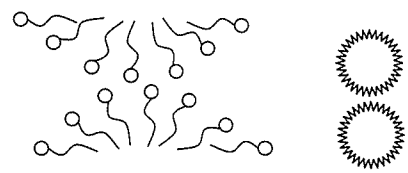
融合タンパク質を組みこんだ人工リポソームが褶曲する様子（上）と褶曲したリポソームが融合する様子（下）[36]





褶曲前の膜が向い合った様子

（右は拡大図）



褶曲後の膜が向い合った様子

膜が折れ曲がるために外系は小さくなる

（右は拡大図）

図 6 膜の褶曲は融合の前段階として必要か？

うわけではないが，「インテグリン重要説」は程なく，インテグリン $\alpha 6$ やインテグリン $\beta 1$ をノックアウトしたマウスの卵子の受精能が正常であった[40]ことから，下火となった．

さて，精子は先体反応を起こさないと卵子と融合できない．これは融合因子が先体反応した精子にだけ表れてくることを示している．もし先体反応精子とだけ反応して，しかも卵子の融合を抑える作用を持つようなモノクローン抗体をみつけることができれば受精の融合因子を明らかにできるかもしれない．こういう考えのもとに我々は抗ヒト先体反応精子特異抗体 MH61[41]及び抗マウス先体反応精子特異抗体[42]を作製した．ヒトの精子が MH61 抗体とどの程度反応するのかを調べることにより不妊の診断に応用することができた．その抗原はいったい何なのかを知るために，ウエスタンブロットでバンドを特定したあと，その部分のゲルからタンパク質を抽出して N 末端分析を行った

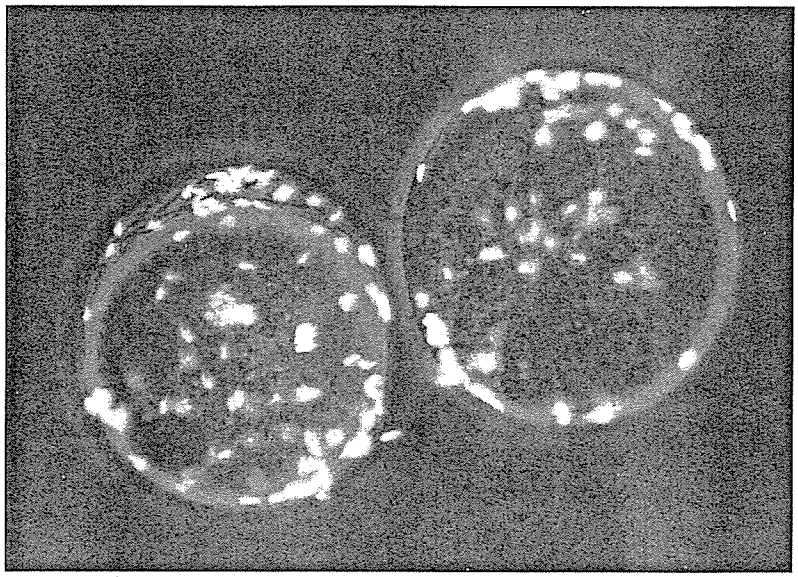


図7 CD9ノックアウト卵子の透明帯の中に蓄積した多くの精子
光っているのはヘキストで染色された精子の核．卵子に融合能が無いために次々と精子が透明帯内へ進入を続けるが精子の核は卵子と融合・膨潤することなくそのままの形を保つ．

ところほとんど全身の細胞に発現している補体制御因子CD46であることがわかった．瀬谷らがマウスのオーソログのクローニングに成功したが，発現部位を調べたところ，マウスにおけるCD46は精巣だけ（正確には精子にだけ）に発現していた[43]．このことからCD46が受精に関わる重要な因子であるに違いないと考え，勇んでCD46欠損マウスの作製を行った．ところがCD46を欠損したマウスは予想を完全に裏切り，生殖能力にはなんの問題も生じなかった[44]．旧セオリーの破壊に一役買ったわけである．再び「抗体による阻害実験」の限界を知ることになった．しかしこれはヒトで明らかになったことをマウスで確認しようとして失敗しただけかも知れない．そこで気を取り直してもともとマウスでとれていたOBF13抗体が認識する抗原についても明らかにして遺伝子をノックアウトすることでその役割を検証することにした．OBF13抗原のスポットを2Dゲル上でウエスタンブロットにより同定し，そのスポットをLC/MS/MSで解析し，抗原を明らかにした．クローニングされた配列は，分子量56 kDaでIgドメインをひとつだけ持つイムノグロブリンスーパーファミリーのタンパク質であった．RT-PCRを試みると，OBF13抗原はCD46の場合と同じく，精子にだけ発現していた[45]．

しかしこれまでの例から分かるようにOBF13抗原が融合に関与する必須の因子であるかどうかは，ノックアウトマウスを作製してその妊孕性をみるまでは判断がつかない．そこでノックアウトマウスを作製し，野生型の雌と交配して，交尾してから数時間経過した雌の輸卵管から卵子を取り出し，受精しているかどうかをおそるおそる見てみたところ，期待通り，受精しておらず，卵子は未受精卵のみであった．体外受精をするとカルメジンの場合と異なり透明帯には鈴なりの精子が結合しており，たくさんの精子が透明帯の中に侵入していた．しかし，卵子と融合している精子は1匹もなく，OBF13抗原を持たない雄は不妊であった．OBF13抗原を持たない精子を人工的に卵子の細胞質内にインジェクションするICSIを行った場合は正常な受精卵が得られ，移植すると健康な産仔が得られたので，この抗原を持たない精子の欠陥は融合のステップに限局されていることが示された[45]．

しかし，気をつけなければならないのは，ノックアウトマウスを作製して，フェノタイプが見えたからといって必ずしもその遺伝子がなくなったからとはいえない場合があることである．たとえばMRF4遺伝子を三つの研究室で別々にノックアウトしたところ，胎生致死，一部死亡，影響なしと三つのフェノタイプが得られている．プリオン遺伝子も複数の研究室で別々にノックアウトされ，影響なし[46]，神経症状あり[47]の二通りのフェノタイプが報告された．MRF4のフェノタイプの出た例は，ノックアウトベクターのせいでMRF4遺伝子の隣にあるMyf5遺伝子が働かなくなったためである[48]．プリオンのノックアウトでフェノタイプが出たほうは，ノックアウトベクターのせいで，プリオン遺伝子が途中から隣の遺伝子にスプライスされ，人工的な複合タンパク質が産生されたためである[49]．

このような例を見ると，これ以外にも，ひとつのコンストラクトでしかノックアウトがなされていない遺伝子の中には，間違ったフェノタイプと結び付けられて報告されているものがあるに違いない．したがって，ノックアウトマウスでフェノタイプが見られても，実は間違っているかもしれないと疑ってかかるほうが賢明である．しかし，倍以上の労力を費やして複数のターゲティングベクターから2種のノックアウトマウスを作製したとしてもそれで絶対に

間違いないと断定することはできない．では，どうすれば遺伝子がフェノタイプの直接の原因であると断定できるのであろうか？ 実は，その遺伝子を同じように発現させるプロモーターがあれば，その遺伝子のトランスジェニックマウスを作り，ノックアウトマウスと交配して，フェノタイプが消えればそれがまちがいなく原因遺伝子であったということが証明できる．

そこで我々は，OBF13ノックアウトに見られた雄の不妊というフェノタイプが，本当にOBF13抗原の欠損に由来するのかどうかを確かめるために，精巣特異的に発現するカルメジンプロモーターにOBF13抗原のcDNAを結合してトランスジェニックマウスを作製し，ノックアウトマウスと交配し，本来の遺伝子の欠損をトランスジーンで補ったところ，見事に不妊のフェノタイプが消失した．ここまでくればOBF13抗原が精子上に存在する卵子との融合に関わる因子であると断定しても間違いはないと考えられる．我々は融合に関わる真の精子側因子を理詰めで見つけ出したことがうれしくて，この遺伝子に和名をつけたくなり，縁結びの神様を祭る出雲大社にちなんでIzumoと命名し発表した[45]．

普通，受精は同種の精子と卵子の間に起こる反応であり，異種の配偶子が出会うことはないし，人工的に混合しても受精は起こらない．ところが，ハムスターの卵子は透明帯を取り除くと異種の精子とでも融合することが知られている．このようにハムスターの卵子はあたかもファゴサイトーシスを起こすように精子を取り込む．そこでIzumoをノックアウトしたマウス精子がハムスターの卵子に対して融合能を持つかどうかを検討したところ，さすがのハムスター卵子もIzumoがなければ精子と融合をできないことがわかった．我々はヒト精子にも存在するIzumoの受精への関与を検討したいと考えていた．そこで，ヒトIzumoに対する抗体を作製したところ，できあがった抗体はマウスIzumo抗体の場合と同様に，新鮮なヒト精子とは反応しないが，先体反応を起こさせて内先体膜を露出させると精子と反応することが確かめられた．さらに，この抗体の存在下にヒト精子をハムスターの卵子に加えたところ，融合が阻害されることが認められ，ヒトでもIzumoは受精に関与していることが示唆された[45]．しかしながら，抗体による阻害はあてにならないというのが，この拙文で主張し続けている点である．残念ながらヒトIzumo抗体だけを例外扱いして，ヒトでも機能していると断定するわけにはいかない．Izumo遺伝子に欠陥を持つために不妊である男性が見つかるまでは，ヒトにおける役割の断定はお預けである．

終わりに

「生化学」誌に生化学的な手法の限界を説くのは気がひけたが，こと受精現象に関しては抗体を添加したり，リガンドを添加したり，あるいは酵素阻害剤を添加したりしながら，その影響を観察し，その結果に基づいて描かれた受精の図式は崩れ去ってしまった．これに対して，受精のようにごくわずかな研究材料でミクロの出来事を追跡するには遺伝子操作動物の使用が非常に効果的であることが実証された．受精のメカニズムに登場する新因子はその半分以上が日本人研究者により発見されている．この事実は少子化に悩む我が国が不妊の診断や治療法を開発してゆく上でまことに喜ばしいことである．

文献

1) Sa-Ardrit, M., Saikhun, J., Thongtip, N., Damyang, M., Mahasawangkul, S., Angkawanish, T., Jansittiwate, S., Faisaikarm, T., Kitiyanant, Y., Pavasuthipaisit, K., & Pinyopummin, A. (2006) *Int. J. Androl.*, **29**, 346-352.
2) Nagy, A. (2002) Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, New York.
3) Leibo, S.P. & Songsasen, N. (2002) *Theriogenology*, **57**, 303-326.
4) Johnson, J., Canning, J., Kaneko, T., Pru, J.K., & Tilly, J. L. (2004) *Nature*, **428**, 145-150.
5) Johnson, J., Bagley, J., Skaznik-Wikiel, M., Lee, H.J., Adams, G.B., Niikura, Y., Tschudy, K.S., Tilly, J.C., Cortes, M.L., Forkert, R., Spitzer, T., Iacomini, J., Scadden, D.T., & Tilly, J.L. (2005) *Cell*, **122**, 303-315.
6) Byskov, A.G., Faddy, M.J., Lemmen, J.G., & Andersen, C.Y. (2005) *Differentiation*, **73**, 438-446.
7) Dyce, P.W., Wen, L., & Li, J. (2006) *Nat. Cell Biol.*, 8, 384-390.
8) Talbot, P., Geiske, C., & Knoll, M. (1999) *Mol. Biol. Cell*, **10**, 5-8.
9) Stewart, C.L., Kaspar, P., Brunet, L.J., Bhatt, H., Gadi, I., Kontgen, F., & Abbondanzo, S.J. (1992) *Nature*, **359**, 76-79.
10) Yoshida, M., Ishikawa, M., Izumi, H., De Santis, R., & Morisawa, M. (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 149-154.
11) Spehr, M., Gisselmann, G., Poplawski, A., Riffell, J.A., Wetzel, C.H., Zimmer, R.K., & Hatt, H. (2003) *Science*, **299**, 2054-2058.
12) Fukuda, N., Yomogida, K., Okabe, M., & Touhara, K. (2004) *J. Cell Sci.*, **117**, 5835-5845.
13) Nakanishi, T., Ikawa, M., Yamada, S., Parvinen, M., Baba, T., Nishimune, Y., & Okabe, M. (1999) *FEBS Lett.*, **449**, 277-283.
14) Kim, K.S. & Gerton, G.L. (2003) *Dev. Biol.*, **264**, 141-152.
15) Lu, Q. & Shur, B.D. (1997) *Development*, **124**, 4121-4131.
16) Ensslin, M.A. & Shur, B.D. (2003) *Cell*, **114**, 405-417.
17) Shur, B.D., Rodeheffer, C., Ensslin, M.A., Lyng, R., & Raymond, A. (2006) *Mol. Cell. Endocrinol.*, **250**, 137-148.
18) Baba, T., Azuma, S., Kashiwabara, S., & Toyoda, Y. (1994) *J. Biol. Chem.*, **269**, 31845-31849.
19) Adham, I.M., Nayernia, K., & Engel, W. (1997) *Mol. Reprod. Dev.*, **46**, 370-376.
20) Honda, A., Yamagata, K., Sugiura, S., Watanabe, K., & Baba, T. (2002) *J. Biol. Chem.*, **277**, 16976-16984.
21) Primakoff, P., Lathrop, W., Woolman, L., Cowan, A., &

Myles, D. (1988) *Nature*, **335**, 543-546.
22) Gmachl, M. & Kreil, G. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 3569-3573.
23) Yudin, A.I., Vandevoort, C.A., Li, M.W., & Overstreet, J. W. (1999) *Mol. Reprod. Dev.*, **53**, 350-362.
24) Baba, D., Kashiwabara, S., Honda, A., Yamagata, K., Wu, Q., Ikawa, M., Okabe, M., & Baba, T. (2002) *J. Biol. Chem.*, **277**, 30310-30314.
25) Blobel, C., Wolfsberg, T., Turck, C., Myles, D., Primakoff, P., & White, J. (1992) *Nature*, **356**, 248-252.
26) Cho, C., Bunch, D.O., Faure, J.E., Goulding, E.H., Eddy, E.M., Primakoff, P., & Myles, D.G. (1998) *Science*, **281**, 1857-1859.
27) Nishimura, H., Kim, E., Nakanishi, T., & Baba, T. (2004) *J. Biol. Chem.*, **279**, 34957-34962.
28) Nishimura, H., Cho, C., Branciforte, D.R., Myles, D.G., & Primakoff, P. (2001) *Dev. Biol.*, **233**, 204-213.
29) Ikawa, M., Wada, I., Kominami, K., Watanabe, D., Toshimori, K., Nishimune, Y., & Okabe, M. (1997) *Nature*, **387**, 607-611.
30) Hagaman, J.R., Moyer, J.S., Bachman, E.S., Sibony, M., Magyar, P.L., Welch, J.E., Smithies, O., Krege, J.H., & O'Brien, D.A. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 2552- 2557.
31) Ikawa, M., Nakanishi, T., Yamada, S., Wada, I., Kominami, K., Tanaka, H., Nozaki, M., Nishimune, Y., & Okabe, M. (2001) *Dev. Biol.*, **240**, 254-261.
32) Yagi, M., Miyamoto, T., Sawatani, Y., Iwamoto, K., Hosogane, N., Fujita, N., Morita, K., Ninomiya, K., Suzuki, T., Miyamoto, K., Oike, Y., Takeya, M., Toyama, Y., & Suda, T. (2005) *J. Exp. Med.*, **202**, 345-351.
33) Vignery, A. (2005) *J. Exp. Med.* **202**, 337-340.
34) Chen, H. & Chan, D.C. (2005) *Hum. Mol. Genet.*, **14** Spec No. 2, R283-289.
35) De Blas, G.A., Roggero, C.M., Tomes, C.N., & Mayorga, L.S. (2005) *PLoS Biol.*, **3**, e323.
36) Nomura, F., Inaba, T., Ishikawa, S., Nagata, M., Takahashi, S., Hotani, H., & Takiguchi, K. (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 3420-3425.
37) Miyado, K., Yamada, G., Yamada, S., Hasuwa, H., Nakamura, Y., Ryu, F., Suzuki, K., Kosai, K., Inoue, K., Ogura, A., Okabe, M., & Mekada, E. (2000) *Science*, **287**, 321-324.
38) Barros, C. & Yanagimachi, R. (1971) *Nature*, **233**, 268-269.
39) Chen, M.S., Tung, K.S., Coonrod, S.A., Takahashi, Y., Bigler, D., Chang, A., Yamashita, Y., Kincade, P.W., Herr, J.C., & White, J.M. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 11830-11835.
40) Miller, B.J., Georges-Labouesse, E., Primakoff, P., & Myles, D.G. (2000) *J. Cell Biol.*, **149**, 1289-1296.
41) Okabe, M., Nagira, M., Kawai, Y., Matzno, S., Mimura, T., & Mayumi, T. (1990) *Fertil. Steril.*, **54**, 1121-1126.
42) Okabe, M., Adachi, T., Takada, K., Oda, H., Yagasaki, M., Kohama, Y., & Mimura, T. (1987) *J. Reprod. Immunol.*, **11**, 91-100.
43) Tsujimura, A., Shida, K., Kitamura, M., Nomura, M., Takeda, J., Tanaka, H., Matsumoto, M., Matsumiya, K., Okuyama, A., Nishimune, Y., Okabe, M., & Seya, T. (1998) *Biochem. J.*, **330** (Pt 1), 163-168.
44) Inoue, N., Ikawa, M., Nakanishi, T., Matsumoto, M., Nomura, M., Seya, T., & Okabe, M. (2003) *Mol. Cell. Biol.*, **23**, 2614-2622.
45) Inoue, N., Ikawa, M., Isotani, A., & Okabe, M. (2005) *Nature*, **434**, 234-238.
46) Bueler, H., Fischer, M., Lang, Y., Bluethmann, H., Lipp, H.P., DeArmond, S.J., Prusiner, S.B., Aguet, M., & Weissmann, C. (1992) *Nature*, **356**, 577-582.
47) Sakaguchi, S., Katamine, S., Nishida, N., Moriuchi, R., Shigematsu, K., Sugimoto, T., Nakatani, A., Kataoka, Y., Houtani, T., Shirabe, S., Okada, H., Hasegawa, S., Miyamoto, T., & Noda, T. (1996) *Nature*, **380**, 528-531.
48) Olson, E.N., Arnold, H.H., Rigby, P.W., & Wold, B.J. (1996) *Cell*, **85**, 1-4.
49) Flechsig, E., Hegyi, I., Leimeroth, R., Zuniga, A., Rossi, D., Cozzio, A., Schwarz, P., Rulicke, T., Gotz, J., Aguzzi, A., & Weissmann, C. (2003) *EMBO J.*, **22**, 3095-3101.
50) Couzin, J. (2005) *Science* **308**, 1862-1863.
51) Hutt, D.M., Baltz, J.M., & Ngsee, J.K. (2005) *J. Biol. Chem.*, **280**, 20197-20203.
52) Meeusen, S.L. & Nunnari, J. (2005) *Curr. Opin. Cell Biol.*, **17**, 389-394.
53) Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T.H., & Rothman, J. E. (1998) *Cell*, **92**, 759-772.

0888-8809/06/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 20(10):2584–2590

doi: 10.1210/me.2006-0099

A Novel Hypothyroid Dwarfism Due to the Missense Mutation Arg479Cys of the Thyroid Peroxidase Gene in the Mouse

Shuji Takabayashi, Kazumi Umeki, Etsuko Yamamoto, Tohru Suzuki, Akihiko Okayama, and Hideki Katoh

Institute for Experimental Animals (S.T., E.Y., H.K.), and Department of Parasitology (T.S.), Hamamatsu University School of Medicine, Hamamatsu, Shizuoka 431-3192, Japan; and Clinical Laboratory (K.U.), University of Miyazaki Hospital, and Department of Rheumatology, Infectious Diseases and Laboratory Medicine, Faculty of Medicine (A.O.), University of Miyazaki, Kihara 5200, Kiyotake, Miyazaki 889-1692, Japan

Recently, we found a novel dwarf mutation in an ICR closed colony. This mutation was governed by a single autosomal recessive gene. In novel dwarf mice, plasma levels of the thyroid hormones, T_3 and T_4, were reduced; however, TSH was elevated. Their thyroid glands showed a diffuse goiter exhibiting colloid deficiency and abnormal follicle epithelium. The dwarfism was improved by adding thyroid hormone in the diet. Gene mapping revealed that the dwarf mutation was closely linked to the thyroid peroxidase (*Tpo*) gene on chromosome 12. Sequencing of the *Tpo* gene of the dwarf mice demonstrated a C to T substitution at position 1508 causing an amino acid change from arginine (Arg) to cysteine (Cys) at codon 479 (Arg479Cys). Western blotting revealed that TPO protein of the dwarf mice was detected in a microsomal fraction of thyroid tissue, but peroxidase activity was not detected. These findings suggested that the dwarf mutation caused a primary congenital hypothyroidism by TPO deficiency, resulting in a defect of thyroid hormone synthesis. (*Molecular Endocrinology* 20: 2584–2590, 2006)

MANY SPONTANEOUS MUTATIONS have been found in inbred strains derived from an ICR closed colony of mice (1–3). Recently, we found that a Jcl:ICR male mouse had an autosomal recessive mutation in our colony, because some of the backcross mice obtained using a DBA/2JJcl female mouse showed dwarfism. Dwarf mice were noticeably smaller than their normal littermates and showed primary congenital hypothyroidism (CH) with goiter.

Six autosomal recessive mutations related to dwarfism have been found in mice, *e.g. dw* [dwarf, chromosome 16, 43.5 centimorgans (cM)] (4, 5), *df* (chromosome 11, 25.0 cM) (6, 7), *lit* (little, chromosome 6, 26.0 cM) (8), *hyt* (hypothyroid, chromosome 12, 37.0 cM) (9), *cog* (congenital goiter, chromosome 15, 36.4 cM) (10), and *grt* (growth-retarded, chromosome 5, 59.0 cM) (11, 12). The *dw* and *df* mutations are a primary hypopituitarism, which does not produce prolactin, TSH, and GH (13–15). The *lit* mutation has a GH deficiency (16). In contrast, the *hyt*, *cog*, and *grt* mutations have primary CH, which show low thyroid hormone (T_3 and T_4) and elevated plasma TSH levels. Recently, *Pax8*−/− created using a homologous recombination technique showed congenital hypothyroidism (17).

In this study, we found a novel dwarf mutant mouse that has different characters from other dwarf mutations. We showed that the responsible gene in our novel dwarf mice was mapped on a position close to the thyroid peroxidase (*Tpo*) gene on chromosome 12 (18) and that sequence analysis demonstrated a missense mutation, Arg479Cys, of the *Tpo* gene. To date, dwarfisms caused by any mutation of the *Tpo* gene have not been reported in mice. The mouse dwarfism reported in this study is the first one to be discovered that is caused by *Tpo* gene mutation. We also showed hormone therapy improved phenotypes of the dwarfism.

First Published Online June 8, 2006

Abbreviations: CH, Congenital hypothyroidism; TPO, thyroid peroxidase.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

RESULTS

Phenotype of Dwarf Mice

Typical phenotypes of adult dwarf mice were characterized by a short trunk and prominent forehead and eyes (Fig. 1). As shown in Fig. 2, dwarf mice (6.46 ± 0.36 g; n = 8) at 15 d after birth were significantly smaller than the normal littermates (8.95 ± 0.32 g; n = 6; $P < 0.01$). At 30 d after birth, dwarf mice (6.54 ± 0.25 g) were extremely small compared with the normal littermates (20.96 ± 0.68 g; $P < 0.001$). Their mean life span was approximately 3 months.

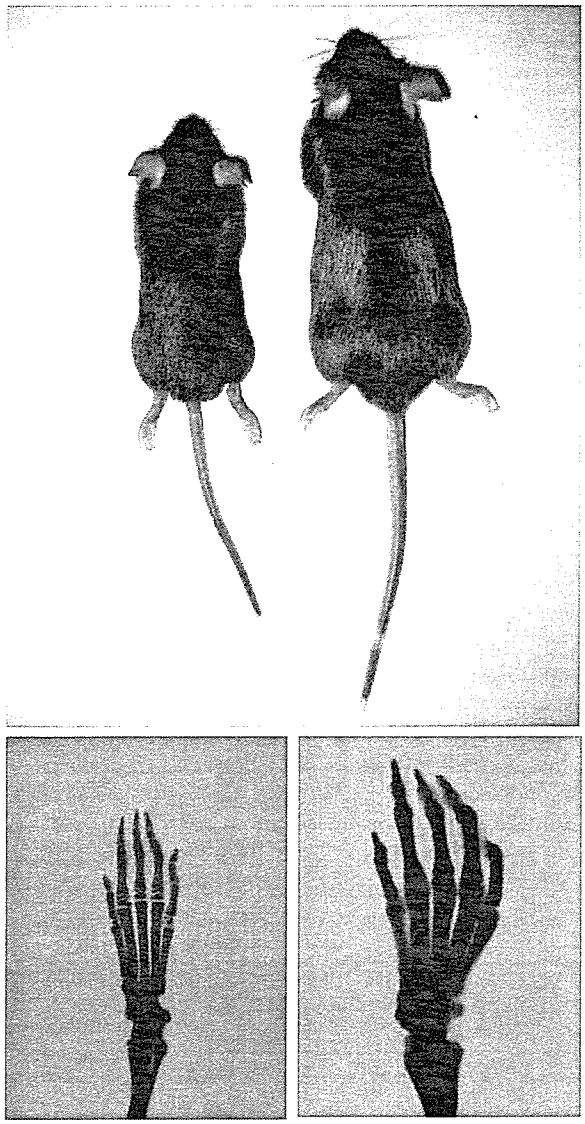


Fig. 1. A Dwarf (*left*) and a Normal (*right*) Mouse at 3 Months
The dwarf mouse is identified by the small body size. Eye opening of the dwarf mouse was delayed for a few days more than that (15 d old) of the normal mouse. Ossification activities in the digit joints of the dwarf mouse were lower than those of the normal.

Bone and spleen, known as target organs of the thyroid hormone, were observed. As shown in Fig. 1, no ossification was observed in dwarf mice compared with normal. Also, spleens of dwarf mice at 3 months after birth were significantly smaller than those of normal mice (Table 1).

Genetics and Chromosomal Mapping of the Dwarf Gene

Genetic crosses revealed that dwarfism was inherited in a Mendelian fashion as an autosomal recessive gene, because no dwarf mice were observed in F1 progeny. The ratio of dwarf and normal mice in the F2

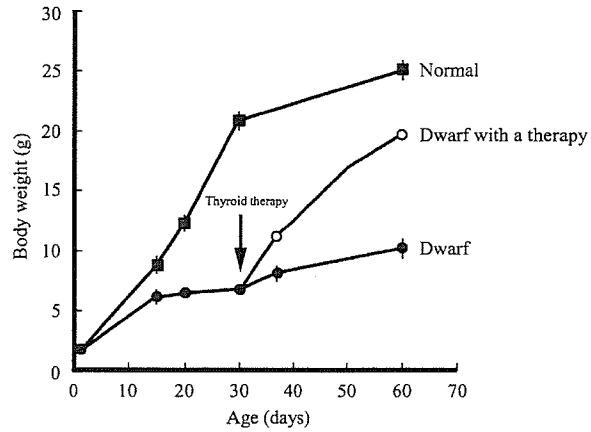


Fig. 2. Growth Curves of Dwarf, Normal, and Dwarf Mice with Thyroid Therapy

generation was 24:74 (1:3). To identify the gene that causes dwarfism, linkage analysis was performed using 24 dwarf mice of the F2 generation. A significant linkage was observed between the dwarf gene and the microsatellite markers on the proximal region approximately 13 cM of chromosome 12. As shown in Fig. 3, no recombination between the dwarf gene and *D12Mit136* marker close to the *Tpo* gene was observed.

Histology of the Thyroid Gland

Figure 4 shows typical thyroid glands of 3-month-old dwarf (A1 and A2) and normal mice (B1 and B2). Thyroid glands of the dwarf mouse displayed hypertrophy and diffuse goiter (A1) compared with thyroid glands of the normal mouse (B1). Weights of the thyroid glands of dwarf and normal mice were 45.0 ± 6.8 mg (n = 4) and 15.0 ± 2.0 mg (n = 5), respectively.

Thyroid follicles of the dwarf mouse showed diminished and disordered colloids, because of hyperproliferation and hypertrophy of the thyrofollicular cells (A2). No lymphoid infiltration was observed in the thyroid of the dwarf mouse.

Hematology and Endocrinology

Hematological data of dwarf and normal mice are shown in Table 1. Hematocrit values and the number of red blood cells of dwarf mice were significantly lower than those of normal mice.

Because dwarf mice showed a typical goiter, concentrations of three thyroid-related hormones (T_3, T_4, and TSH) in plasma were measured (Table 1). T_3 and T_4 were extremely reduced in dwarf mice. However, TSH of dwarf mice was approximately 20 times higher than that of normal mice. These results strongly suggested that TPO is an etiology of goitrous hypothyroidism.

TPO protein and TPO oxidation activity were measured by Western blotting and guaiacol assay, respec-

Table 1. Morphological, Hematological, and Endocrinological Values of the Normal and Dwarf Mice at 3 Months after Birth

Mice	Spleen (mg)	Hematocrit (%)	RBC ($\times 10^6$/ml)	T_3 (ng/ml)	T_4 (ng/ml)	TSH (ng/ml)	TPO Activity (mU/mg)
Dwarf	22.7 ± 1.8[a] (n = 4)	38.5 ± 1.6[a] (n = 7)	71.8 ± 3.6[a] (n = 7)	0.22 ± 0.02[a] (n = 5)	<10.00 (n = 5)	90.4 ± 19.5[a] (n = 8)	<2.0[a] (n = 3)
Normal	83.1 ± 7.6 (n = 5)	47.8 ± 1.4 (n = 7)	89.6 ± 3.4 (n = 7)	0.59±0.06 (n = 5)	17.1 ± 1.9 (n = 5)	4.8 ± 0.8 (n = 8)	164.5 ± 50 (n = 3)

RBC, Red blood cells.
[a] Mean values for the dwarf mice are significantly different from those of the normal mice by *t* test at $P < 0.01$.

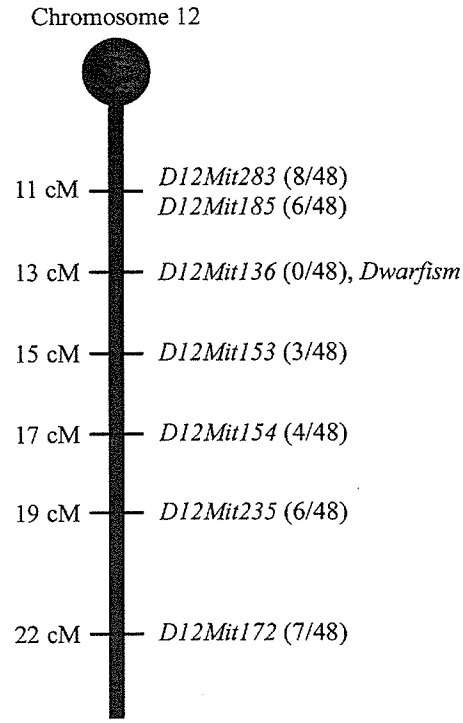


Fig. 3. Map Position of the Gene Responsible for a Novel Dwarfism on Chromosome 12

The number of recombinants between the responsible gene and the microsatellite markers observed in dwarf mice (n = 24) at the F2 generation is indicated.

tively. TPO protein was detected in the microsomal fraction of both mice (Fig. 5). TPO oxidation activity in the microsomal fraction of normal mice was detected, but that of dwarf mice was undetectable (Table 1).

Sequencing of the *Tpo* Gene

The whole genomic *Tpo* gene length including 18 exons is approximately 150 kb. mRNA of the *Tpo* gene is 3281 bp long (19). In this study, *Tpo* cDNA of dwarf and normal mice was amplified by RT-PCR, and nucleotide sequences were compared. There was no difference in the lengths of their fragments. Therefore, this suggests that there are no gross changes of nucleotide sequence in the mutant allele such as insertion or deletion.

Sequence analyses revealed that the *Tpo* gene of the dwarf mouse has a nucleotide change from C to T at position 1508 in exon 9. This missense mutation leads to an amino acid exchange from Arg to Cys at amino acid residue 479 (Fig. 6, *middle*). The male ICR no. 8 mouse, which is the founder, was heterozygous for the *Tpo* gene (Fig. 6, *right*).

Hormone Therapy in Dwarf Mice

Dwarf mice were given a thyroid powder-supplemented diet at 30 d after birth. After 1 wk, the body weight of dwarf mice was significantly increased, as

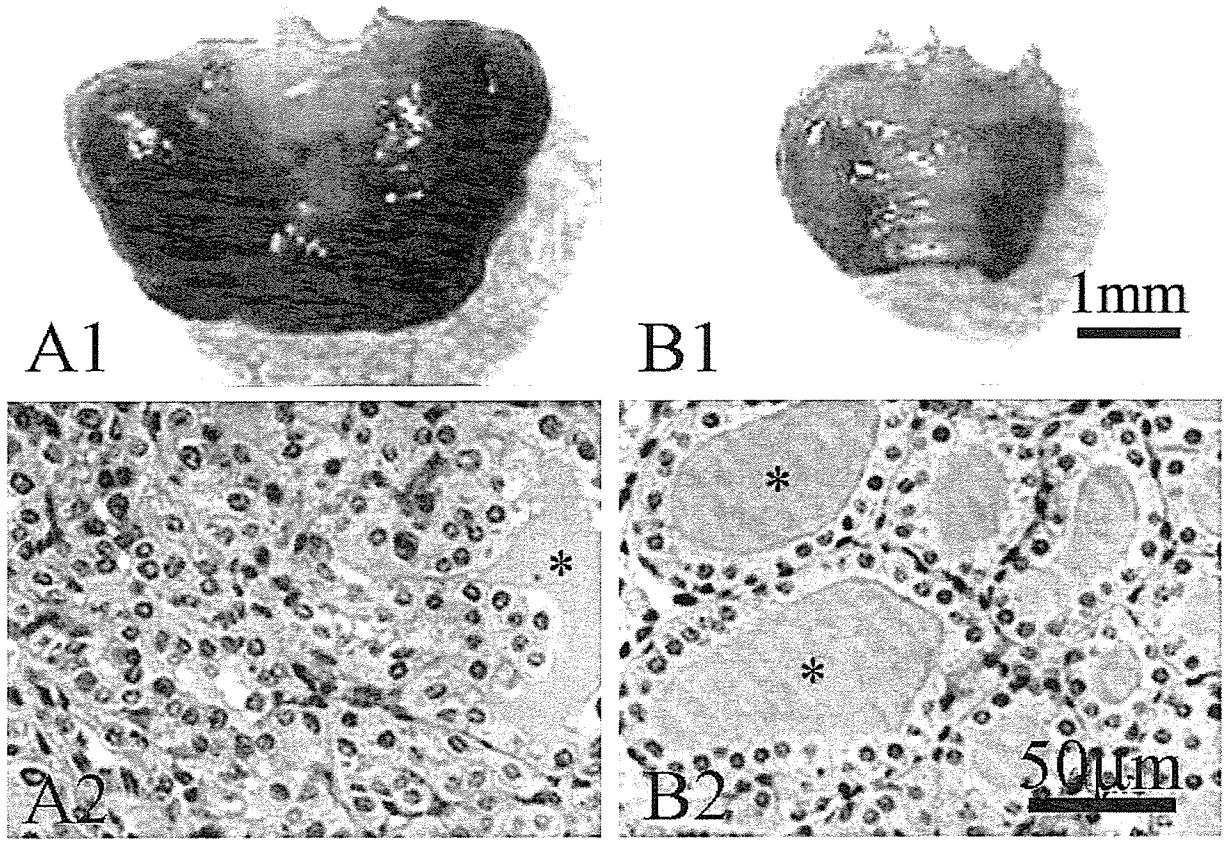


Fig. 4. Thyroid Glands of Dwarf (A1 and A2) and Normal Mice (B1 and B2)
Enlarged thyroid glands due to a diffuse goiter were observed in the dwarf mice. The *asterisks* show colloids.

shown in Fig. 2. Body weight of dwarf mice with the diet was 11.25 ± 0.06 g (n = 4), whereas that of untreated dwarf mice was significantly lower (7.03 ± 0.35 g, n = 3; $P < 0.01$). Body weights of untreated dwarf mice, treated dwarf mice, and normal mice, 30 d after the beginning of hormone therapy, were 10.73 ± 0.52 g (n = 4), 19.75 ± 0.06 g (n = 4), and 24.76 ± 0.35 g (n = 5), respectively. Improved dwarf mice showed normal fertility.

DISCUSSION

Flamant *et al.* (17) reported that *Pax8−/minus]* mice show congenital hypothyroid and that the development of bone, spleen, and brain, which are target organs of thyroid hormone, was not observed in these mice. We observed that our dwarf mice lacking T_4 and T_3 showed the same phenotypes in bone and spleen.

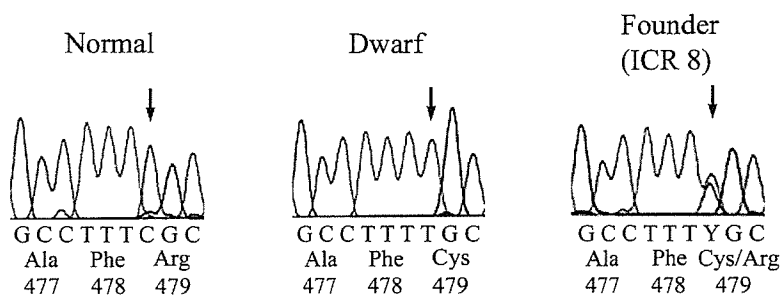


Fig. 5. Detection of TPO Protein in Thyroid Microsomal Fractions Using Western Blotting with Antihuman TPO Antibody
Lane 1, Positive control (Chinese hamster ovary-K1 cells transfected with human TPO cDNA); lane 2, the novel dwarf mouse; lane 3, control (normal mouse)

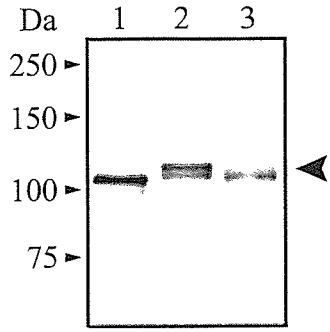


Fig. 6. Nucleotide Sequence (1502–1510) of the *Tpo* Gene in the Novel Dwarf Mouse, Normal Mice, and Their Founder (ICR no. 8)

Arrows in chromatograms show the nucleotide substitution of C to T at the codon.

TPO is a member of the peroxidase superfamily and a key protein in the biosynthesis of thyroid hormone. TPO, which is located on the apical membrane surface of the thyroid follicular cell, mainly catalyzes tyrosine iodination and the coupling of iodotyrosine on trans-Golgi network to form T_4 and T_3 (20). TPO functions as a membrane enzyme containing a heme protein, which is essential for the catalytic site. Ambrugger *et al.* (21) reported that exon 9 of the *TPO* gene plays an important role for the proper structure and function of the TPO enzyme in humans. Many missense mutations of exon 9 have been reported in human CH patients (21–23). The first CH mouse, reported in this study, had a missense Arg479Cys mutation of the *Tpo* gene. In the peroxidase superfamily, the amino acid sequence from Arg 479 to His 482 is completely conserved. A substitution from Arg to Cys at 479 could lead to a change of tertiary structure of TPO that does not bind heme and does not have enzyme activity. Generally, Cys plays an important role in the tertiary structure of protein through a disulfide bond. The major extracellular portion of the TPO molecule corresponds to amino acid residues 1–745 in humans and 1–733 in mice. The sequence from 473Phe to 484Thr in mice is conserved in humans, rats, pigs, and dogs. The codon Arg479 in mice corresponds to the codon Arg491 in humans. In human CH patients, a missense mutation at codon 491 (Arg491His) has been reported (21), and mutations in the coding region of the *TPO* gene have been also described (24–26).

Primary CH in the human is a well-documented syndrome that is a common endocrine disease in neonates and leads to reduced growth and mental retardation. Newborn screening data in humans revealed that 1 in 4000 neonates has CH and 15–20% of CH patients show functional disorders in hormone synthesis (27). In human CH patients, thyroid hormone therapy is given to improve their disease symptoms. We attempted this therapy using dwarf mice. As a result, dwarf mice gained almost normal body weight (80% of normal mice) and sexual maturity. This mutant mouse could be useful for future studies such as gene therapy and thyrocyte transplantation that should be undertaken for improvement of a patient's quality of life.

MATERIALS AND METHODS

Genetic Crosses and Gene Mapping

ICR, DBA/2JJcl, and C57BL/6JJcl mice were purchased from CLEA Japan (Tokyo, Japan). Genetic crosses were performed to find spontaneous recessive mutations existing in ICR closed colony mice. Dwarf mice were segregated from the normal mice in litters of backcrossing of F1 (DBA/2JJcl × ICR no. 8) × ICR no. 8.

Linkage analysis between the dwarf gene and microsatellite markers on autosomes was performed using the F2 progeny derived from the cross ICR no. 8 with C57BL/6JJcl. The F2 progeny were produced by intercrossing of F1 (C57BL/6JJcl × ICR no. 8) mice. Eighty-three markers on chromosome 1 to chromosome 19 showing genetic polymorphisms between ICR no.8 and C57BL/6JJcl were selected (Table 2). Microsatellite DNA markers were amplified by PCR followed by agarose gel electrophoresis. The procedures were described elsewhere (3). Primer sets for microsatellite markers were purchased from Invitrogen (Carlsbad, CA).

Anatomical and Histological Phenotyping

Body and tissue weights of dwarf and normal mice were measured using an electronic balance. Thyroid tissues were removed and fixed in Bouin's solution for 24 h. They were embedded in paraffin and were sectioned 5 μm in thickness. After staining in hematoxylin and eosin solution, histological diagnosis was performed.

The removed skin and all organs and tissues of dwarf and normal mice were fixed in 95% ethanol overnight and then stained by alcian blue followed by alizarin red. After destaining for 72 h in 1% KOH, samples were subsequently treated in 20%, 40%, 60%, and 80% (vol/vol) glycerol solutions prepared in 1% KOH for 48–72 h before soaking in 100% glycerol.

Measurement of Hormones

Heparinized blood samples were collected from 3-month-old dwarf and normal mice to measure plasma TSH and thyroid hormones. The plasma was stored at −30 C until use. Plasma levels of T_3, T_4, and TSH were measured using RIA kits purchased from Abbott Japan Corp. (Tokyo, Japan), Diagnostic Products Corp. (Los Angeles, CA) and Amersham Biosciences (Piscataway, NJ), respectively. TPO activity was measured using 30 mmol/liter Guaiacol and 0.1 mmol/liter $HB_{2B}OB_{2B}$ in 0.1 mol/liter potassium phosphate buffer (pH 7.4) at 30 C. Guaiacol oxidation of 1 μmol/min was defined as one unit (28).

Sequencing of the *Tpo* Gene

Entire coding regions corresponding to exons 2–18 of the *Tpo* gene of dwarf and normal mice were amplified using the five following primer pairs: Tpo-F1/R1, caaaggctggaaccctaa/tggacacagtagggttca; Tpo-F2/R2, tacaaccccactgtgaac/gcacaaagttcccattgtcc; Tpo-F3/R3, gccttccgtattggaaag/cacatgagatggaagctac; Tpo-F4/F4, cctcctgtgcgaatagaggt/gtgactggaccgtaacgaga; and Tpo-F8/R8, gctctagaatgagaacacttgg/gccgtggtataagaaattaggg. Nucleotide sequences of these primers were obtained from NCBI/GenBank accession no. X60703.

Table 2. Microsatellite Markers Used for Gene Mapping

Chromosome	cM	Markers	Chromosome	cM	Markers	Chromosome	cM	Markers
1	15.0	*D1Mit 211*	7	15.0	*D7Mit155*		28.0	*D12Mit190*
	34.8	*D1Mit303*		52.4	*D7Mit220*		29.0	*D12Mit33*
	43.1	*D1Mit46*	8	37.0	*D8Mit249*		37.0	*D12Mit14*
	64.1	*D1Mit217*		43.0	*D8Mit248*		37.0	*D12Mit157*
	96.3	*D1Mit36*	9	18.0	*D9Mit205*		50.0	*D12Mit277*
2	41.4	*D2Mit92*		28.0	*D9Mit229*		53.0	*D12Mit262*
	52.5	*D2Mit274*		61.0	*D9Mit212*	13	75.0	*D13Mit78*
	86.0	*D2Mit285*	10	44.0	*D10Mit42*	14	15.0	*D14Mit141*
	91.8	*D2Mit346*		51.0	*D10Mit95*		28.3	*D14Mit203*
3	0.0	*D3Mit60*	11	1.1	*D11Mit71*		44.4	*D14Mit194*
	45.8	*D3Mit100*		20.0	*D11Mit236*	15	23.0	*D15Mit184*
	64.1	*D3Mit110*	11	28.0	*D11Mit86*		54.5	*D15Mit171*
4	20.8	*D4Mit12*		50.0	*D11Mit212*	16	3.4	*D16Mit154*
	60.0	*D4Mit203*		59.5	*D11Mit99*		27.3	*D16Mit4*
	66.0	*D4Mit251*	12	11.0	*D12Mit185*		66.8	*D16Mit51*
5	54.0	*D5Mit10*		11.0	*D12Mit283*	17	6.5	*D17Mit113*
	68.0	*D5Mit95*		13.0	*D12Mit171*		17.7	*D17Mit175*
	72.0	*D5Mit30*		13.0	*D12Mit136*	18	57.0	*D18Mit4*
	81.0	*D5Mit101*		15.0	*D12Mit153*	19	6.0	*D19Mit68*
6	15.0	*D6Mit86*		17.0	*D12Mit154*		41.0	*D19Mit89*
	35.2	*D6Mit8*		19.0	*D12Mit235*		47.0	*D19Mit91*
	74.0	*D6Mit15*		22.0	*D12Mit172*			

Marker's position was taken from the Mouse Genome database.

Nucleotide sequences were determined by the dideoxy chain terminating method with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and then applied to an automated DNA sequencer ABI PRISM 3100 (Applied Biosystems).

Measurement of TPO Activity in Thyroid Tissue

Microsomal and supernatant fractions of thyroid tissues of 3-month-old dwarf and normal mice were prepared by the method of Hosoya and Morrison (29) as modified by Nakagawa *et al.* (30). Concentration of protein in the fractions was measured using the Bradford method with bovine serum as a standard (31).

Western Blots

Thyroid microsomes and Chinese hamster ovary-K1 cells expressing recombinant human TPO (32) were electrophoresed using a 7.5% sodium dodecyl sulfate polyacrylamide gel. The gel was transferred to a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences). After blocking with 5% skim milk, the membrane was incubated in a solution containing affinity-purified rabbit antihuman TPO antibody (5 μg/ml) and then reacted with 1:5000 diluted antirabbit IgG antibody conjugated with alkaline phosphatase (Promega Corp., Madison, WI) (33). Antihuman TPO antibody (primary antibody) showed cross-reaction with murine and porcine TPOs (data not shown).

Thyroid Hormone Therapy

To observe effects of thyroid hormone on dwarf mice, the animals were given a diet containing 0.01% thyroid powder (Sigma-Aldrich, St. Louis, MO) for 30 d after weaning according to the method of Beamers *et al.* (9, 10). Body weights were recorded for 30 d after the beginning of the therapy.

Acknowledgments

We thank Jiro Kimura for technical assistance.

Received March 1, 2006. Accepted May 30, 2006.

Address all correspondence and requests for reprints to: Hideki Katoh, Institute for Experimental Animals, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan. E-mail: Hideki-k@hama-med.ac.jp.

This work was partially supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Young Scientists (B), no. 16700347, 2004–2005.

Disclosure statement: The authors have nothing to disclose.

REFERENCES

1. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y 1980 Breeding of a non-obese, diabetic strain of mice. Jikken Dobutsu 29:1–13
2. Katoh H, Endo F, Suzuki K, Matsuda I 1991 Hereditary hypertyrosinemia mouse. Mouse Genome 89:572
3. Takabayashi T, Katoh H 2005 A mutant mouse with severe anemia and skin abnormalities controlled by a new allele of the flaky skin (*fsn*) locus. Exp Anim 54: 339–347
4. Snell GD 1929 Dwarf, a new Mendelian recessive character of the house mouse. Proc Natl Acad Sci USA 15: 733–734
5. Eicher EM, Beamer WG 1980 New mouse dw allele: genetic location and effects on lifespan and growth hormone levels. J Hered 71:187–190
6. Schaible RH, Gowen JW 1961 df– Ames dwarf. Mouse News Lett 25:35
7. Buckwalter MS, Katz RW, Camper SA 1991 Localization of the panhypopituitary dwarf mutation (*df*) on mouse

chromosome 11 in an intersubspecific backcross. Genomics 10:515–526
8. Eicher EM, Beamer WG 1976 Inherited ateliotic dwarfism in mice: characteristics of the mutation, little, on chromosome 6. J Hered 67:87–91
9. Beamer WJ, Eicher EM, Maltais LJ, Southard JL 1981 Inherited primary hypothyroidism in mice. Science 212: 61–63
10. Beamer WG, Maltais LJ, DeBeats MH, Eicher EM 1987 Inherited congenital goiter in mice. Endocrinology 120: 838–840
11. Yoshida T, Yamanaka K, Atsumi S, Tsumura H, Sasaki R, Tomita K, Ishikawa E, Ozawa H, Watanabe K, Totsuka T 1994 A novel hypothyroid 'growth-retarded' mouse derived from Snell's dwarf mouse. J Endocrinol 142: 435–446
12. Agui T, Miyamoto T, Tsumura H, Yoshida T 1997 Mapping of the *grt* locus to mouse chromosome 5. Mamm Genome 18:944
13. Camper SA, Saunders TL, Katz RW, Reeves RH 1990 The Pit-1 transcription factor gene is a candidate for the murine Snell dwarf mutation. Genomics 8:586–590
14. Ingraham HA, Albert VR, Chen R, Crenshaw III EB, Elsholtz HP, He X, Kapiloff MS, Mangalam HJ, Swanson LW, Treacy MN, Rosenfeld MG 1990 A family of POU-domain and Pit-1 tissue-specific transcription factors in pituitary and neuroendocrine development. Annu Rev Physiol 52:773–791
15. Karin M, Theill L, Castrillo J-L, McCormic A, Brady H 1990 Tissuespecific expression of the growth hormone gene and its control by growth hormone factor-1. Recent Prog Horm Res 46:43–58
16. Lin SC, Lin CR, Gukovsky I, Lusis AJ, Sawchenko PE, Rosenfeld MG 1993 Molecular basis of the little mouse phenotype and implications for cell type-specific growth. Nature 364:208–213
17. Flamant F, Poguet A-L, Plateroti M, Chassande O, Gauthier K, Streichenberger N, Mansouri A, Samarut J 2002 Congenital hypothyroid. Pax8(−/−) mutant mice can be rescued by inactivating the TRα gene. Mol Endocrinol 16:24–32
18. Taylor BA, Reifsnyder PC, DiLauro R 1992 Linkage of the thyroid peroxidase locus (*Tpo*) to markers in the proximal part of chromosome 12 of the mouse. Cytogenet Cell Genet 60:250–251
19. Kotani T, Umeki K, Yamamoto I, Takeuchi M, Takechi S, Nakayama T, Ohtaki S 1993 Nucleotide sequence of the cDNA encoding mouse thyroid peroxidase. Gene 123: 289–290
20. Ohtaki S, Nakagawa H, Nakamura M, Kotani T 1996 Thyroid peroxidase: experimental and clinical integration. Endocr J 43:1–14
21. Ambrugger P, Stoeva I, Biebermann H, Torresani T, Leitner C, Gruters A 2001 Novel mutations of the thyroid peroxidase gene in patients with permanent congenital hypothyroidism. Eur J Endocrinol. 145:19–24
22. Bakker B, Bikker H, Vulsma T, de Randamie JS, Wiedijk BM, De Vijlder JJ 2000 Two decades of screening for congenital hypothyroidism in The Netherlands: TPO gene mutations in total iodide organification defects. J Clin Endocrinol Metab 85:3708–3712
23. Wu JY, Shu SG, Yang CF, Lee CC, Tsai FJ 2002 Mutation analysis of thyroid peroxidase gene in Chinese patients with total iodide organification defect: identification of five novel mutations. J Endocrinol 172:627–635
24. Mangklabruks A, Billerbeck AE, Wajchenberg B, Knobel M, Cox NJ, DeGroot LJ, Medeiros-Neto G 1991 Genetic linkage studies of thyroid peroxidase (TPO) gene in families with TPO deficiency. J Clin Endocrinol Metab 72: 471–476
25. Kotani T, Umeki K, Yamamoto I, Maesaka H, Tachibana K, Ohtaki S 1999 A novel mutation in the human thyroid peroxidase gene resulting in a total iodide organification defect. J Endocrinol 160:267–273
26. Santos CL, Bikker H, Rego KG, Nascimento AC, Tambascia M, De Vijlder JJ, Medeiros-Neto G 1999 A novel mutation in the TPO gene in goitrous hypothyroid patients with iodide organification defect. Clin Endocrinol (Oxf) 51:165–172
27. Gruters A 1992 Congenital hypothyroidism. Pediatr Ann 21:15, 18–21:24–28
28. Hosoya T 1960 Turnip peroxidase. II. The reaction mechanisms of turnip peroxidase A1, A2 and D. J Biochem 47:794
29. Hosoya T, Morrison M 1967 A study of the hemoprotein of thyroid microsomes with emphasis on the thyroid peroxidase. Biochemistry 6:1021–1026
30. Nakagawa H, Kotani T, Ohtaki S, Kawano J, Aikawa E, Imagawa M, Hashida S, Ishikawa E 1985 Characterization of a monoclonal antibody to hog thyroid peroxidase and its use for immuno-histochemical localization of the peroxidase in the thyroid gland. J Biochem 97: 1709–1718
31. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
32. Hata J, Yamashita S, Yagihashi S, Kato H, Kabeno S, Hirai K, Kuma K, Kimura S, Umeki K, Kotani T, Ohtaki S 1989 Stable high level expression of human thyroid peroxidase in cultured Chinese hamster ovary cells. Biochem Biophys Res Commun 164:1268–1273
33. Umeki K, Kotani T, Kawano J, Suganuma T, Yamamoto I, Aratake Y, Furujo M, Ichiba Y 2002 Two novel missense mutations in the thyroid peroxidase gene, R665W and G771R, result in a localization defect and cause congenital hypothyroidism. Eur J Endocrinol 146:491–498

Molecular Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

0888-8809/06/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 20(10):2584–2590

doi: 10.1210/me.2006-0099

A Novel Hypothyroid Dwarfism Due to the Missense Mutation Arg479Cys of the Thyroid Peroxidase Gene in the Mouse

Shuji Takabayashi, Kazumi Umeki, Etsuko Yamamoto, Tohru Suzuki, Akihiko Okayama, and Hideki Katoh

Institute for Experimental Animals (S.T., E.Y., H.K.), and Department of Parasitology (T.S.), Hamamatsu University School of Medicine, Hamamatsu, Shizuoka 431-3192, Japan; and Clinical Laboratory (K.U.), University of Miyazaki Hospital, and Department of Rheumatology, Infectious Diseases and Laboratory Medicine, Faculty of Medicine (A.O.), University of Miyazaki, Kihara 5200, Kiyotake, Miyazaki 889-1692, Japan

Recently, we found a novel dwarf mutation in an ICR closed colony. This mutation was governed by a single autosomal recessive gene. In novel dwarf mice, plasma levels of the thyroid hormones, T_3 and T_4, were reduced; however, TSH was elevated. Their thyroid glands showed a diffuse goiter exhibiting colloid deficiency and abnormal follicle epithelium. The dwarfism was improved by adding thyroid hormone in the diet. Gene mapping revealed that the dwarf mutation was closely linked to the thyroid peroxidase (*Tpo*) gene on chromosome 12. Sequencing of the *Tpo* gene of the dwarf mice demonstrated a C to T substitution at position 1508 causing an amino acid change from arginine (Arg) to cysteine (Cys) at codon 479 (Arg479Cys). Western blotting revealed that TPO protein of the dwarf mice was detected in a microsomal fraction of thyroid tissue, but peroxidase activity was not detected. These findings suggested that the dwarf mutation caused a primary congenital hypothyroidism by TPO deficiency, resulting in a defect of thyroid hormone synthesis. (*Molecular Endocrinology* 20: 2584–2590, 2006)

MANY SPONTANEOUS MUTATIONS have been found in inbred strains derived from an ICR closed colony of mice (1–3). Recently, we found that a Jcl:ICR male mouse had an autosomal recessive mutation in our colony, because some of the backcross mice obtained using a DBA/2JJcl female mouse showed dwarfism. Dwarf mice were noticeably smaller than their normal littermates and showed primary congenital hypothyroidism (CH) with goiter.

Six autosomal recessive mutations related to dwarfism have been found in mice, *e.g. dw* [dwarf, chromosome 16, 43.5 centimorgans (cM)] (4, 5), *df* (chromosome 11, 25.0 cM) (6, 7), *lit* (little, chromosome 6, 26.0 cM) (8), *hyt* (hypothyroid, chromosome 12, 37.0 cM) (9), *cog* (congenital goiter, chromosome 15, 36.4 cM) (10), and *grt* (growth-retarded, chromosome 5, 59.0 cM) (11, 12). The *dw* and *df* mutations are a primary hypopituitarism, which does not produce prolactin, TSH, and GH (13–15). The *lit* mutation has a GH deficiency (16). In contrast, the *hyt*, *cog*, and *grt* mutations have primary CH, which show low thyroid hormone (T_3 and T_4) and elevated plasma TSH levels. Recently, *Pax8*−/− created using a homologous recombination technique showed congenital hypothyroidism (17).

In this study, we found a novel dwarf mutant mouse that has different characters from other dwarf mutations. We showed that the responsible gene in our novel dwarf mice was mapped on a position close to the thyroid peroxidase (*Tpo*) gene on chromosome 12 (18) and that sequence analysis demonstrated a missense mutation, Arg479Cys, of the *Tpo* gene. To date, dwarfisms caused by any mutation of the *Tpo* gene have not been reported in mice. The mouse dwarfism reported in this study is the first one to be discovered that is caused by *Tpo* gene mutation. We also showed hormone therapy improved phenotypes of the dwarfism.

RESULTS

Phenotype of Dwarf Mice

Typical phenotypes of adult dwarf mice were characterized by a short trunk and prominent forehead and eyes (Fig. 1). As shown in Fig. 2, dwarf mice (6.46 ± 0.36 g; n = 8) at 15 d after birth were significantly smaller than the normal littermates (8.95 ± 0.32 g; n = 6; $P < 0.01$). At 30 d after birth, dwarf mice (6.54 ± 0.25 g) were extremely small compared with the normal littermates (20.96 ± 0.68 g; $P < 0.001$). Their mean life span was approximately 3 months.

First Published Online June 8, 2006

Abbreviations: CH, Congenital hypothyroidism; TPO, thyroid peroxidase.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

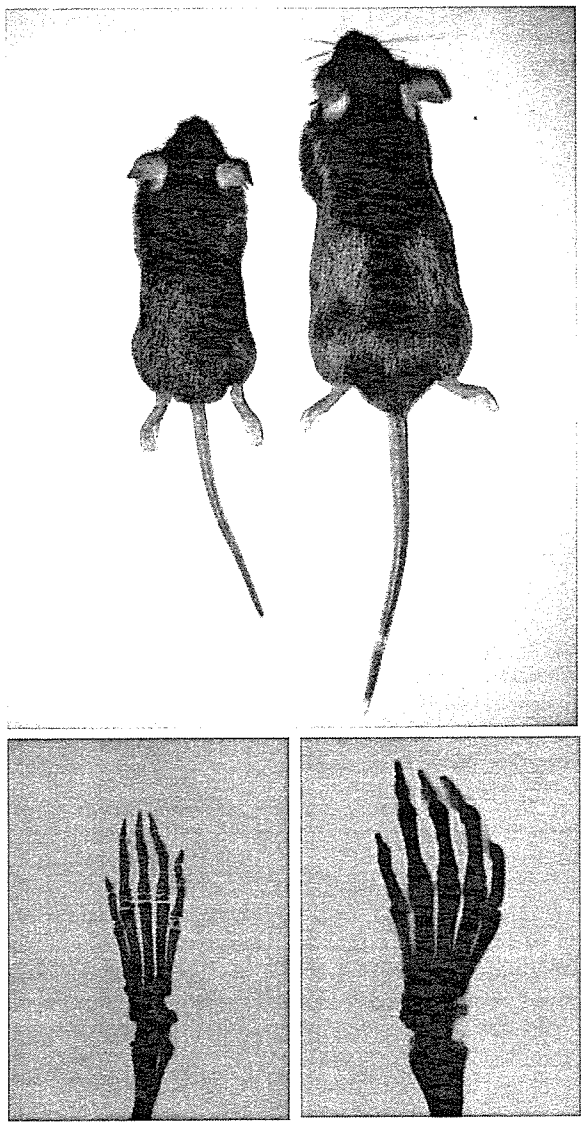


Fig. 1. A Dwarf (*left*) and a Normal (*right*) Mouse at 3 Months
The dwarf mouse is identified by the small body size. Eye opening of the dwarf mouse was delayed for a few days more than that (15 d old) of the normal mouse. Ossification activities in the digit joints of the dwarf mouse were lower than those of the normal.

Bone and spleen, known as target organs of the thyroid hormone, were observed. As shown in Fig. 1, no ossification was observed in dwarf mice compared with normal. Also, spleens of dwarf mice at 3 months after birth were significantly smaller than those of normal mice (Table 1).

Genetics and Chromosomal Mapping of the Dwarf Gene

Genetic crosses revealed that dwarfism was inherited in a Mendelian fashion as an autosomal recessive gene, because no dwarf mice were observed in F1 progeny. The ratio of dwarf and normal mice in the F2

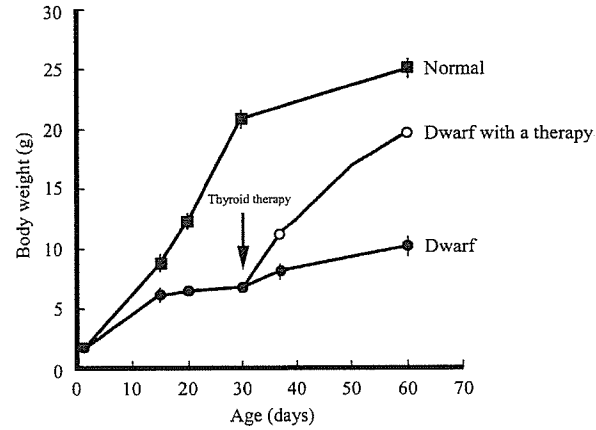


Fig. 2. Growth Curves of Dwarf, Normal, and Dwarf Mice with Thyroid Therapy

generation was 24:74 (1:3). To identify the gene that causes dwarfism, linkage analysis was performed using 24 dwarf mice of the F2 generation. A significant linkage was observed between the dwarf gene and the microsatellite markers on the proximal region approximately 13 cM of chromosome 12. As shown in Fig. 3, no recombination between the dwarf gene and *D12Mit136* marker close to the *Tpo* gene was observed.

Histology of the Thyroid Gland

Figure 4 shows typical thyroid glands of 3-month-old dwarf (A1 and A2) and normal mice (B1 and B2). Thyroid glands of the dwarf mouse displayed hypertrophy and diffuse goiter (A1) compared with thyroid glands of the normal mouse (B1). Weights of the thyroid glands of dwarf and normal mice were 45.0 ± 6.8 mg (n = 4) and 15.0 ± 2.0 mg (n = 5), respectively.

Thyroid follicles of the dwarf mouse showed diminished and disordered colloids, because of hyperproliferation and hypertrophy of the thyrofollicular cells (A2). No lymphoid infiltration was observed in the thyroid of the dwarf mouse.

Hematology and Endocrinology

Hematological data of dwarf and normal mice are shown in Table 1. Hematocrit values and the number of red blood cells of dwarf mice were significantly lower than those of normal mice.

Because dwarf mice showed a typical goiter, concentrations of three thyroid-related hormones (T_3, T_4, and TSH) in plasma were measured (Table 1). T_3 and T_4 were extremely reduced in dwarf mice. However, TSH of dwarf mice was approximately 20 times higher than that of normal mice. These results strongly suggested that TPO is an etiology of goitrous hypothyroidism.

TPO protein and TPO oxidation activity were measured by Western blotting and guaiacol assay, respec-

Table 1. Morphological, Hematological, and Endocrinological Values of the Normal and Dwarf Mice at 3 Months after Birth

Mice	Spleen (mg)	Hematocrit (%)	RBC ($\times 10^6$/ml)	T_3 (ng/ml)	T_4 (ng/ml)	TSH (ng/ml)	TPO Activity (mU/mg)
Dwarf	22.7 ± 1.8[a] (n = 4)	38.5 ± 1.6[a] (n = 7)	71.8 ± 3.6[a] (n = 7)	0.22 ± 0.02[a] (n = 5)	<10.00 (n = 5)	90.4 ± 19.5[a] (n = 8)	<2.0[a] (n = 3)
Normal	83.1 ± 7.6 (n = 5)	47.8 ± 1.4 (n = 7)	89.6 ± 3.4 (n = 7)	0.59±0.06 (n = 5)	17.1 ± 1.9 (n = 5)	4.8 ± 0.8 (n = 8)	164.5 ± 50 (n = 3)

RBC, Red blood cells.
[a] Mean values for the dwarf mice are significantly different from those of the normal mice by *t* test at $P < 0.01$.

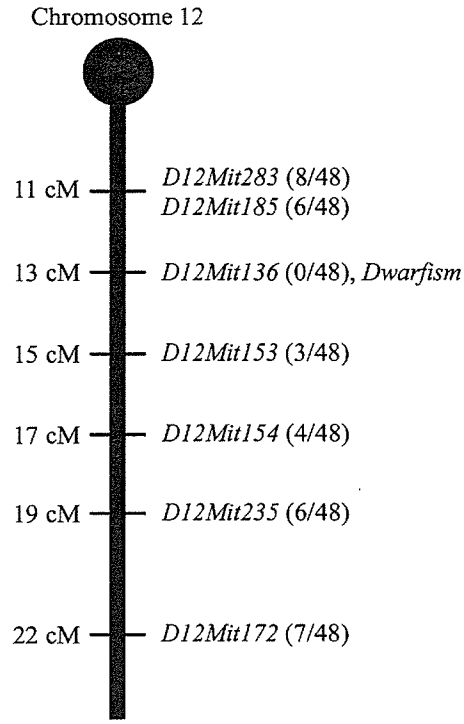


Fig. 3. Map Position of the Gene Responsible for a Novel Dwarfism on Chromosome 12

The number of recombinants between the responsible gene and the microsatellite markers observed in dwarf mice (n = 24) at the F2 generation is indicated.

tively. TPO protein was detected in the microsomal fraction of both mice (Fig. 5). TPO oxidation activity in the microsomal fraction of normal mice was detected, but that of dwarf mice was undetectable (Table 1).

Sequencing of the *Tpo* Gene

The whole genomic *Tpo* gene length including 18 exons is approximately 150 kb. mRNA of the *Tpo* gene is 3281 bp long (19). In this study, *Tpo* cDNA of dwarf and normal mice was amplified by RT-PCR, and nucleotide sequences were compared. There was no difference in the lengths of their fragments. Therefore, this suggests that there are no gross changes of nucleotide sequence in the mutant allele such as insertion or deletion.

Sequence analyses revealed that the *Tpo* gene of the dwarf mouse has a nucleotide change from C to T at position 1508 in exon 9. This missense mutation leads to an amino acid exchange from Arg to Cys at amino acid residue 479 (Fig. 6, *middle*). The male ICR no. 8 mouse, which is the founder, was heterozygous for the *Tpo* gene (Fig. 6, *right*).

Hormone Therapy in Dwarf Mice

Dwarf mice were given a thyroid powder-supplemented diet at 30 d after birth. After 1 wk, the body weight of dwarf mice was significantly increased, as

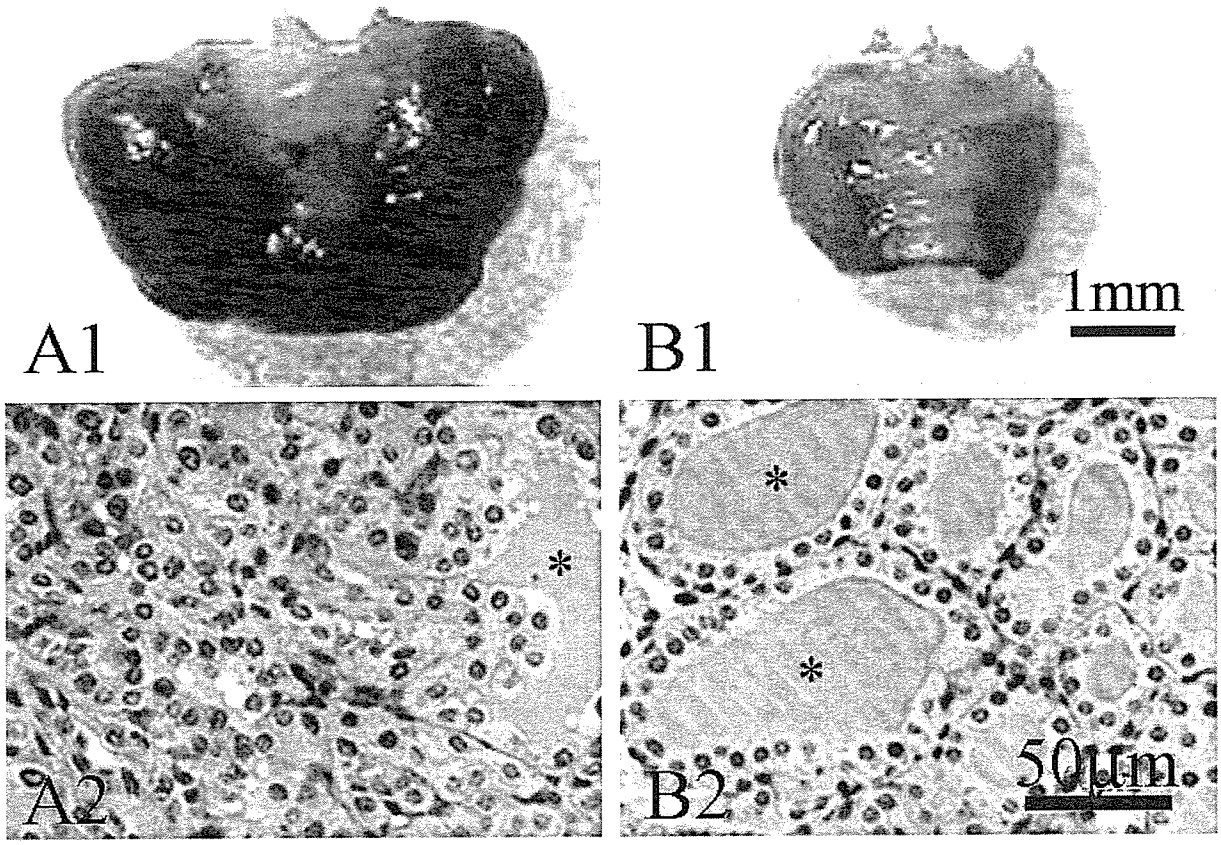


Fig. 4. Thyroid Glands of Dwarf (A1 and A2) and Normal Mice (B1 and B2)
Enlarged thyroid glands due to a diffuse goiter were observed in the dwarf mice. The *asterisks* show colloids.

shown in Fig. 2. Body weight of dwarf mice with the diet was 11.25 ± 0.06 g (n = 4), whereas that of untreated dwarf mice was significantly lower (7.03 ± 0.35 g, n = 3; $P < 0.01$). Body weights of untreated dwarf mice, treated dwarf mice, and normal mice, 30 d after the beginning of hormone therapy, were 10.73 ± 0.52 g (n = 4), 19.75 ± 0.06 g (n = 4), and 24.76 ± 0.35 g (n = 5), respectively. Improved dwarf mice showed normal fertility.

DISCUSSION

Flamant *et al.* (17) reported that *Pax8*−/minus] mice show congenital hypothyroid and that the development of bone, spleen, and brain, which are target organs of thyroid hormone, was not observed in these mice. We observed that our dwarf mice lacking T_4 and T_3 showed the same phenotypes in bone and spleen.

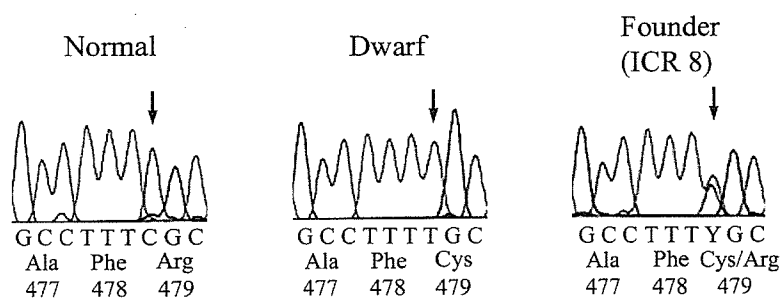


Fig. 5. Detection of TPO Protein in Thyroid Microsomal Fractions Using Western Blotting with Antihuman TPO Antibody
Lane 1, Positive control (Chinese hamster ovary-K1 cells transfected with human TPO cDNA); lane 2, the novel dwarf mouse; lane 3, control (normal mouse)

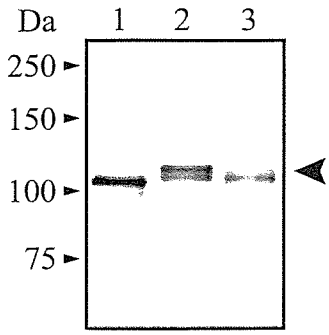


Fig. 6. Nucleotide Sequence (1502–1510) of the *Tpo* Gene in the Novel Dwarf Mouse, Normal Mice, and Their Founder (ICR no. 8)

Arrows in chromatograms show the nucleotide substitution of C to T at the codon.

TPO is a member of the peroxidase superfamily and a key protein in the biosynthesis of thyroid hormone. TPO, which is located on the apical membrane surface of the thyroid follicular cell, mainly catalyzes tyrosine iodination and the coupling of iodotyrosine on trans-Golgi network to form T_4 and T_3 (20). TPO functions as a membrane enzyme containing a heme protein, which is essential for the catalytic site. Ambrugger *et al.* (21) reported that exon 9 of the *TPO* gene plays an important role for the proper structure and function of the TPO enzyme in humans. Many missense mutations of exon 9 have been reported in human CH patients (21–23). The first CH mouse, reported in this study, had a missense Arg479Cys mutation of the *Tpo* gene. In the peroxidase superfamily, the amino acid sequence from Arg 479 to His 482 is completely conserved. A substitution from Arg to Cys at 479 could lead to a change of tertiary structure of TPO that does not bind heme and does not have enzyme activity. Generally, Cys plays an important role in the tertiary structure of protein through a disulfide bond. The major extracellular portion of the TPO molecule corresponds to amino acid residues 1–745 in humans and 1–733 in mice. The sequence from 473Phe to 484Thr in mice is conserved in humans, rats, pigs, and dogs. The codon Arg479 in mice corresponds to the codon Arg491 in humans. In human CH patients, a missense mutation at codon 491 (Arg491His) has been reported (21), and mutations in the coding region of the *TPO* gene have been also described (24–26).

Primary CH in the human is a well-documented syndrome that is a common endocrine disease in neonates and leads to reduced growth and mental retardation. Newborn screening data in humans revealed that 1 in 4000 neonates has CH and 15–20% of CH patients show functional disorders in hormone synthesis (27). In human CH patients, thyroid hormone therapy is given to improve their disease symptoms. We attempted this therapy using dwarf mice. As a result, dwarf mice gained almost normal body weight (80% of normal mice) and sexual maturity. This mutant mouse could be useful for future studies such as gene therapy and thyrocyte transplantation that should be undertaken for improvement of a patient's quality of life.

MATERIALS AND METHODS

Genetic Crosses and Gene Mapping

ICR, DBA/2JJcl, and C57BL/6JJcl mice were purchased from CLEA Japan (Tokyo, Japan). Genetic crosses were performed to find spontaneous recessive mutations existing in ICR closed colony mice. Dwarf mice were segregated from the normal mice in litters of backcrossing of F1 (DBA/2JJcl × ICR no. 8) × ICR no. 8.

Linkage analysis between the dwarf gene and microsatellite markers on autosomes was performed using the F2 progeny derived from the cross ICR no. 8 with C57BL/6JJcl. The F2 progeny were produced by intercrossing of F1 (C57BL/6JJcl × ICR no. 8) mice. Eighty-three markers on chromosome 1 to chromosome 19 showing genetic polymorphisms between ICR no.8 and C57BL/6JJcl were selected (Table 2). Microsatellite DNA markers were amplified by PCR followed by agarose gel electrophoresis. The procedures were described elsewhere (3). Primer sets for microsatellite markers were purchased from Invitrogen (Carlsbad, CA).

Anatomical and Histological Phenotyping

Body and tissue weights of dwarf and normal mice were measured using an electronic balance. Thyroid tissues were removed and fixed in Bouin's solution for 24 h. They were embedded in paraffin and were sectioned 5 μm in thickness. After staining in hematoxylin and eosin solution, histological diagnosis was performed.

The removed skin and all organs and tissues of dwarf and normal mice were fixed in 95% ethanol overnight and then stained by alcian blue followed by alizarin red. After destaining for 72 h in 1% KOH, samples were subsequently treated in 20%, 40%, 60%, and 80% (vol/vol) glycerol solutions prepared in 1% KOH for 48–72 h before soaking in 100% glycerol.

Measurement of Hormones

Heparinized blood samples were collected from 3-month-old dwarf and normal mice to measure plasma TSH and thyroid hormones. The plasma was stored at −30 C until use. Plasma levels of T_3, T_4, and TSH were measured using RIA kits purchased from Abbott Japan Corp. (Tokyo, Japan), Diagnostic Products Corp. (Los Angeles, CA) and Amersham Biosciences (Piscataway, NJ), respectively. TPO activity was measured using 30 mmol/liter Guaiacol and 0.1 mmol/liter $HB_{2B}OB_{2B}$ in 0.1 mol/liter potassium phosphate buffer (pH 7.4) at 30 C. Guaiacol oxidation of 1 μmol/min was defined as one unit (28).

Sequencing of the *Tpo* Gene

Entire coding regions corresponding to exons 2–18 of the *Tpo* gene of dwarf and normal mice were amplified using the five following primer pairs: Tpo-F1/R1, caaaggctggaaccctaa/tggacacagtagggttca; Tpo-F2/R2, tacaaccccactgtgaac/gcacaaagttcccattgtcc; Tpo-F3/R3, gccttccgtattggaaag/cacatgagatggaagctac; Tpo-F4/F4, cctcctgtgcgaatagaggt/gtgactggaccgtaacgaga; and Tpo-F8/R8, gctctagaatgagaacacttgg/gccgtggtataagaaattaggg. Nucleotide sequences of these primers were obtained from NCBI/GenBank accession no. X60703.

Table 2. Microsatellite Markers Used for Gene Mapping

Chromosome	cM	Markers	Chromosome	cM	Markers	Chromosome	cM	Markers
1	15.0	*D1Mit 211*	7	15.0	*D7Mit155*		28.0	*D12Mit190*
	34.8	*D1Mit303*		52.4	*D7Mit220*		29.0	*D12Mit33*
	43.1	*D1Mit46*	8	37.0	*D8Mit249*		37.0	*D12Mit14*
	64.1	*D1Mit217*		43.0	*D8Mit248*		37.0	*D12Mit157*
	96.3	*D1Mit36*	9	18.0	*D9Mit205*		50.0	*D12Mit277*
2	41.4	*D2Mit92*		28.0	*D9Mit229*		53.0	*D12Mit262*
	52.5	*D2Mit274*		61.0	*D9Mit212*	13	75.0	*D13Mit78*
	86.0	*D2Mit285*	10	44.0	*D10Mit42*	14	15.0	*D14Mit141*
	91.8	*D2Mit346*		51.0	*D10Mit95*		28.3	*D14Mit203*
3	0.0	*D3Mit60*	11	1.1	*D11Mit71*		44.4	*D14Mit194*
	45.8	*D3Mit100*		20.0	*D11Mit236*	15	23.0	*D15Mit184*
	64.1	*D3Mit110*	11	28.0	*D11Mit86*		54.5	*D15Mit171*
4	20.8	*D4Mit12*		50.0	*D11Mit212*	16	3.4	*D16Mit154*
	60.0	*D4Mit203*		59.5	*D11Mit99*		27.3	*D16Mit4*
	66.0	*D4Mit251*	12	11.0	*D12Mit185*		66.8	*D16Mit51*
5	54.0	*D5Mit10*		11.0	*D12Mit283*	17	6.5	*D17Mit113*
	68.0	*D5Mit95*		13.0	*D12Mit171*		17.7	*D17Mit175*
	72.0	*D5Mit30*		13.0	*D12Mit136*	18	57.0	*D18Mit4*
	81.0	*D5Mit101*		15.0	*D12Mit153*	19	6.0	*D19Mit68*
6	15.0	*D6Mit86*		17.0	*D12Mit154*		41.0	*D19Mit89*
	35.2	*D6Mit8*		19.0	*D12Mit235*		47.0	*D19Mit91*
	74.0	*D6Mit15*		22.0	*D12Mit172*			

Marker's position was taken from the Mouse Genome database.

Nucleotide sequences were determined by the dideoxy chain terminating method with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and then applied to an automated DNA sequencer ABI PRISM 3100 (Applied Biosystems).

Measurement of TPO Activity in Thyroid Tissue

Microsomal and supernatant fractions of thyroid tissues of 3-month-old dwarf and normal mice were prepared by the method of Hosoya and Morrison (29) as modified by Nakagawa *et al.* (30). Concentration of protein in the fractions was measured using the Bradford method with bovine serum as a standard (31).

Western Blots

Thyroid microsomes and Chinese hamster ovary-K1 cells expressing recombinant human TPO (32) were electrophoresed using a 7.5% sodium dodecyl sulfate polyacrylamide gel. The gel was transferred to a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences). After blocking with 5% skim milk, the membrane was incubated in a solution containing affinity-purified rabbit antihuman TPO antibody (5 μg/ml) and then reacted with 1:5000 diluted antirabbit IgG antibody conjugated with alkaline phosphatase (Promega Corp., Madison, WI) (33). Antihuman TPO antibody (primary antibody) showed cross-reaction with murine and porcine TPOs (data not shown).

Thyroid Hormone Therapy

To observe effects of thyroid hormone on dwarf mice, the animals were given a diet containing 0.01% thyroid powder (Sigma-Aldrich, St. Louis, MO) for 30 d after weaning according to the method of Beamers *et al.* (9, 10). Body weights were recorded for 30 d after the beginning of the therapy.

Acknowledgments

We thank Jiro Kimura for technical assistance.

Received March 1, 2006. Accepted May 30, 2006.

Address all correspondence and requests for reprints to: Hideki Katoh, Institute for Experimental Animals, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan. E-mail: Hideki-k@hama-med.ac.jp.

This work was partially supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Young Scientists (B), no. 16700347, 2004–2005.

Disclosure statement: The authors have nothing to disclose.

REFERENCES

1. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y 1980 Breeding of a non-obese, diabetic strain of mice. Jikken Dobutsu 29:1–13
2. Katoh H, Endo F, Suzuki K, Matsuda I 1991 Hereditary hypertyrosinemia mouse. Mouse Genome 89:572
3. Takabayashi T, Katoh H 2005 A mutant mouse with severe anemia and skin abnormalities controlled by a new allele of the flaky skin (*fsn*) locus. Exp Anim 54: 339–347
4. Snell GD 1929 Dwarf, a new Mendelian recessive character of the house mouse. Proc Natl Acad Sci USA 15: 733–734
5. Eicher EM, Beamer WG 1980 New mouse dw allele: genetic location and effects on lifespan and growth hormone levels. J Hered 71:187–190
6. Schaible RH, Gowen JW 1961 df– Ames dwarf. Mouse News Lett 25:35
7. Buckwalter MS, Katz RW, Camper SA 1991 Localization of the panhypopituitary dwarf mutation (*df*) on mouse