pro-MMP9, active-MMP9, 2 量体 MMP9)と比較することにより、各検体の MMP それぞれの濃度を、Inagaki らの方法により半定量(スコアリング)した。5 点以上を陽性とし、陽性例には抗生物質とプレドニゾロン 10mg を 10 日間投与した。

胚移植は新鮮胚移植を原則としたが、OHSS のリスクの高い症例や子宮内膜の菲薄化が顕著な例では全胚凍結し、翌周期以降で自然排卵周期あるいはホルモン補充周期において融解胚移植を行った。移植胚数については、平成 18 年より原則として単一胚盤胞移植を目指した。しかし、胚の状態によっては 2 個移植を行うこともあった。また、採卵数の少ない場合は採卵後培養3日目に移植を行った。胚移植 10 日後に血中 hCG を測定して妊娠の有無を判定した。採卵後 3 週後に子宮内に胎嚢を確認できた場合を臨床的妊娠と診断した。

統計処理には、$\chi 2$ 乗検定を用いた。

C. 研究結果 (表1)

MMP 陰性例は 496 例 [90.3%](I 群)、陽性例は 53 例 [9.7%] であった。陽性例のうち、内服治療後の再検で陰性となった例が 50 例 [94.3%](II 群)、治療後も陰性であった例は 3 例 [5.7%](III 群)。

平均年齢は、I 群で 36.71±4.11、II 群で 36.34±4.12、III 群で 38.33±0.57 と有意な差は認められなかった。

検査後に妊娠した例は、I 群で 223 例 [45.0%]、II 群で 27 例 [54.0%]、III 群で 1 例 [33.3%]であったが、

妊娠までの移植回数および平均移植胚数に差は認められなかった。しかし、流産率は、I 群で 123 例 [55.2%]、II 群で 7 例 [25.9%]、III 群で 0 例 であった。すなわち、MMP 陰性例よりも、MMP 陽性で治療後に陰性化した例の方が流産率は低かった。

D. 考察

Inagaki らは、16 例の反復着床不全例に対し、子宮内腔洗浄液中の MMPs 活性が高いことを報告している(Inagaki N et al, Hum Reprod 2003;18(3):608-615)。今回我々は、子宮内腔洗浄液中の MMP 活性が高い症例に対して、抗生剤とプレドニゾロンの内服治療が MMP 活性の正常化に著効を示し(94.3%で陰性化)、妊娠率の改善にも有用である可能性を示した(妊娠率:I 群, 45.0%;II 群, 54.0%;III 群, 33.3%)。興味深いことに、妊娠率あるいは流産率のいずれについても、I 群で II 群よりも良好な結果が得られている(流産率:I 群, 55.2%;II 群, 25.9%)。このことから、I 群では MMP 以外に反復着床不全の原因が存在すると考えられるものの、II 群では、MMP 活性の上昇が反復着床不全に寄与していたが、MMP の正常化により反復着床不全が一部あるいは完全に解消できたことを示していると考えられる。今後は、子宮筋腫、子宮内膜症、内膜ポリープ、卵管因子などの存在と、MMP 陽性率および妊娠率との相関についても検討する予定である。

また、今後は、子宮腔内におけるCD9あるいは HB-EGF の濃度をウェスタンブロットにより定量し、妊娠予後の相関について検討する予定である。

E. 結論

子宮内腔洗浄液中の液性因子と妊娠予後の相関を検討することにより、着床における新たな臨床的指標を発見できると考えられた。

F. 健康危険情報　　　なし

G. 研究発表

論文投稿準備中

学会発表: 未発表

H. 知的財産権の出願・登録状況　　なし

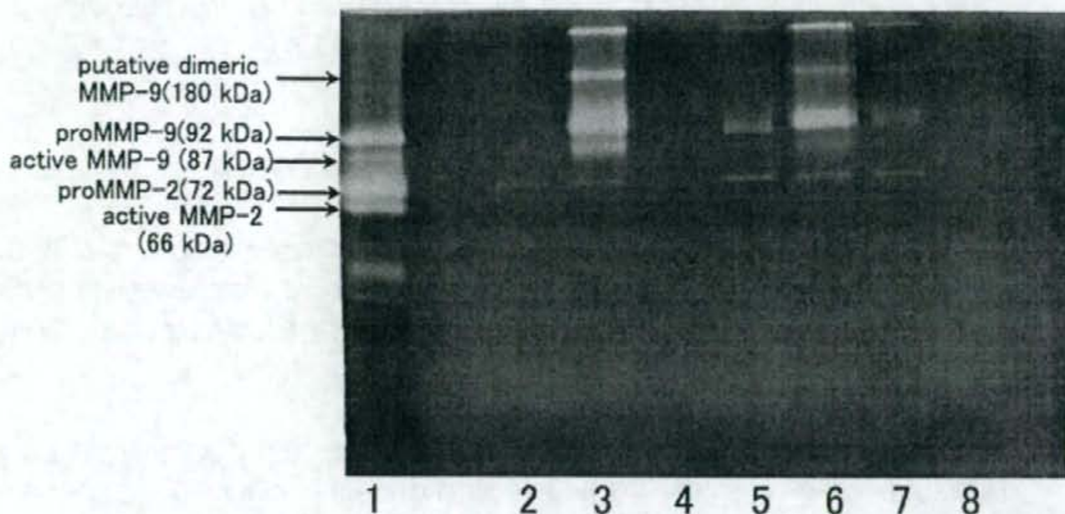


図1 MMP2 および MMP9 に関する zymography (Inagaki N et al, Hum Reprod 2003;18(3):608-615 より引用)
Lane 1 は、ポジティブ・コントロールとして BHK9 細胞培養上清。Lanes 2-8 は、患者子宮内腔洗浄液上清で、MMP スコアはそれぞれ、1, 12, 0, 3, 7, 4, 0 と判定された。それぞれのスコアは、下記の通り MMP2 および MMP6 の活性スコアの合計により算出された。

Lane 2: proMMP-2: 1, activeMMP-2: 0, proMMP-9: 0, activeMMP-9: 0, dimericMMP-9: 0.

Lane 3: proMMP-2: 1, activeMMP-2: 0, proMMP-9: 5, activeMMP-9: 2, dimericMMP-9: 4.

Lane 4: proMMP-2: 0, activeMMP-2: 0, proMMP-9: 0, activeMMP-9: 0, dimericMMP-9: 0.

Lane 5: proMMP-2: 1, activeMMP-2: 0, proMMP-9: 2, activeMMP-9: 0, dimericMMP-9: 0.

Lane 6: proMMP-2: 1, activeMMP-2: 0, proMMP-9: 3, activeMMP-9: 1, dimericMMP-9: 2.

Lane 7: proMMP-2: 1, activeMMP-2: 0, proMMP-9: 2, activeMMP-9: 0, dimericMMP-9: 1.

Lane 8: proMMP-2: 0, activeMMP-2: 0, proMMP-9: 0, activeMMP-9: 0, dimericMMP-9: 0.

MMP スコア(5 点以上が陽性)	陰性(I 群)	陽性→陰性(II 群)	陽性→陽性(III 群)	p
平均年齢(歳)	36.71±4.11	36.34±4.12	38.33±0.57	N.S.
n	496	50	3	N.S.
妊娠(%)[例数]	45.0 [223]	54.0 [27]	33.3 [1]	N.S.
妊娠までの胚移植回数(回)	1.64±0.84	1.85±1.16	1	N.S.
平均移植胚数(個)	1.82±0.59	1.85±0.54	1	N.S.
流産率(%)[例数]	55.2 [123]	25.9 [7]	0	<0.05

表1 MMP 活性と妊娠予後

課題番号: H20－子ども－若手－011

課題名: 子ども家庭総合研究事業「女性生殖器における妊孕能の客観的な評価法の確立」

主任研究者: 吉野 修 (東京大学 産婦人科)

分担課題: 子宮内膜の評価

分担研究者:大須賀 穣 (東京大学医学部 産婦人科 講師)

(要約) 胚の着床機転において、胎児と母体の相互作用が関わっていることが想定されている。我々は胚成分より産生されるサイトカイン interleukin-1 (IL-1)が子宮内膜細胞における IL-8 を誘導し、IL-8 が胎児成分である絨毛細胞の子宮側への遊走および子宮内膜における生存に関与していることを見出した。

A. 研究目的

妊娠成立にむけて 胚の子宮内膜への着床、という母体および胎児側の協調的な作用が必須である。本年度は、基礎的実験として、胎児成分である絨毛細胞と、母体側細胞、即ち子宮内膜細胞との相互関係を介した絨毛細胞の生着および生存に関して検討を行った。

特に、胚および絨毛細胞から分泌されることが知られている interleukin-1 (IL-1)に注目した。

B. 研究方法

倫理委員会の承認を得、文書同意のもと、婦人科良性疾患手術患者より正常子宮内膜組織を、また人工妊娠中絶患者より絨毛組織を採取した。胚および絨毛細胞から分泌することがしられている IL-1 (0-10ng/ml) を子宮内膜上皮細胞 (endometrial epithelial cell: EEC)および子宮内膜間質細胞(endometrial stromal cell:ESC)に添加し、24 時間後の上清中の IL-8 濃度を ELISA 法を用いて測定した。同上清を用いて、絨毛細胞の migration assay および、生存細胞数の測定を専用チャンバーを用いて行った。また。IL-8 のブロッキング抗体を同上清に添加した。

C. 研究結果

IL-1 は子宮内膜上皮細胞(EEC)および子宮内膜間質細胞(ESC)からの IL-8 産生を濃度依存性に上昇させた。(図1) 特に子宮内膜上皮細胞(EEC)から IL-8 の産生をみとめ、以下の実験は子宮内膜上皮細胞(EEC)を用いた。Migration assay において、IL-1で刺激した子宮内膜上皮細胞(EEC)から得られた上清は、絨毛細胞の遊走能および、生存細胞数を優位に上昇させた。(図2および図3)また、同上清を抗 IL-8 抗体で処置を行うと、絨毛細胞の遊走能および、生存細胞数はコントロールレベルに減少した。(図2および図3)

D. 考察

これまで IL-8 は種々の細胞における遊走に関与することが報告されているが、そのほかにも血管新生や細胞の生存にも関与することが知られている。これらの事象、即ち細胞の遊走、生存、血管新生は胎児成分の着床へも重要なプロセスである。本年度の研究では、胚および絨毛細胞より産生される IL-1 が子宮内膜細胞に働きかけて IL-8 を誘導させ、その IL-8 の作用により絨毛細胞の遊走能および生存を促進することを見出した。また、子宮内膜上皮細胞は間質細胞よりも IL-8 を産生することを見出した。これまで我々は、絨毛細胞の遊走に関与する因子としてケモカイン CXCL11 の子宮内膜における存在を見出しているが、同ケモカインも上皮細胞にのみ発現しており、間質細胞には発現していなかった(Hirota Y,

Osuga Y et al. The expression and possible roles of chemokine CXCL11 and its receptor CXCR3 in the human endometrium. J Immunol 177: 8813-21)。このことから、胎児側成分と母体側細胞の相互作用は主に上皮細胞において営まれている可能性が想定される。

E. 結論

胚の着床機転において、胎児と母体の相互作用が関わっていることが想定されている。我々は胚成分より産生されるサイトカイン interleukin-1 (IL-1)が子宮内膜細胞における IL-8 を誘導し、IL-8 が胎児成分である絨毛細胞の子宮側への遊走および子宮内膜における生存に関与していることを見出した。今後、着床に関わる因子について基礎的、臨床的に発展させていく予定である。

F. 健康危険情報　　なし

G. 研究発表

論文投稿 Y Hirota, Y Osuga, A Hasegawa et al. Interleukin(IL)-1beta stimulates migration and survival of first trimester villous cytotrophoblast cells through endometrial epithelial cell-derived IL-8. Endocrinology 150: 350-356 2009

学会発表：未発表

H. 知的財産権の出願・登録状況　　なし

図1

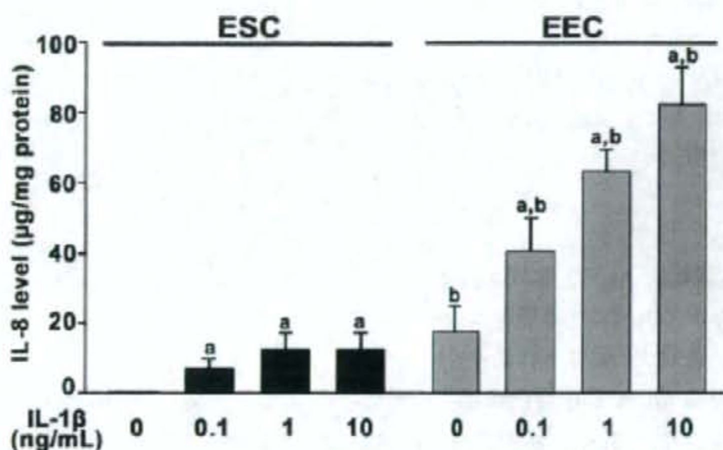


図1．子宮内膜上皮（EEC）および子宮内膜間質細胞(ESC)をIL-1β（0-10ng/ml）にて刺激し
上清中のIL-8濃度をELISAにて測定した。上皮細胞の方が間質細胞よりもよりIL-8を産生した。

図2

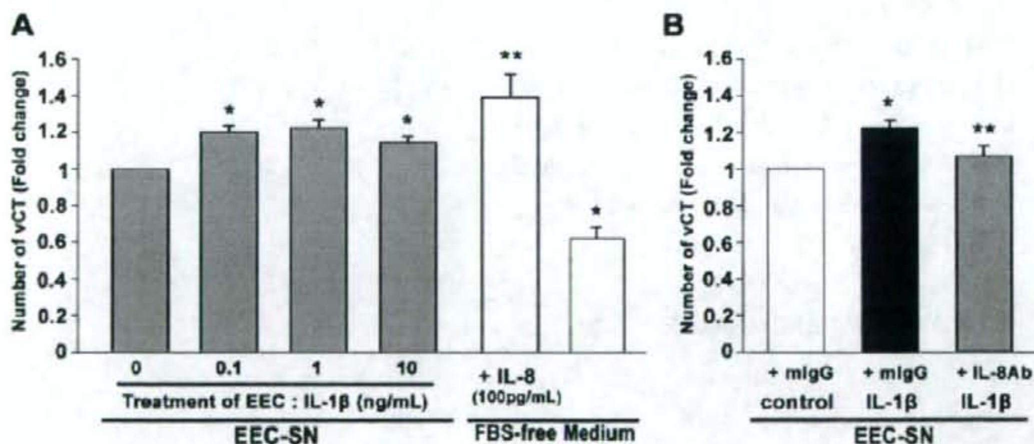


図2A　子宮内膜上皮細胞(EEC)をIL-1にて24時間刺激し、上清をもちいて絨毛細胞のmigration assayを行った。ポジティブコントロールとして100pg/mlのIL-8を含んだメディウムを用いた。
図2B　同上清にコントロール抗体もしくは抗IL-8抗体を添加したところ、絨毛細胞の遊走が低下した。

図3

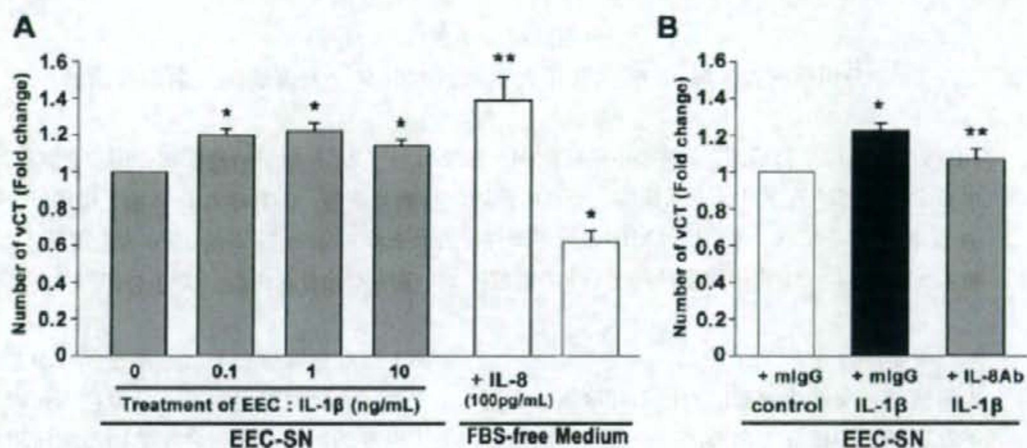


図3A　子宮内膜上皮細胞（EEC）をIL-1にて24時間刺激し、上清をもちいて絨毛細胞数を培養し、生存細胞数を検討した。ポジティブコントロールとして100pg/mlのIL-8を含んだメディウムを用いた。
図3B　同上清にコントロール抗体もしくは抗IL-8抗体を添加したところ、絨毛細胞の生存数が低下した。

課題番号: H20－子ども－若手－011

課題名: 子ども家庭総合研究事業「女性生殖器における妊孕能の客観的な評価法の確立」

主任研究者: 吉野 修（東京大学 産婦人科）

分担課題: 子宮内膜の評価

分担研究者: 堀 正明（東邦大学医療センター大森病院放射線科講師）

（要約） おもに子宮筋腫を有する不妊症症例を対象として、あらたなMRI撮影法の開発を試みた。子宮全体の三次元ボリューム撮像、子宮の連続的時間差撮像，いわゆるシネMRI撮像、子宮内膜のT2値測定を試み、今年度はMRIの設定条件を設定することができ、今後、データを蓄積する環境を整えることができた。来年度以降のデータの集積により、新たな知見を得ることが十分に期待される。

A. 研究目的

本研究は，女性生殖器における妊孕能の客観的な評価法の確立であるが，そのうち画像診断，特に磁気共鳴画像（以下，MRI）における評価法の確立を主たる目的とした．画像診断の側からみれば，骨盤部女性生殖器（子宮，卵巣等）はその疾患の評価において，MRIの良い適応となり，実際臨床の現場でも多数施行されている．その理由としては，MRIにより得られる画像が，他の画像検査（CT，あるいは超音波，X線写真等）に比し，組織内のコントラストに優れ（例：子宮の内膜と筋層の境界），遊離X線の被曝は皆無であり，検査を行う術者の力量に影響されることが少ない，客観的評価可能なものであることが挙げられる．従って，本研究の主題に沿った評価方法の確立として，MRIによる手法による確立を目指したのは自然といえる．また，日本とはWHO（世界保健機構）が認定する世界一，人口あたりのMRI装置数が多い国であり，他の先進国と比しても，MRIを用いた研究は相対的に容易に可能であることも本研究遂行のための現実的な要素として指摘できる．

前述した理由により，MRIを妊孕能の客観的な評価法に用いることに対しては何ら問題ないと思われるが，では実際の遂行においてどのような撮像プロトコールを行うかという点では，大いに逡巡がある．現在本邦のみならず世界的に広く施行されている臨床現場におけるMRI検査は，例えば子宮を例に挙げると，その目的としては，子宮筋腫の大きさ，位置を調べる，子宮筋腫と腺筋症の鑑別診断を行う，腫瘍があった場合にその伸展範囲や浸潤の程度を確認するといったものであり，まず病的背景ありきであり．いわば正常子宮に対して通常では存在しない異常信号あるいは異常構造といったものの，正常と思われる構造との対比あるいはその関わりを検査するというのが本質である．しかし，妊孕能というものは，勿論子宮筋腫が存在すれば病的状態としての低下という状態がありうると思われるが，そうでない場合が多数在ると思われる．むしろ，いわゆる器質的疾患がなく，妊孕能の低下がある場合における MRI による画像診断による評価の確立，いわば正常である被験者の評価方法を考慮しなければならない．従来の子宮の画像診断における検査手法においては，著しく欠如していた観点あるいは考え方であるといわざるを得ない．

さらに，上記の従来の診断法を目的とした撮像では様々な場面でその限界を露呈している．翻って，近年 MRI を用いた疾患の評価方

法として，視覚的評価ではなく，画像統計解析や，PC 上における自動処理，あるいは定量的評価といった手法が用いられている．順に例示していくと，まず画像統計解析に関しては，いわゆる標準といわれる状態から，信号強度あるいは形態的変化が統計学的にどの程度の偏位があるのかを評価する手法である．
具体的には，脳神経の分野で特に顕著な発展を認め，アルツハイマー病における早期発見，あるいは高リスク群における統合失調症の発症前評価が可能であるとされている．これら疾患の脳の形態的変化はごく軽微なもので人間の目による視覚的評価では，仮に経時的データが存在しても認識しえない程度のものである．また，PC 上における自動処理としては，特に周期的な運動を行う器官において，視覚的評価以上の情報を同一データより取得することができる．この分野では，特に心拍動時における心筋の奇異運動がそれに相当する．
最後に定量的評価としては，従来から MR spectroscopy による成分分析や T2 値の測定といった手法がある．過去では，これら撮像方法は施行に要する時間がかなり長く，研究レベルならともかく日常臨床においてはあまり現実的な検査手法ではなかったが，近年の MRI の進歩により，かなり短時間で施行可能となっている．例えば T2 値の測定は関節（膝関節等）において，早期より軟骨の変性を指摘可能であるといった用いられ方をしている．
したがって本分担研究の目的は，上記のような従来とは異なるアプローチを用いつつ，MRI を使用して女性生殖器における妊孕能の客観的な評価法の確立を目指すことである．

B. 研究方法

目的の項で示したとおり，本研究では骨盤部撮像の MRI として，臨床で多く用いられている骨盤部撮像法とは異なる撮像，解析方法でのデータ取得を目指す．

1 対象

不妊症を有する女性被験者であれば，特に制限はなし．ただし，一般的な MRI 撮像の禁忌がないこと（閉所恐怖症，体内金属等）

2 撮像方法

まず，通常の臨床で多く用いられているルーチン撮像，具体的には骨盤部の T2 強調像矢状断，横断像，T1 強調像の横断像にてそれぞれ数イメージ撮像を行い，MRI で指摘可能な器質的疾患の除外を行う．（ただし，子宮筋腫は不妊症と大きく関係しているので，筋腫があっても対象群からは除外しない）

その後研究の撮像手法として

① 子宮全体の三次元ボリューム撮像
② 子宮の連続的時間差撮像，いわゆるシネ MRI 撮像
③ 子宮内膜の T2 値測定

を行う．それぞれの撮像手法に関しては，実際の撮像開始前に撮像パラメーターの至適化を行う

3 データ解析

上記により得られた画像について，可能であれば画像統計解析を行うが，そもそもその解析手法そのものが確立されたものはなく，そこから検討を行う必要がある．また，シネ MRI に関しても，子宮にての定量的解析手法は現時点ではなく，その検討も必要であり．T2 値測定も正常子宮内膜の正常値というものは存在せず，撮像手法の妥当性（正しく T2 値が測定されているか，被験者周囲に生理食塩水等の対照物を置く）のみが確認可能であり，その結果の解釈は他の要素との比較検討を行う

（倫理面への配慮）

被験者より得られたデータは全て匿名化し，いわゆるインターネットと接続のない専用のPC内に保存してあり，解析を行っている．すわなち，将来的には，本研究の結果は個々の事例において益するべき必要性があるが，現時点，すなわち検査方法の確立段階においては，個々の差異よりは共通すべき点を抽出し，かつ統計学的な普遍なベースラインとしての性格をより有していると思われ，それ以上の意味を持たないため匿名化されたデータで研究遂行上，実際的な支障は来たさないものと確信できる．

C. 研究結果

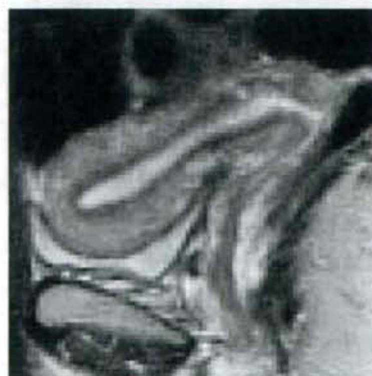
1　撮像可能であった被験者は，本年度中で２０人であった

2　撮像した結果，被験者全員で子宮筋腫以外の器質的疾患は MRI 画像上，指摘できなかった．

3　研究的撮像手法の各々について

①子宮全体の三次元ボリューム撮像

子宮内膜，筋層，筋腫（もしあれば）が比較的明瞭に区別できる三次元 T2 強調像の撮像が可能であった．ただし，この画像から各部分を抽出し，その容積を統計学的に処理するような手法の開発は、現在検討中である。



図：約 1.3×1.3×1.3mm のボクセルサイズで撮像

②子宮の連続的時間差撮像，いわゆるシネ MRI 撮像

子宮の矢状断面像のシネ MRI の撮像は可能であり，データの収集を行うことができた．本年度は適切なシネＭＲＩ撮影を行うことができる条件を設定することができた。

③子宮内膜の T2 値測定

得られた値に関しては偏差が大きく，本研究の目的に合致するような傾向，意義は現時点では見出せなかった．

D. 考察

個々の撮像方法について 限られたデータのみであるが考察する．まず子宮全体の三次元ボリューム撮像であるが，約 1.3×1.3×1.3mm のボクセルサイズで撮像可能であり，画質も良好であった（結果にある画像を参照）．現在，画像統計解析を目的とした脳の volumetric scan が約 1×1×1mm の T1 強調像で撮像されていることを考えると今回の撮像で得られたものも十分高空間分解能といえる．問題は，脳の解析においては，いわゆる標準脳なるものが存在し，それからの偏差が異常として捉えられることができるが，蠕動により大きく動き，かつ性周期により形態を動的に変化させる子宮では，群の標準としての標準子宮の設定は当初から困難であることは容易に推測できる．今後の方針としては，同一被験者を複数回撮像し，その差分上の変化を解析する，あるいは筋腫がある場合に筋腫の子宮全体に対する容積等による検討であれば十分可能であるし，そこから何らかの定量的評価可能な基準が見出しうると確信している．

また，いわゆるシネ MRI 撮像に関しては，既報がいくつか存在しうるが，蠕動運動の有

無あるいは異常運動（全体的，部分的）なものが妊孕能に関連しているのは根拠のある推測であり，解析手法の確立を迅速に行うのを目標とすべきである

子宮内膜のT2値測定に関しては，その値そのものでは解釈も解析も困難であり，撮像時の他の条件，すなわち女性ホルモン値等の相関が問題となると思われる，ただし，相当数のデータが収集できれば，妊孕能との相関も見出しうると思われる．

総括として，MRIの新たな撮像手法とその画像の妥当性に関しては，現時点で問題ないものと考える．データの収集方法や他の要素との相関に関して，代表研究者や他の分担研究者との検討が必要である．

E. 結論

本年度においては，データ収集方法としてのMRIの撮像方法に確立はほぼなしえたと考える．今後は，統計処理に必要な数の被験者のデータ収集，および得られた画像からの解析手法の確立の研究を推進すべきである．

F. 健康危険情報 なし

G. 研究発表

論文投稿 なし

学会発表：なし

H. 知的財産権の出願・登録状況

なし

III. 主な研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
阿部宏之	ヒト卵・胚のクオリティー評価	森 崇英	卵子学	京都大学学術出版会		2009	印刷中

雑誌

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Abe H., Hoshi H.	Morphometric and ultrastructural changes in ciliated cells of the oviductal epithelium in prolific Chinese Meishan and Large White pigs during the estrous cycle	Reproduction in Domestic Animals	Vol. 43	66-73	2008
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Utsunomiya T., Goto K., Nasu M., Kumasako Y., Araki Y., Yokoo M., Itoh-Sasaki T., Abe H.	Evaluating the quality of human embryos with a measurement of oxygen consumption by scanning electrochemical microscopy	Journal of Mammalian Ova Research	Vo. 25	2-7	2008
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Hirobe T., Ishizuka K., Ogawa S., Abe H.	Mitochondria are well developed in pink-eyed dilution melanoblasts in the neonatal mouse epidermis	Zoological Science	Vol. 25	1057-1065	2008

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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ZOOLOGICAL SCIENCE 25: 1057–1065 (2008)
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Mitochondria are More Numerous and Smaller in Pink-Eyed Dilution Melanoblasts and Melanocytes Than in Wild-Type Melanocytes in the Neonatal Mouse Epidermis

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The mouse pink-eyed dilution (*p*) locus is known to control the melanin content in melanocytes. However, it was not known whether the *p* gene is involved in regulating the proliferation and differentation of melanocytes during development, especially the biogenesis of melanosomes and other organelles. Epidermal cell suspensions of neonatal dorsal skin derived from mice wild type for the *p* locus (black, C57BL/10JHir-*P/P*) and their congenic mutant phenotype (pink-eyed dilution, C57BL/10JHir-*p/p*) were cultured in serum-free melanocyte-proliferation medium (MDMD). The supplement of additional L-tyrosine (Tyr) into the MDMD stimulated the differentiation of *p/p* melanoblasts into melanocytes. Electron microscopy revealed that in *p/p* melanoblasts and melanocytes treated with L-Tyr, the number of stage II and III melanosomes dramatically increased. Moreover, *p/p* melanoblasts possessed smaller but more numerous mitochondria than *P/P* melanocytes. The treatment of *p/p* melanoblasts and melanocytes with L-Tyr decreased the number of mitochondria. The supplement of 2, 4-dinitrophenol (DNP), an inhibitor of mitochondrial function, into the MDMD stimulated both the proliferation and differentiation of *p/p* melanoblasts. Simultaneous treatment of DNP and L-Tyr dramatically stimulated the differetiation of *p/p* melanocytes. These results suggest that L-Tyr and some unknown factors related to mitochondrial function may influence the differentiation of melanoblasts in the epidermis of *p/p* mice.

Key words: pink-eyed dilution, melanocyte, melanoblast, mitochondria

INTRODUCTION

Mouse epidermal melanocytes differentiate around the time of birth (Hirobe, 1984) from undifferentiated precursors, melanoblasts, which originate from the neural crest in the embryo (Rawles, 1947; Mayer, 1973). They increase in number until 3 or 4 days after birth, and then their numbers decrease (Hirobe, 1984). Most epidermal melanocytes migrate into hair bulbs, and pigment-accumulating organelles, melanosomes (Seiji et al., 1963), are transported to surrounding keratinocytes to produce pigmented hairs. Differentiated melanocytes produce two types of melanin: brownish-black eumelanin and reddish-yellow pheomelanin (Prota, 1980; Ito, 2003).

Numerous coat-color genes are involved in regulating the development of murine melanocytes (Silvers, 1979). The mouse pink-eyed dilution (*p*) locus controls the melanin content in melanocytes and in the retinal pigment epithelium (Silvers, 1979). In mouse hairs, the *p* mutation drastically reduces the eumelanin content (Silvers, 1979). In addition to a reduction in the amount of eumelanin deposited (Ozeki et al., 1995), melanin granules within the hair shafts of *p/p* mice are smaller than those in *P/P* mice (Russell, 1949). The *p/p* melanocytes contain smaller and rounder (Markert and Silvers, 1956, Orlow and Brilliant, 1999), immature (Sidman and Pearlstein, 1965; Moyer, 1966; Hearing et al., 1973; Hirobe and Abe, 1999) melanosomes, and the numbers of stage III and IV melanosomes are much fewer than in *P/P* melanocytes (Hirobe et al., 2002b).

Levels of tyrosinase, the rate-limiting enzyme in melanin synthesis, are greatly decreased in the skin of *p/p* mice (Tamate et al., 1989) and in cultured epidermal melanocytes of newborn *p/p* mice (Hirobe et al., 1998). The product of the *p* gene is an integral membrane protein that localizes in melanosomes (Rosemblat et al., 1994); its predicted secondary structure is a 12-transmembrane domain protein similar to a channel or transporter (Gardner et al., 1992; Rinchik et al., 1993). Excess tyrosine (Tyr) in a culture medium can greatly stimulate the pigmentation of retinal pigment epithelial cells and choroidal melanocytes from *p/p*

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mice (Sidman and Peatstein, 1965), suggesting that *p* protein functions as a Tyr transporter. Other groups suggest that the *p* protein is involved in regulating the maturation of melanosomes and the stabilization or trafficking of melanosomal membrane proteins (Lamoreux et al., 1995; Rosemblat et al., 1998; Orlow and Brilliant, 1999). Another theory that the *p* protein is a transporter that controls melanosomal acidification (Puri et al., 2000; Brilliant, 2001) has also been presented. The *p* protein also controls the processing and transport of tyrosinase (Chen et al., 2002; Toyofuku et al., 2002). Moreover, the *p* protein is reported to increase cellular sensitivity to arsenicals and other metalloids and to modulate intracellular glutathione metabolism (Staleva et al., 2002). It has also been proposed that the *p* protein mediates neutralization of melanosomal pH (Ancas et al., 2001). In addition, the proliferation and differentiation of neonatal mouse melanocytes are greatly inhibited by the *p* mutation (Hirobe and Abe, 1999; Hirobe et al., 2002a, b), and excess L-Tyr supplemented to the culture medium rescues both proliferative and differentiative activities of *p/p* melanoblasts (Hirobe et al., 2002b). Furthermore, the differentiation of melanoblasts into melanocytes is gradually induced by L-Tyr as the age of the donor mice advances, even though eumelanin and pheomelanin fail to accumulate in *p/p* melanocytes and are released from them at all stages of skin development (Hirobe et al., 2003a). This observation was confirmed by a study that showed that in 7.5-day-old *p/p* mice, the plasma levels of a eumelanin-related metabolite, 6-hydroxy-5-methoxyindole-2-carboxylic acid, and a pheomelanin-related metabolite, 5-*S*-cysteinyldopa, were nine-fold and four-fold greater, respectively, than in 7.5-day-old *P/P* mice (Wakamatsu et al., 2007).

However, it was not known whether the *p* mutation affects the biogenesis of melanosomes and other organelles in melanocytes in serum-free primary culture. In this study, we observed in detail, by using electron microscopy, the biogenesis of melanosomes and other organelles in cultured *p/p* melanoblasts with or without L-Tyr.

MATERIALS AND METHODS

Mice

All animals used in this study belonged to the strain C57BL/10JHir (B10, black, *P/P*) and its congenic strain, B10-*p/p* (pink-eyed dilution, *p/p*) of the house mouse, *Mus musculus*. They were given water and a commercial diet, OA-2 (Clea Japan, Tokyo, Japan) ad libitum. Congenic B10-*p/p* mice were established in our laboratory (Hirobe, 1986). Originally, the mutation (*p*) was introduced into B10 animals with nine generations of continued backcrossing followed by sib mating (Hirobe, 1986). After the 20th generation of sib mating, the congenic mice were continuously backcrossed with B10 for at least three generations, followed by sib mating. This procedure was then repeated. The genic constitution of the line differs only in the *p* locus. They were maintained at 24±1°C with 40–60% relative humidity; 12 hr of fluorescent light were provided daily. This study was approved by the ethics committee of the National Institute of Radiological Sciences in accordance with the guidelines of the National Institute of Health.

Melanocyte primary culture

The source of tissue for the culture of melanoblasts and melanocytes was dorsal skin from 0.5-day-old *P/P* and *p/p* mice. Unless stated otherwise, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The method for obtaining epidermal cell suspensions was reported previously (Hirobe et al., 2002a, b). Disaggregated epidermal cell suspensions were pelleted by centrifugation and suspended in Ham's F-10 medium (Gibco, Grand Island, NY, USA). The cell pellet after centrifugation was resuspended in melanocyte-proliferation medium (MDMD) consisting of Ham's F-10 plus 10 µg/ml bovine insulin, 0.5 mg/ml bovine serum albumin (Fraction V), 1 µM ethanolamine, 1 µM phosphoethanolamine, 10 nM sodium selenite, 0.5 mM dibutyryl adenosine 3':5'-cyclic monophosphate (DBcAMP), 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 50 µg/ml gentamycin sulfate, and 0.25 µg/ml amphotericin B. The same lots of these supplements were used in this study. Cells in each epidermal cell suspension were counted in a hemocytometer and were plated onto dishes coated with type-I collagen (Becton Dickinson, Bedford, MA, USA) an initial density of 1×10^6 cells/35 mm dish (1.04×10^5 cells/cm^2). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air (pH 7.2). The medium was replaced with fresh medium four times a week. After 14 days, pure cultures of melanoblasts (*p/p*) or melanocytes (*P/P*) were obtained. In some cases, additional L-Tyr (2 mM) was added to the medium from initiation of the primary culture. The standard concentration of L-Tyr in Ham's F-10 is 10 µM. Moreover, 2, 4-dinitrophenol (DNP) was added to the medium at concentrations of 0.01, 0.1, and 1 µM from initiation of the primary culture.

Assays for proliferation and differentiation

The number of melanoblasts and melanocytes was determined per dish by phase-contrast and bright-field microscopy; the calculation was based on the average number of cells from 10 randomly chosen microscopic fields covering an area of 0.581 mm^2. Bipolar, tripolar, dendritic, polygonal, or epithelioid cells, as seen in phase-contrast, that contained brown or black pigment granules, as observed by bright-field microscopy, were scored as pigmented melanocytes. In contrast, bipolar, tripolar, dendritic, or polygonal cells, as seen in phase-contrast, that contained no pigments and were negative (no tyrosinase activity) to 3, 4-dihydroxyphenylalanine (dopa) were scored as melanoblasts. These cells were stained by the combined dopa-premelanin reaction (combined dopa-ammoniacal silver nitrate staining; Mishima, 1960; Hirobe, 1984). This preferential staining reveals undifferentiated melanoblasts that contain stage I and II melanosomes without tyrosinase activity, in addition to tyrosinase-containing differentiated melanocytes. The ammoniacal silver nitrate reaction specifically reveals both unmelanized melanosomes and melanized melanosomes in melanocytes, the metallic silver particles being deposited with a high degree of selectivity (Mishima, 1960; Hirobe, 1984). Melanoblasts were also stained by antibodies to the tyrosinase-related proteins TRP-1 and TRP-2 (Hirobe et al., 2002a). A "melanoblast" is defined here as an unpigmented cell that possesses no tyrosinase activity. The statistical significance of differences was determined by Student's *t*-test for comparisons of groups of equal size.

Electron microscopy

Pure primary *P/P* and *p/p* melanoblasts/melanocytes cultured for 3, 7, and 14 days were treated with a solution of 0.05% trypsin (Difco, Sparks, MD, USA) and 0.02% ethylenediaminetetraacetate in Ca^{2+}-, Mg^{2+}-free phosphate buffered saline at 37°C for 10 min. After trypsinization was inhibited by addition of 2000 U/ml of soybean trypsin inhibitor, the cell suspensions were centrifuged at 1500 rpm for 5 min. The cell pellets were fixed in chilled (2°C) 2.5% glutaraldehyde (Taab Laboratories Equipment Ltd., Berkshire, UK) in 0.1 M phosphate buffer (pH 7.4). After washing with chilled 0.1 M phosphate buffer, the cells were postfixed in chilled 1% osmium tetroxide (Taab Laboratories Equipment Ltd.) in 0.1 M phosphate buffer. After washing again with chilled 0.1 M phosphate buffer, the

cells were dehydrated in a graded ethanol series and embedded in epoxy resin (Taab Laboratories Equipment Ltd.). Ultrathin sections were cut with a diamond knife on an ultramicrotome (Leica, Heerbrugg, Switzerland), stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEM-2000 EX, JEM, Tokyo, Japan).

One hundred electron micrographs of cells in each group were surveyed for the presence of stage I, II, III, and IV melanosomes, Golgi apparatus, mitochondria, and lysosomes, which were scored, and the numbers of these organelles per unit area (100 μm^2) were calculated. The size of mitochondria was estimated as the area of an ellipse (πab), calculated after measurement of the major (a) and minor (b) axes. The total number of mitochondria measured was 100 for each group.

Melanosome development was categorized in the four stages defined by Fitzpatrick et al. (1969): Stage I melanosomes initiate the accumulation of intralumenal fibrils; stage II melanosomes possess completed intralumenal fibrils without pigment; stage III melanosomes possess longitudinal depositions of pigment in intralumenal fibrils; stage IV melanosomes are fully deposited with pigment.

RESULTS

Light-microscopic observations

Within 1 day after the initiation of epidermal cell suspensions derived from 0.5-day-old *P/P* mice in MDMD, small keratinocyte colonies could be seen. Small bipolar or tripolar cells (melanoblasts) were scattered between the keratino-

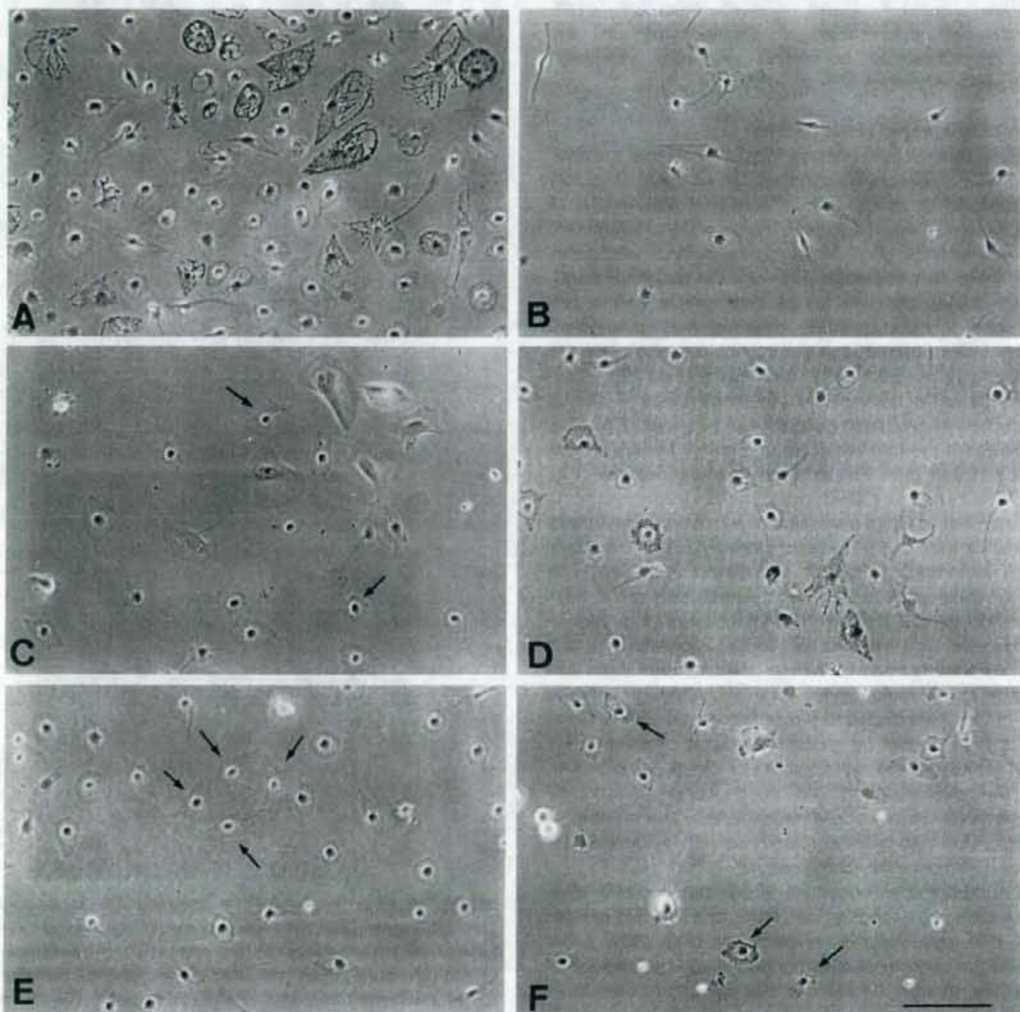

Fig. 1. Melanoblasts and melanocytes derived from epidermal cell suspensions of 0.5-day-old *P/P* **(A, D)** and *p/p* **(B, C, E, F)** mice. They were cultured with **(C)** or without **(A, B)** 2 mM L-Tyr. They were also cultured with 1 µM DNP **(D, E)** or with 1 µM DNP + 2 mM L-Tyr **(F)**. L-Tyr stimulated the differentiation of *p/p* melanoblasts into melanocytes after 14 days in culture. **(C)**; arrows indicate differentiated *p/p* melanocytes. DNP **(E)** as well as DNP + L-Tyr **(F)** stimulated the differentiation of *p/p* melanocytes. Arrows indicate differentiated melanocytes **(E, F)**. Phase-contrast microscopy. Scale bar, 100 µm.

cyte colonies. After 2 or 3 days, pigment granules appeared in the cytoplasm and processes of melanoblasts. After 4 or 5 days, melanocytes increased in number. They were more pigmented than before and extended dendrites into the surrounding keratinocytes. After 8 or 9 days, the keratinocyte colonies gradually decreased in number, and by 12–14 days, cultures contained pure melanocytes with intense pigmentation (Fig. 1A). When epidermal cell suspensions from *p/p* mice were cultured in MDMD, a similar proliferation of keratinocytes was observed. However, the proliferation and differentiation of melanoblasts were greatly inhibited. No pigmented melanocytes were found during the culture (Fig. 1B). When epidermal cell suspensions from *p/p* mice were cultured with L-Tyr (2 mM), keratinocyte proliferation showed no change. However, many differentiated melanocytes appeared; the percentage of melanocytes in the melanoblast-melanocyte population gradually increased (Fig. 1C), and exceeded 40% at 14 days.

Electron-microscopic observations

Electron microscopic observations showed that melanosomes of *P/P* melanocytes cultured for 14 days (Fig. 2A) were ellipsoidal or ovoid, with intralumenal depositions of melanin. Mature stage IV melanosomes were predominant in *P/P* melanocytes (Fig. 2A). Golgi apparatus and mitochondria were also observed (Fig. 2A). On the other hand, *p/p* melanoblasts cultured for 14 days possessed a few stage I and II melanosomes, whereas they possessed numerous Golgi apparatus and mitochondria (Fig. 2B). No stage III and IV melanosomes were found (Fig. 2B). However, excess L-Tyr dramatically increased stage II and III melanosomes in *p/p* cells cultured for 14 days (Fig. 2C). This increase in the number of stage II and III melanosomes was much higher than that in stage IV melanosomes (Fig. 2C).

The number of Golgi apparatus in *p/p* melanoblasts was much greater than in *P/P* melanocytes (Fig. 3A). The number (*p/p*) increased as culture proceeded (Fig. 3A). The number of Golgi apparatus in *p/p* melanoblasts at 14 days was four-fold greater than in *P/P* melanocytes (Fig. 3A). L-Tyr decreased the number of Golgi apparatus in *p/p* melanoblasts/melanocytes, though the number was still higher than in *P/P* melanocytes (Fig. 3A). Although why the number of Golgi apparatus in *p/p* cells cultured with L-Tyr at 3 days is high cannot be explained well at present, L-Tyr possibly stimulates the development of Golgi apparatus to induce the differentiation (de novo stage III and IV melanosome formation) of *p/p* melanocytes. The number of Golgi apparatus may gradually decrease with increasing formation of melanosomes during culture.

The numbers of mitochondria in *p/p* melanoblasts at 3, 7, and 14 days were roughly two-, four-, and five-fold greater than in *P/P* melanocytes, respectively (Fig. 3B). L-Tyr decreased the number of mitochondria in *p/p* melanoblasts/melanocytes, though the number was still higher than in *P/P* melanocytes (Fig. 3B). The size of mitochondria in *p/p* melanoblasts was smaller than in *P/P* melanocytes (Fig. 4), suggesting that mitochondria in *p/p* melanoblasts may divide, and this division may produce more numerous and smaller mitochondria. L-Tyr failed to increase the size of mitochondria in *p/p* melanoblasts/melanocytes.

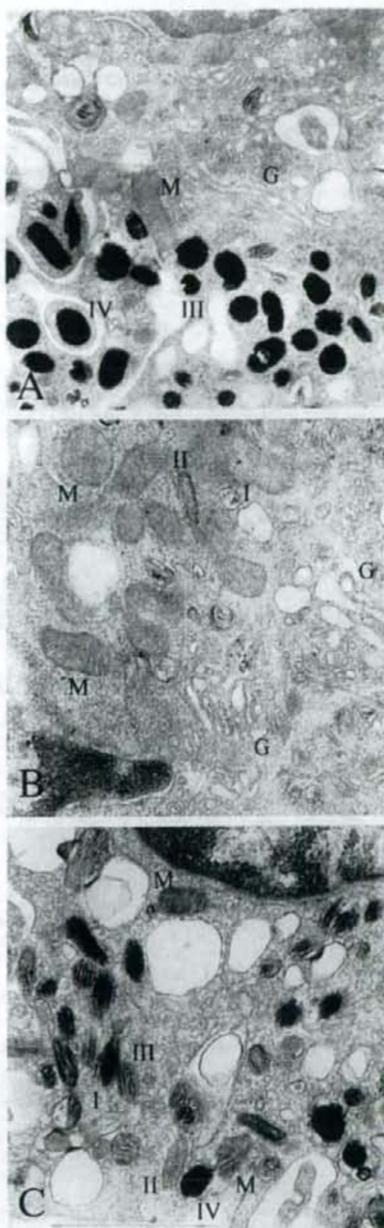

Fig. 2. Electron micrographs of epidermal *P/P* melanocytes **(A)** and *p/p* melanoblasts **(B)** or melanocytes **(C)** cultured for 14 days in MDMD with **(C)** or without **(A, B)** 2 mM L-Tyr. **(A)** Numerous stage III and IV melanosomes (indicated in the figure by corresponding roman numberals) are seen in *P/P* melanocytes. **(B)** In contrast, only a small number of stage I and II melanosomes are seen in *p/p* melanoblasts. In *p/p* melanoblasts **(B)**, well-developed Golgi apparatus (G) and mitochondria (M) are seen. However, in *p/p* melanocytes cultured with 2 mM L-Tyr **(C)**, all stages (I, II, III, and IV) of melanosomes are seen, including numerous stage III melanosomes. Scale bar, 1 μm.

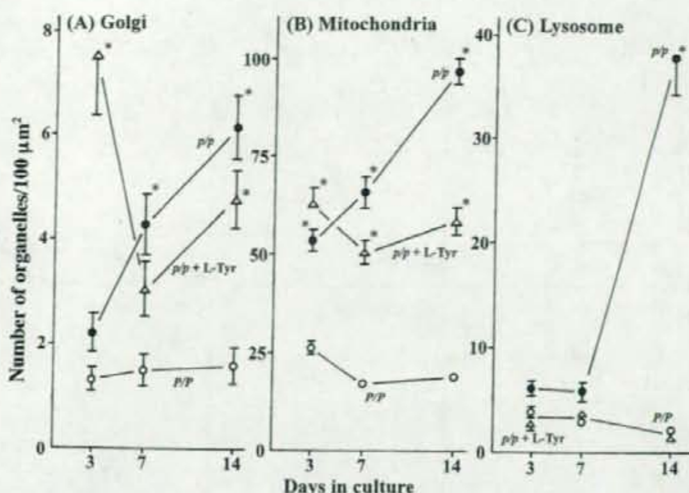

Fig. 3. Changes in the number of **(A)** Golgi apparatus, **(B)** mitochondria, and **(C)** lysosomes in cultured *P/P* melanocytes, *p/p* melanoblasts, and *p/p* melanoblasts/melanocytes in the presence of 2 mM L-Tyr. Epidermal cell suspensions were derived from 0.5-day-old mice and cultured for 14 days. The cells were fixed at 3, 7, and 14 days, and 100 melanoblasts or melanocytes for each group were recorded for the detection of organelles. The number of Golgi apparatus **(A)** in *p/p* melanoblasts is more numerous than in *P/P* melanocytes. The number of Golgi apparatus in *p/p* melanoblasts increased dramatically as the culture proceeded. L-Tyr reduced the number of Golgi apparatus, though the number still exceeded that of *P/P* melanocytes. The number of mitochondria **(B)** in *p/p* melanoblasts is much greater than in *P/P* melanocytes. The number of mitochondria in *p/p* melanoblasts increased dramatically as the culture proceeded. L-Tyr decreased the number of mitochondria, though the number was still higher than in *P/P* melanocytes. The number of lysosomes **(C)** in *p/p* melanoblasts is higher than in *P/P* melanocytes, especially at 14 days. L-Tyr completely reduced the number of lysosomes in *p/p* melanoblasts/melanocytes, which was comparable to that in *P/P* melanocytes. The data are the averages of results from 100 electron micrographs of cells. Bars indicate the standard error of the mean (SEM) and are shown only when they were larger than the symbols. $^*P<0.05$.

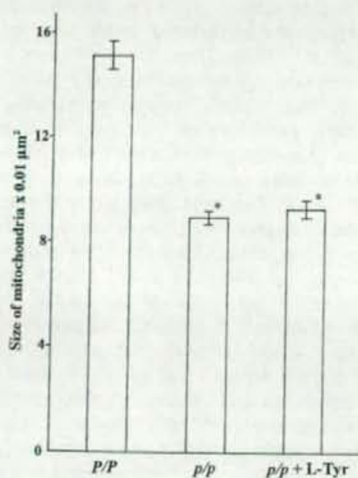

Fig. 4. Size of mitochondria in *P/P* melanocytes, *p/p* melanoblasts, and *p/p* melanoblasts/melanocytes cultured with 2 mM L-Tyr. Epidermal cell suspensions were derived from 0.5-day-old mice and cultured for 7 days. The cells were fixed, and size measurements were made for 100 mitochondria. The size of mitochondria in *p/p* melanoblasts is smaller than in *P/P* melanocytes. L-Tyr failed to increase the size of mitochondria in *p/p* melanoblasts/melanocytes. The data are the averages of results from 100 mitochondria in electron micrographs of cells. Bars indicate SEM. $^*P<0.05$.

The number of lysosomes in *p/p* melanoblasts was greater than in *P/P* melanocytes, especially at 14 days (Fig. 3C). L-Tyr completely reduced the number of lysosomes in *p/p* melanoblasts/melanocytes, and the number was comparable to that in *P/P* melanocytes (Fig. 3C).

These results suggest the possibility that changes in Golgi apparatus, mitochondria, and lysosomes in *p/p* melanoblasts may influence the formation and maturation of melanosomes.

Effects of DNP on melanocytes

In *P/P* melanocytes, the proliferation of melanocytes was inhibited by DNP, an inhibitor of mitochondrial function (Figs. 1D, 5A), though the differentiation of melanocytes was not affected (Figs. 1D, 5D). In contrast, in *p/p* melanoblasts, both proliferation (Figs. 1E, 5B) and differentiation (Figs. 1E, 5E) were stimulated by DNP. The number of melanoblasts and melanocytes was significantly increased at 14 days by DNP treatment at a concentration of 1 µM (Fig. 5B, $P<0.05$), and many mitotic figures of *p/p* melanoblasts and melanocytes cultured with DNP were observed. Moreover, the percentage of melanocytes in the melanoblast-melanocyte population was also increased at 7 and 14 days at all concentrations tested (0.01, 0.1, 1 µM; $P<0.05$). Ca. 20% of cells were induced to differentiate at 1 µM DNP at 14 days (Fig. 5E). Simultaneous treatment with DNP and L-Tyr resulted in a greater increase in the percentage of melanocytes in the melanoblast-melanocyte population (Figs. 1F,

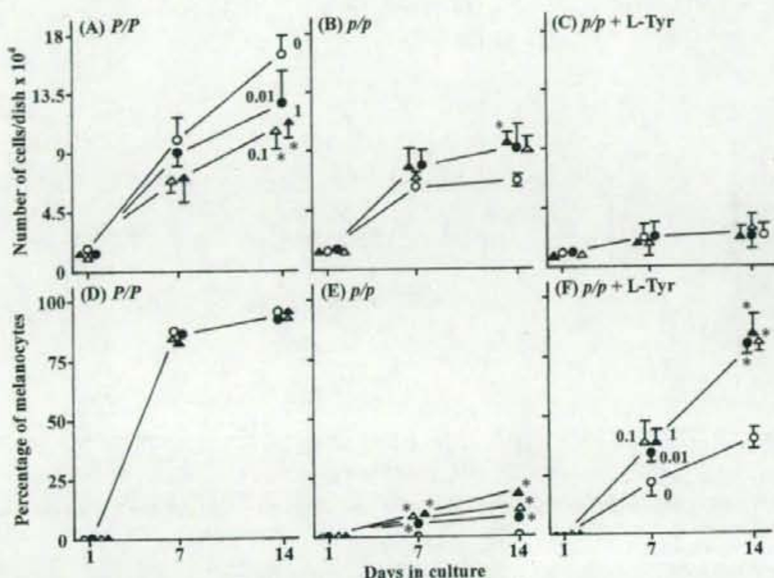

Fig. 5. Kinetics of the proliferation **(A, B, C)** and differentiation **(D, E, F)** of epidermal *P/P* melanocytes **(A, D)**, *p/p* melanoblasts **(B, E)**, and *p/p* melanoblasts/melanocytes cultured with 2 mM L-Tyr **(C, F)**. Epidermal cell suspensions were derived from 0.5-day-old mice. DNP at concentrations of 0 (○), 0.01 (●), 0.1 (△), and 1 (▲) μM was added to each culture from initiation of the primary culture. Pure cultures of melanoblasts or melanocytes were obtained after 14 days. The number of melanoblasts and melanocytes was counted by phase-contrast and bright-field microscopy at 1, 7, and 14 days. The data are the averages of results from three experiments. Each experiment was performed with different litters of mice. Bars indicate the SEM and are shown only when they were larger than the symbols. $^*P<0.05$.

5F). More than 80% of cells were differentiated at 14 days at all concentrations tested (Fig. 5F; $P<0.05$). However, the proliferation of melanoblasts and melanocytes was not stimulated by simultaneous treatment with DNP and L-Tyr (Fig. 5C). These results suggest that DNP may rescue the reduced proliferation and differentiation of *p/p* melanoblasts, and that DNP may restore the reduced differentiation of *p/p* melanoblasts in cooperation with L-Tyr.

DISCUSSION

Our present study demonstrated that the proliferative and differentiative activity of *p/p* melanoblasts cultured from neonatal murine epidermis was greatly reduced compared with that of *P/P* melanocytes. The differentiative activity of *p/p* melanoblasts in culture was increased by the addition of L-Tyr to MDMD in this study. Our previous study showed that L-Tyr increased the tyrosinase activity of *p/p* melanocytes in primary culture (Hirobe et al., 2002b). It is possible that increased tyrosinase activity in cultured *p/p* melanocytes by L-Tyr stimulates their differentiation. The question arises as to what mechanisms are involved in regulation by L-Tyr of the differentiation of *p/p* melanocytes. One possible explanation is that L-Tyr acts directly on melanocytes and activates factors involved in regulating signal transduction pathways required for cell differentiation, such as the protein kinase A (PKA; Hirobe, 1992), tyrosine kinase (TK, Coughlin et al., 1988), and PKC (Imokawa et al., 1997) pathways. Indeed, in mouse melanoma cells, L-Tyr stimulates the capacity of melanocyte-stimulating hormone (MSH) to bind the MSH receptor, and consequently the PKA pathway is activated through the elevation of cAMP level in the cells (Slominski et al., 1989). Thus, the activation of PKA is thought to stimulate the differentiation of *p/p* melanocytes. Another explanation is that L-Tyr acts on the tissue environment, especially keratinocytes, and induces or stimulates the synthesis of melanogens, such as MSH (Thody et al., 1983), basic fibroblast growth factor (Halaban et al., 1988), nerve growth factor (Yaar et al., 1991), endothelins (Yada et al., 1991; Imokawa et al., 1992; Yohn et al., 1993; Hirobe, 2001), granulocyte macrophage colony-stimulating factor (Imokawa et al., 1996; Hirobe et al., 2004a), steel factor (Kunisada et al., 1998; Hirobe et al., 2003b), hepatocyte growth factor (Kunisada et al., 2000; Hirobe et al., 2004b), and leukemia inhibitory factor (Hirobe, 2002). This hypothesis may be partially supported by the observation that L-Tyr failed to stimulate the differentiation of cultured melanocytes in the absence of keratinocytes (Hirobe et al., 2002b).

Our present study showed that the formation and maturation of melanosomes was greatly inhibited in *p/p* melanoblasts, but that L-Tyr induced the de-novo formation and maturation of melanosomes. Although L-Tyr dramatically increased stage II and III melanosomes, mature stage IV melanosomes were not dramatically increased. These results suggest that the rescue by L-Tyr of the impaired melanocyte differentiation is incomplete, and that other, additional factors are required for the complete rescue.

Our present study demonstrated that *p/p* melanoblasts possess much more Golgi apparatus than *P/P* melanocytes.

The number of Golgi apparatus in *P/P* melanocytes in the epidermis of newborn mice is known to decrease as the developmental age advances (Hirobe and Takeuchi, 1978). Since melanosomes may at least in part originate from Golgi apparatus (Seiji et al., 1963; Novikoff et al., 1968; Maul, 1969; Imokawa and Mishima, 1981; Hirobe, 1982), the latter is thought to gradually decrease in number with increasing formation of melanosomes. However, in this study, L-Tyr failed to reduce the number of Golgi apparatus in *p/p* melanoblasts/melanocytes. Although this discrepancy between *P/P* melanocytes and *p/p* melanoblasts/melanocytes cannot be fully explained at present, it might be attributed to differences in the formation and maturation of melanosomes between *P/P* melanocytes and *p/p* melanoblasts/melanocytes. In *P/P* melanocytes, the formation and maturation of melanosomes may be fully activated, and thus a small number of Golgi apparatus may exist under normal circumstances. In contrast, in *p/p* melanoblasts, the formation and maturation of melanosomes may not be fully activated, and thus a large number of Golgi apparatus may be present under normal circumstances. Moreover, the stimulation of melanosome formation and maturation by L-Tyr in *p/p* melanoblasts/melanocytes was incomplete (stage IV melanosomes did not increase greatly), and thus the number of Golgi apparatus failed to reach the level of *P/P* melanocytes. This hypothesis may be partially supported by the finding that the total numbers of both melanosomes and Golgi apparatus were not affected by the treatment with L-Tyr in cultured *P/P* melanocytes (Hirobe et al., 2007). Therefore, it is reasonable to assume that the number of Golgi apparatus in *p/p* melanoblasts is greater than in *P/P* melanocytes because of the reduced formation and maturation of melanosomes.

In our present study, *p/p* melanoblasts possessed many more lysosomes than *P/P* melanocytes, especially at 14 days in culture. In contrast, L-Tyr dramatically decreased the number of lysosomes at all days tested, and their numbers were comparable to those of *P/P* melanocytes. These results suggest that the increased melanosome formation and maturation induced by L-Tyr in *p/p* melanoblasts/melanocytes may elicit the decrease in the number of lysosomes. Melanosomes are thought to be specialized organelles of lysosomes (Orlow, 1995). Indeed in the *P/P* mouse epidermis, differentiated melanocytes possess a small number of lysosomes, whereas undifferentiated melanoblasts possess many lysosomes (Hirobe and Takeuchi, 1978). The relationship between melanosome formation and lysosome formation should be precisely investigated in a future study.

In our present study, many more mitochondria were present in *p/p* melanoblasts than in *P/P* melanocytes. Moreover, the number of mitochondria increased as the culture proceeded. L-Tyr decreased the number of mitochondria in *p/p* melanoblasts and melanocytes, though the number was still greater than in *P/P* melanocytes. From these results, we assume that the mitochondria in *p/p* melanoblasts influence the formation and maturation of melanosomes. This assumption may be partially supported by the findings that L-Tyr decreased the number of mitochondria in *p/p* melanoblasts/melanocytes, whereas L-Tyr increased the number of melanosomes. The size of mitochondria in *p/p* melanoblasts was smaller than in *P/P* melanocytes. Therefore, it is reasonable to conclude that the division of mitochondria may occur in *p/p* melanoblasts, resulting in numerous small-sized mitochondria. Although the theory that melanosomes originate from mitochondria (du Buy et al., 1963) was not supported by the study of Seiji et al. (1963), in the present study we propose the new theory that mitochondria may influence the formation and maturation of melanosomes in melanocytes by an unknown mechanism.

DNP inhibits oxidative phosphorylation in mitochondria (Han et al., 2008). In our present study, this inhibition of mitochondrial function by DNP stimulated the proliferation and differentiation of *p/p* melanoblasts. In contrast, DNP inhibited the proliferation of *P/P* melanocytes, whereas it did not affect the differentiation of *P/P* melanocytes. Therefore, it is reasonable to conclude that the dysfunction of mitochondria caused by DNP in *P/P* melanocytes may inhibit cell proliferation, due to a reduced energy supply. In contrast, the dysfunction of mitochondria caused by DNP in *p/p* melanoblasts may stimulate the proliferation of melanocytes, without a severe reduction in the energy supply, since *p/p* melanoblasts possess numerous mitochondria. DNP possibly stimulates the signaling pathway of PKA, PKC, and TK, and consequently the proliferation and differentiation of *p/p* melanoblasts are increased. This hypothesis may be partially supported by our findings that DNP and L-Tyr synergistically stimulated the differentiation of *p/p* melanoblasts.

Taken together, our present results suggest that the *p* gene exerts its influence by affecting the proliferation and differentiation of melanocytes through regulating melanosome formation and maturation, or through regulating the uptake of L-Tyr and the function of other organelles.

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