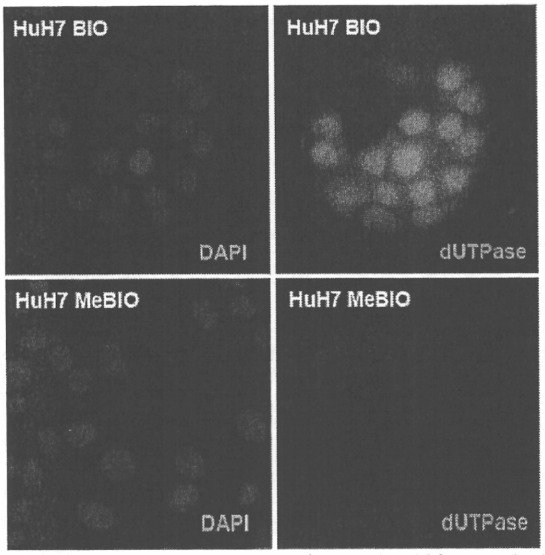

BIO 処理を行い Wnt シグナルを活性化したところ、nuclear dUTPase の著しい発現増加が認められる。

さらに nuclear dUTPase に特異的なプロモーター領域を pGL3 vector に組み込んだ pGL3-DUT を用いてプロモーター活性(nDUT)につき検討したところ、BIO 投与で nDUT プロモーターが活性化し、逆にβ-catenin に対する si-RNA を用いて Wnt シグナル伝達を抑制したところ nDUT プロモーター活性の低下が認められた。

最後に、dUTPase の発現そのものが EpCAM 陽性肝がん幹細胞の 5-FU 感受性に関与するかについて siRNA を用いた knock down 実験で検討した。Control siRNA を投与した HuH7 細胞に 2.5μM の 5-FU で 3 日間処理したところ、EpCAM 陽性細胞分画の増加が認められたが、異なる 2 つの領域を標的とした DUT siRNAs を投与したところ、両者ともに 5-FU 投与による EpCAM 陽性細胞の増加が抑えられており、EpCAM 陽性肝がん幹細胞の 5-FU 感受性が改善していることが示唆された。

D. 考察

近年のがん幹細胞仮説においては、正常幹細胞が正常臓器の維持に重要な役割を果たしているのと同様に、がん幹細胞はがん組織の維持に重要な役割を果たしていると考えられている。これまでに我々は EpCAM 陽性 AFP 陽性の幹細胞型の肝細胞がんでは Wnt シグナルの活性化と肝幹細胞マーカーの発現亢進、若年発症、強い門脈浸潤傾向、予後不良傾向が認められ、特に EpCAM 陽性細胞が強い腫瘍形成能、浸潤能、5-FU 抵抗性を有し、がん幹細胞の特徴を有していることを報告してきた。本研究において、さらに我々は EpCAM 陽性がん幹細胞では nuclear dUTPase の発現が亢進していること、nuclear dUTPase の発現調節は少なくとも一部は Wnt シグナル伝達系を介していること、DUT 遺伝子の発現抑制を行うことで EpCAM 陽性がん幹細胞の 5-FU 抵抗性が改善することを見出した。

DUT 遺伝子はウイルス、結核菌、寄生虫から哺乳類まで幅広く保存されており、ヌクレオチドプールを調節することで DNA ダメージを回避する重要な遺伝子である。ヒトでは DUT は splicing により nuclear dUTPase と mitochondrial dUTPase の少なくとも 2 つの isoform をコードしており、その転写調節には p53 の関与が報告されている。正常胎児肝において nuclear dUTPase の活性化が認められることに関しては、dUTP pool を低く保つことで DNA ダメージを抑え、遺伝子情報を正確に娘細胞に伝えていくことが可能になることから、遺伝子変異を最小に抑えるという点で発生段階においては生物学的に有利であると考えられる。一方、がん幹細胞でも同様のメカニズムが働くことで抗がん剤抵抗性を獲得している可能性が高く、両者に共通して認められるシグナル伝達系として Wnt シグナルの活性化が示唆された。DUT 遺伝子発現抑制により EpCAM 陽性がん幹細胞の抗がん剤感受性が改善していることから、nuclear dUTPase をターゲットにした薬剤開発も含め、EpCAM 陽性細胞の生物学的悪性度を規定する分子機構の解明は、肝がん幹細胞に対する治療法開発における重要な課題であると考えられた。

E. 結論

肝細胞がんは EpCAM と AFP により幹細胞型と肝細胞型に分類可能であり、特に幹細胞型は若年発症、予後不良、門脈浸潤傾向などがんとしての生物学的悪性度が高いこと、EpCAM 陽性細胞はがん幹細胞としての特徴を有し 5-FU に対する抵抗性を示すこと、そのメカニズムとして Wnt シグナル伝達系を介した nuclear dUTPase の活性化が関与している可能性が示唆された。DUT の遺伝子発現抑制により EpCAM 陽性がん幹細胞の 5-FU 抵抗性が改善することから、特に nuclear dUTPase はがん幹細胞を標的とする治療法の開発において重要な分子の一つであると考えられた。

F. 研究発表

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G. 知的所有権の出願・取得状況

1. 特許取得
なし
2. 実用新案登録
なし
3. その他
特記事項なし

厚生労働科学研究費補助金（第3次対がん総合戦略研究事業）
分担研究報告書

肝部分切除後の間葉系幹細胞移植による肝再生の検討

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研究要旨
前年度に脂肪由来の間葉系幹細胞をマウスに静脈注射して腫瘍組織や炎症部への遊走能を検討した所、間葉系幹細胞の明らかな腫瘍組織や炎症部への集積は認められなかったが、免疫抑制作用が認められた。今回は脂肪由来の間葉系幹細胞と腫瘍細胞を同時に移植した場合の相互作用を検討し、腫瘍増殖は促進されることを確認した。また脂肪由来の間葉系幹細胞を無血清培地で効率よく内皮細胞に分化させる方法を見いだした。

A.研究目的

骨髄をはじめとする様々な組織に間葉系幹細胞が存在しており、筋肉や骨、軟骨などへの多分化能をもつことや免疫調節作用が報告されている。骨髄由来の間葉系幹細胞研究がすすんでいるが、近年、脂肪組織にも骨髄と同様な多分化能をもった間葉系幹細胞の存在が報告され、採取の容易さと比較的大量に採取できることから注目されるようになってきた。これまで我々もヒト脂肪組織由来の間葉系幹細胞から肝細胞を誘導することに成功している。一般的に、間葉系幹細胞は創傷部や炎症部に集積しやすいとの報告があるが、腫瘍組織への遊走に関しては集積しやすいという報告や逆に集積しないという報告もみられて、定説がない。また間葉系幹細胞が産生する種々の因子により血管新生作用も指摘されている。間葉系幹細胞を治療手段として移植に用いる際に、血管新生作用による腫瘍の増殖促進効果が危惧される。特に癌切除後の場合は腫瘍細胞の残存の可能性を常に考慮する必要がある。したがって本研究では骨髄や脂肪由来の間葉系幹細胞と腫瘍組織や炎症部への遊走能や相互作用を検討することを目的とする。

B.研究方法

1 腫瘍細胞との相互作用の検討

前年度ヒト膵臓癌由来の細胞株MiaPaCa-2細胞を100万個 ヌードマウスの皮下に接種したのち、ヒト脂肪組織由来の間葉系幹細胞をCM-Dilで標識して150万個をマウスの尾静脈から注入したが、腫瘍の大きさはPBSのみの対照群と有意な差は認められなかった。そこで今回はMiaPaCa-2細胞100万個とCM-Dilで標識したヒト脂肪組織由来の間葉系幹細胞150万個を混合したものをヌードマウスの皮下に接種した。腫瘍の大きさを経時的に測定するとともに２週間後に組織標本を作製して移植細胞の生着を検討した。

2 皮膚炎症に対する免疫調整作用の検討

前年度にBalb/Cマウスの接触皮膚炎モデルを用いて脂肪組織由来の間葉系幹細胞の炎症部への遊走を検討したところ、特に炎症部への集積は認められなかったが、炎症反応自体は抑制され、何らかの免疫調整作用が認められた。DNFBを感作物質として用い、高濃度の物(0.5%, 50ul)をマウス腹部に塗布した後、惹起は５日後片方の耳に低濃度の物を塗布し(0.2%, 10ul)接触皮膚炎モデルを作成した。惹起６時間後に骨髄ないし脂肪組織由来の間葉系幹細胞を20, 50, 100, 150万個、尾静脈より注入した。接触皮膚炎の反応の強さは惹起後の経時的な耳の厚さの測定ならびに病理組織像で評価した。

3 内皮細胞への分化の検討

マウス（C57BL/6Njcl, ♀ 10 週齢）の鼠径部の脂肪塊を摘出して、0.12 % type1 collagenase を加え、37 ℃で振盪（ 30 min ）し、細胞を解離した。遠心（1300 rpm , 6 min , room temperature）し、1 × 10^6 / 10 cm dishに播種した。
内皮細胞の培地であるEGM-2 には FCS が 2 % 含まれているので、 基本培地のEBM-2 の改良を行った。EBM-2 中の FCS をそれぞれ ES 細胞の血清代替物である 2 % Knock out serum replacement（ KSR ） , 神経細胞の血清代替物である 2 % B27 , 1 % G5, 1 % N2 に置換して培養した。さらにDMEM/F12を基本培地とし、FGF2およびEGF, Angiotensin, VEGFの濃度を変えて添加し、血管内皮細胞の典型的なマーカーであるtie-2とvWF (von Willebrand Factor) の遺伝子発現を定量的PCR (real-time PCR) 法で検討した。DiIで蛍光ラベルしたアセチルLDL (2.5 μg/ml) を分化培地で2週間培養した細胞に4時間37°Cで作用させ、アセチルLDL-FITCの細胞内への取り込みを検討した。またC57BL6Jマウスの大腿筋に液体窒素で障害を与え、そこにGFPマウス由来脂肪由来の幹細胞から分化誘導した血管内皮細胞を筋注した。細胞は1x10^6注射し、7日後、14日後に組織を回収して、凍結切片を作成し、4%パラホルムアルデヒドで固定した後、GFP抗体、Vectastain ABC Rabit IgG Kitで反応させ、DABを用いて発色させた。

（倫理面への配慮）
ヒト由来の細胞は市販されているものあるいは手術時に同意の得られた組織から細胞を分離して用いた。マウス由来の細胞は施設の動物実験委員会において動物実験計画の承認を受け、動物の愛護上の配慮をもって、実験を行った。

C. 研究結果

1 腫瘍細胞との相互作用の検討

ヒト膵臓癌由来の細胞株MiaPaCa-2細胞とヒト脂肪組織由来の間葉系幹細胞を同時にヌードマウスへ移植し、経時的に腫瘍の大きさを計測したところ、MiaPaCa-2細胞単独の場合に比べて、腫瘍の増大が

認められた。（図１） ２週間後に組織切片を作製して、腫瘍部においてヒト脂肪組織由来の間葉系幹細胞の生着を検討した。細胞は腫瘍組織の中にDifuseに分布していたが、明らかに血管になっている所見は認められなかった。

２皮膚炎症対する免疫調整作用の検討

DNFBを腹部に塗布し、５日後に片方の耳に再塗布して炎症を惹起した。再塗布の６時間後に脂肪組織または骨髄由来の間葉系幹細胞を尾静脈より、投与して経過を観察した。対照群はPBSの投与とした。対照群では再塗布２日目に耳の厚さは最大となったが、脂肪組織または骨髄由来の間葉系幹細胞、20, 50, 100, 150万個いずれの投与群も耳の腫脹は抑制された。特に細胞数による抑制の程度に有意差は認められなかった。

３内皮細胞への分化の検討

脂肪組織由来の間葉系幹細胞を5回以上継代培養を繰り返すと、CD34陽性細胞は増殖せず、ほぼ均一な脂肪由来の幹細胞（繊維芽細胞様細胞）となった。

DMEM/F12を基本培地とし、FGF2（10ng/ml），2%ITS, EGM-2 BulletKitの添加によって、血清がない条件でも血管内皮様に分化誘導することができ、その約80%以上の細胞がアセチルLDL-FITCを細胞内に取り込んだ。（図２）

分化誘導していない脂肪由来の間葉系幹細胞は移植7日後では筋肉中に細胞塊として存在していたが、14日後にはほとんどその存在が認められなかった。一方、血管内皮細胞へと分化誘導した細胞は7日後には集合体として筋束外部に認められ、14日後には筋束外部で毛細血管様の環状構造を形成していることが分かった。（図３）

D. 考察

ヒト膵臓癌由来の細胞株を用いた研究では脂肪組織由来の間葉系幹細胞を同時に投与すると明らかな腫瘍の増大がみられ、増殖促進作用が認められた。間葉系細胞から細胞外マトリックスの産生がおこり、足場が確保された可能性と間葉系細胞からの何らかのサイトカインによる増殖刺激が考えられた。移植後２週間で腫瘍組織の間に間葉系細胞はdiffuseに分布していたが、明らかな血管内皮細胞様構造は認められなかった。我々はin vitroで脂肪由来の間葉系細胞を高率に血管内皮細胞に分化させることに成功しているが、今回のin vivoでの条件では血管内皮細胞に分化するのに適した条件ではなかったと思われた。

炎症部への間葉系幹細胞のホーミングを検討するために耳の接触性皮膚炎モデルを用いたが、脂肪組織由来の細胞と骨髄由来の細胞を移植した場合に著明に耳の腫脹が抑制されたので、移植細胞による免疫反応抑制作用が示唆された。しかし予想に反して、炎症を起こしている皮膚の局所には移植細胞がほとんど検出されなかった。これらの結果から、間葉系幹細胞に免疫反応を抑制する能力があることが示唆されたが、局所においての直接作用というよりは液性因子を介した間接的な作用である可能性が示唆された。そこで炎症細胞のサイトカイン産生などに影響を与えているのではないかと考え、移植細胞数による影響を検討したが、20万個と150万個を移植した場合でも明らかな差は認められなかった。免疫抑制作用についてはさらなる検討が必要だと思われた。

間葉系幹細胞は条件によって骨芽細胞や軟骨細胞になることが知られており、今回無血清培地で高率に血管内皮細胞に分化する系を構築できた。FGF2の添加が鍵を握っており、FGF2の存在下では、他の細胞成長因子であるEGFやVEGFをさらに加えても、内皮細胞の分化には影響を及ぼさなかった。誘導した細胞はin vitro とin vivoにおいて内皮細胞としての機能も確認することができた。ヒトの間葉系幹細胞にも応用できると血管新生療法に使用できる可能性が期待できる。

E. 結論

脂肪組織由来の間葉系細胞は膵臓がん由来の細胞に対して同時投与により腫瘍増殖を促進する作用が認められた。脂肪組織由来の間葉系細胞に抗炎症作用を認めたが、局所における直接作用は証明できなかった。効率よく内皮細胞に分化させる系を構築した。

F. 健康危険情報

なし。

G. 研究発表

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H. 知的財産権の出願・登録状況（予定を含む。）

1.特許取得

なし。

2. 実用新案登録

なし。

厚生労働科学研究費補助金（第3次対がん総合戦略研究事業）
分担研究報告書

がん幹細胞のエピゲノムプロファイリング

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研究要旨
MIAMI 網羅的なメチル化解析をおこない、GREM1 が乳癌幹細胞で脱メチル化され発現上昇していることがわかった。この遺伝子は血管新生作用があり浸潤性の乳癌で発現が高い。またメチル化がまばらな部位も検出できる MBD1DIP 法を開発した。

A．研究目的
iPS細胞の研究からもわかるようにがん幹細胞の特徴はエピゲノムにより裏打ちされている。この研究では独自のエピゲノムプロファイリング法を用いてがん幹細胞のエピゲノムの特徴を探る。

B．研究方法
自ら開発した網羅的メチル化解析法(MIAMI法)を用いて乳癌幹細胞を調べる。またメチル化がまばらなところも検索できる方法の確立をMBD1を用いておこなう。
（倫理面への配慮）

乳癌幹細胞で脱メチル化され発現上昇しているGREM1は正常細胞での発現が低く癌で発現が高いこと、血管新生作用があり浸潤性の乳癌で発現が高いこと、大腸癌のリスクを上げることが知られている。またMBD1DIP法は従来法で検出できないメチル化疎領域が検出できた。

E．結論
MIAMI法のスクリーニングでみつかったGREM1は癌幹細胞で脱メチル化され発現上昇していた。また開発したMBD1DIP法はメチル化のまばらなところの検出が可能である。

F．健康危険情報
（分担研究報告書には記入せずに、総括研究報告書にまとめて記入）

G．研究発表
1.　論文発表

必要な場合は群馬大学ゲノム倫理委員会の規定に従っておこなう。

C．研究結果
乳癌幹細胞をMCF7ADR細胞からCD44+/CD24-の細胞（癌幹細胞）とCD44+/CD24+の細胞（非癌幹細胞）をソーティングし、MIAMI法でメチル化が変化している遺伝子をスクリーニングした。その結果GREM1が脱メチル化され発現上昇していることがわかった。またメチル化結合タンパク質のMBD1を用いてメチル化がまばらな部位でもメチル化を検出できるMBD1DIP法を開発した。

D．考察

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H．知的財産権の出願・登録状況
（予定を含む。）
1. 特許取得
なし
2. 実用新案登録
なし
3.その他
なし

厚生労働科学研究費補助金（第3次対がん総合戦略研究事業）
分担研究報告書

がん幹細胞モデルの構築とそれを用いての薬剤スクリーニング

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研究要旨

発がん過程で重要な役割を担うことおよび幹細胞の機能維持にも関わることが知られているテロメレースの作用機序を解明することで、がん幹細胞を標的とした診断治療法の開発が期待されている。がん幹細胞機能維持に必須の複合体を同定し、がん幹細胞モデルを確立した（人工がん幹細胞モデル）。作製した人工がん幹細胞モデルは、顕著な造腫瘍能の亢進とがん幹細胞マーカーである、CD133、 CD44 の発現量が増加していた。今年度は、この人工がん幹細胞が有意な転移能および ES 細胞様の遺伝子発現プロファイルを有すること、および放射線感受性の低下を認めたことより、人工がん幹細胞モデルはがん幹細胞としての特徴を有することが確認された。次年度の薬剤スクリーニングのための基盤が確立された。

A．研究目的

人工がん幹細胞モデルは、がん幹細胞を標的とした治療薬のスクリーニングには必須のtoolである。昨年度までに、テロメレース触媒活性領域であるTERTが幹細胞因子であるnucleostemminおよびクロマチンリモデリング因子であるBrg1と複合体を形成しがん幹細胞の機能維持に関わることを見出していた。またこれらの因子を過剰発現して作製した人工細胞は造腫瘍能の亢進を示すことを確認していた。しかしながら、がん幹細胞としての特徴である転移能の亢進およびES細胞様の遺伝子発現様式、および抗がん剤や放射線への抵抗性の確認は行われていなかった。今年度は、がん幹細胞を標的とした薬剤スクリーニングを行うための人工がん幹細胞モデルの構築を完成させることを目的とする。

B．研究方法

すでに確立されたがん細胞株(HeLa細胞やMCF細胞)、あるいは正常細胞から人為的遺伝子導入により作製したがん細胞に対してレトロウイルスを用いてTERT-NS-Brg1複合体を導入することで人工がん幹細胞モデルを作製した。作製した人工がん幹細胞を用いて免疫不全マウスへの経尾静脈注射による肺転移能を評価。加えてがん浸潤転移において重要と考えられるEMT(epithelial-mesenchymal transition)関連タンパク質の発現の解析を行なった。ES細胞様の遺伝子発現様式については人工がん幹細胞を用いて、iPS因子であるc-Myc、Oct3/4、Klf4、Sox2の発現解析を行ない、放射線抵抗性については放射線照射後の細胞の増殖曲線を作製し放射性感受性の検討を行った。

（倫理面への配慮）特記事項なし。

C．研究結果

人工がん幹細胞では、免疫不全マウスにおける著明な肺転移能の亢進及びEMTマーカーであるTWIST、SNAIL、Vimentinの発現上昇、iPS因子であるc-Myc、Oct3/4、Klf4の発現上昇を認めた。加えて放射線療法抵抗性における検討では放射線感受性が約20%〜30%低下していることを確認した。

D．考察

がん幹細胞は造腫瘍能及び転移能の亢進を認めること、ES細胞様の遺伝子発現パターンを呈すること（Nagahama *et al.*, Cancer Research 2010）や、乳がんにおけるがん幹細胞分画がEMT関連タンパク質を高発現していること（Mani *et al.*, Nature ）、がん幹細胞の放射線療法抵抗性の分子機序としてDNA損傷応答機構の亢進が関わる（Bao *et al.*, Nature 2006）などの報告があり、転移能の亢進およびES細胞様の遺伝子発現プロファイル、放射線療法抵抗性はがん幹細胞の代表的な特徴であると考えられている。今年度の研究結果より、作製したがん幹細胞モデルも免疫不全マウスにおける転移能の亢進およびEMT関連ンパク質の発現上昇、ES細胞様の遺伝子発現パターン、放射線感受性の低下というがん幹細胞としての特徴を有することが確認された。

E．結論

確立した人工がん幹細胞モデル細胞株が顕著な造腫瘍能・転移能の亢進およびES細胞様遺伝子発現プロファイル、放射線療法抵抗性というがん幹細胞の特徴を有することが確認されたことより、この人工がん幹細胞モデルはがん幹細胞を標的とした治療薬のスクリーニングに資するtoolとして活用できることが示唆される。

F．健康危険情報

特記事項なし。
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(予定を含む。)

該当無し

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Generation of genetically modified rats from embryonic stem cells

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At present, genetically modified rats have not been generated from ES cells because stable ES cells and a suitable injection method are not available. To monitor the pluripotency of rat ES cells, we generated *Oct4*-Venus transgenic (Tg) rats via a conventional method, in which Venus is expressed by the *Oct4* promoter/enhancer. This monitoring system enabled us to define a significant condition of culture to establish authentic rat ES cells based on a combination of 20% FBS and cell signaling inhibitors for Rho-associated kinase, mitogen-activated protein kinase, TGF-β, and glycogen synthase kinase-3. The rat ES cells expressed ES cell markers such as *Oct4*, *Nanog*, *Sox2*, and *Rex1* and retained a normal karyotype. Embryoid bodies and teratomas were also produced from the rat ES cells. All six ES cell lines derived from three different rat strains successfully achieved germline transmission, which strongly depended on the presence of the inhibitors during the injection process. Most importantly, high-quality Tg rats possessing a correct transgene expression pattern were successfully generated via the selection of gene-manipulated ES cell clones through germline transmission. Our rat ES cells should be sufficiently able to receive gene targeting as well as Tg manipulation, thus providing valuable animal models for the study of human diseases.

genetic engineering | rat | embryonic stem cells

The laboratory rat was the earliest mammalian species domesticated for scientific research and has been used as an animal model in physiology, toxicology, nutrition, behavior, immunology, and neoplasia for over 150 y (1). Despite this history, rats lag far behind mice in functional genetic studies and the generation of knockout animal models reflecting human diseases because of the absence of germline-competent rat ES cells, which are vital in a reverse genetics approach (2, 3). Recently, gene-targeting rats were created by the zinc finger nuclease strategy (4). However, the system is not available for most researchers because a special technique is required to make algorithm-based sequence-specific DNA nucleases. Thus, establishment of rat ES cells has been desired to produce gene-targeting rats, such as mutant mice, routinely.

Although we established rat ES cell lines with chimeric contribution, none could complete germline transmission (5). Soon after our report, other groups succeeded in establishing rat ES cells with germline transmission by using 2i, mitogen-activated protein kinase (MEK) inhibitor PD0325901, and glycogen synthase kinase-3 (GSK3) inhibitor CHIR99021 (6, 7). The 2i is widely used in the establishment of ES cells or induced pluripotent stem (iPS) cells in mice (8, 9), rats (6, 7, 10), and humans (10). Thus, the inhibition of MEK and GSK3 has been thought to maintain a ground state of pluripotency in various species. Rat iPS cells with chimeric contribution were established by using an inhibitor of type 1 TGF-β receptor Alk5 (A-83-01) with the 2i, although germline transmission was not accomplished (10). Furthermore, a combination of MEK and ALK5 inhibitors dramatically improved the efficiency of iPS cell generation from human fibroblasts (11). These reports indicate that the inhibition of TGF-β signaling also plays a key role in pluripotency.

It is known that rat ES cells present critical problems in that undifferentiated cells cannot proliferate from single cells after enzymatic dissociation (5) and that chromosomal instability is caused by long-term culture, resulting in the failure of germline transmission (5–7). Recently, Watanabe et al. found that a Rho-associated kinase inhibitor Y-27632 (12) blocks apoptosis and enhances the proliferation of human ES cells after their dissociation into single cells by enzymatic treatment (13). The propagated ES cells cultured by Y-27632 were positive for alkaline phosphatase (ALP) and marker genes such as E-cadherin, Oct4, and SSEA4, and the number of chromosomes was normally maintained during long-term culture (13). These recent reports indicate the suitability of cell signaling inhibitors in the establishment of rat ES cells.

To generate genetically modified rats, highly potent ES cells that can stably contribute to germline chimeras have to be established. As a first step, we generated *Oct4*-Venus transgenic (Tg) rats, in which Venus [YFP mutant (14)] is expressed by the *Oct4* promoter/enhancer. This Tg line enables us to monitor the pluripotency of rat ES cells during the process of establishment. We addressed suitable combinations of the signaling inhibitors based on a culture medium that included 20% FBS. As a result, the use of a combination of four inhibitors, Y-27632, PD0325901, A-83-01, and CHIR99021 (termed YPAC), allowed the establishment of authentic rat ES cells and appeared necessary in the blastocyst injection process for the generation of germline chimeras. Finally, we report that high-quality Tg rats retaining reproductive ability can be generated from rat ES cells.

Results

YPAC Maintains Pluripotency in the Outgrowths of *Oct4*-Venus Tg Blastocysts. We first generated a Tg rat carrying an *Oct4*-Venus fluorescence reporter to monitor pluripotency during establishment of rat ES cells and to investigate development of the ES cells into germ cells in fetal gonads of chimeras. The 3.9-kb *Oct4* (also known as *Pou5f1*) promoter includes both the proximal enhancer and distal enhancer, which gives *Oct4* expression in morula, inner cell mass (ICM), epiblast, primordial germ cells (PGCs), and ES cells (15). In the Tg embryo, Venus was detected specifically in PGCs in the gonad (Fig. S1). This result corresponds to previous reports regarding *Oct4*-reporter Tg mice (16).

Outgrowths were examined from the Tg blastocysts in a basic medium containing 20% FBS, which is generally used for mouse ES cell culture, with or without YPAC. In its absence, Venus fluorescence was decreased at day 3 after plating and disappeared at day 7 despite the fact that ES-like cells propagated and formed a domed structure similar to the mouse ES cell colony (Fig. 1*A*). In the presence of YPAC, ICM cells rapidly propagated while maintaining Venus fluorescence even at day 7. The fluorescence was not observed in differentiated cells (Fig. 1*B*). The expression levels of ES cell marker genes *Oct4*, *Nanog*, *Sox2*, and *Rex1* in ICM cells with YPAC were higher than those without YPAC (Fig. 1*C*).

Author contributions: M.K. and T.O. designed research; M.K. performed research; M.K. analyzed data; and M.K. and T.O. wrote the paper.

The authors declare no conflict of interest.

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DEVELOPMENTAL BIOLOGY

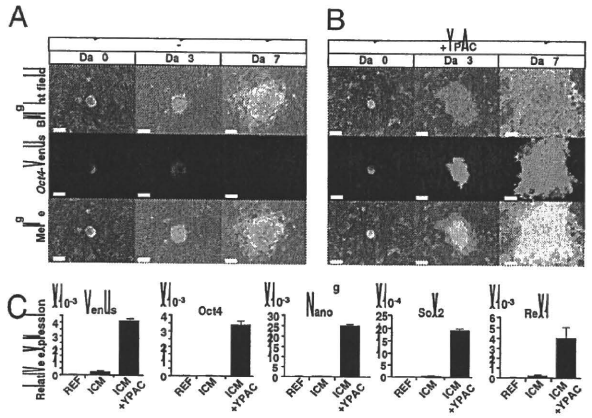

Fig. 1. Outgrowth of ICM cells in YPAC medium. Outgrowth of blastocysts in −YPAC (*A*) or +YPAC (*B*) medium. E4.5 blastocysts were plated onto mitotically inactivated MEFs. (*C*) qPCR analysis of Venus, *Oct4*, *Nanog*, *Sox2*, and *Rex1* in ICM cells. Seven days after plating, RNAs were extracted from domed segments of ICM cells derived from seven or four blastocysts in −YPAC or +YPAC medium, respectively. Transcript levels were normalized to *Gapdh* levels. Data are the mean ± SD of one biological sample assayed in three independent experiments and represent relative expression levels of indicated genes in REFs, ICM (−YPAC), and ICM (+YPAC). (Scale bars: 100 μm.)

In its absence, the decrease of *Oct4* mRNA was parallel to that of Venus mRNA and fluorescence. In the YPAC condition, blastocyst outgrowth was observed in 51 samples for all the tested embryos regardless of the strains (Table 1). The blastocyst strains were derived from a hybrid of Tg Wistar and wild-type Wistar (TgWW, albino), wild-type Wistar (WW, albino), Long–Evans agouti [LEA (LL, agouti)], or a hybrid of Tg Wistar and LEA (TgWL, agouti).

Small Molecules Enable Efficient Derivation and Maintenance of Rat ES Cells. The outgrowths were dissociated into small pieces and replated in the same mouse embryonic fibroblasts (MEFs)/YPAC condition. After undifferentiated colonies appeared, they were split into single cells by Accutase (Innovative Cell Technologies, Inc.). These cells attached on the MEFs and formed domed colonies, which can be passaged continuously (Fig. 2*A Upper Left*). Although most of the ES cells showed ALP activity (Fig. 2*B Left*) and Oct4 protein expression (Fig. 2*D Left*) even after long passages, Venus fluorescence became weak or negative (Fig. 2*A Lower Left*). The expression pattern of Venus mRNA was not parallel to that of *Oct4* between TgWL1 and TgWW1 cell lines (Fig. 2*C*). These results suggest that the function of the *Oct4*-Venus transgene is unavailable in rat ES cell lines. The long-passaged rat ES cells might receive epigenetic silencing effects.

The ES cell lines maintained higher mRNA levels of ES cell marker genes *Oct4*, *Sox2*, *Nanog*, and *Rex1* compared with rat embryonic fibroblasts (REFs) (Fig. 2*C*). Microarray analyses also indicated that global gene expression was remarkably different between ES cells and REFs but similar between the three ES cell lines TgWL1, TgWW1, and LL1 (Fig. S2). Nanog and Sox2 proteins were also detected in ES cells (Fig. 2*D*). The karyotypes of 50 cells were analyzed by G-band staining. Most of the cells exhibited a normal chromosomal number of 42 in TgWL1 (70%, XX, P14), TgWL2 (84%, XX, P7; Fig. 2*E*), TgWW1 (92%, XX, P5), and LL1 (84%, XX, P6). TgWW1 cells (2.6×10^6) could form a teratoma 34 d after transplantation under the skin of an immunodeficient SCID mouse. A histological examination showed that the tumor contained all three germ layers, including the intestinal epithelium (endoderm), cartilage (mesoderm), and neuronal rosette (ectoderm) (Fig. 2*F*).

Table 1. Establishment of rat ES cells from blastocysts in YPAC medium

Strain	No. ICMs	Outgrowth†	Continue	Cell line‡
TgWL	2	2	2	2
TgWW	15	15	1	1
WW	9	9	1	1
LL	19	19	2	2
TgWW*	3	3	2	2
WW*	3	3	0	0
Total	51	51 (100%)	8	8 (100%)

*Specific serum was used (FBS for MEF culture; EQUITECH-BIO, Inc.).
†Outgrowth refers to the expansion of the ICM.
‡Cell line refers to continuous culture of at least seven passages. Single-cell passage was begun at passages 1–3. Domed colonies with undifferentiated cells are continuously formed from single cells.

To confirm the effect of Y, the rat ES cells were cultured in a PAC medium. Under this condition, sparse colonies appeared because of a failure in the adherence process of single cells on MEFs, although the colonies kept undifferentiated morphology and ALP activity (Fig. 2*A Middle* and *B Middle*). Although only Y enabled most of the single cells to adhere on MEFs and to proliferate, they were differentiated and did not show ALP activity (Fig. 2*A Right* and *B Right*).

The classic method to induce ES cell differentiation is to allow the cells to grow in suspension and to form 3D aggregates known as embryoid bodies (EBs) (17). Dissociated ES cells were plated into low-cell-binding dishes in the basal medium. EBs could be formed from the ES cells at a much lower efficiency compared with their formation from mouse ES cells (Fig. 2*E*). The expression of marker genes was decreased during the process of EB differentiation (Fig. 2*I*). In the presence of PAC, cells aggregated with high efficiency and formed a clear 3D structure (Fig. 2*F*). The EBs with PAC at day 7 still sustained high expression levels of the marker genes (Fig. 2*I*). These results suggest that PAC enables ES cells to maintain pluripotency, whereas for rat ES cells to adhere on MEFs, Y is necessary.

YPAC Injection Engenders Germline Chimeras. First, we produced stable transfectant ES cells expressing cyan fluorescence from a CAG-AmCyan1 transgene to monitor cell fate in the blastocyst after injection or chimerism in fetuses (Fig. 3*A*). Before generation of the chimera, the potential of YPAC was investigated during the injection and blastocyst incubation processes because the rat ES cells tended to differentiate easily in the absence of inhibitors (Fig. 2*A*, *B*, and *E*). There was no difference between normal and YPAC injection 5 h after incubation; in both cases, several cyan-positive cells adhered on the ICM and trophectoderm. However, 30 h after incubation, few cyan-positive cells existed in the blastocysts in the absence of YPAC, whereas in its presence, several cells remained on the ICM surface. Furthermore, blastocyst shape was maintained by the addition of YPAC even after incubation for 30 h (Fig. 3*B*). This result suggests that administration of YPAC during the injection process causes both ES cells and recipient blastocysts to block differentiation or apoptosis. This YPAC injection method enabled generation of chimeric embryos showing positivity for cyan but negativity for Venus in the surface of skin and kidney. Venus-positive cells were detected specifically in the gonads, showing the successful development of the ES cells into germ cells (Fig. 3*C*). We also succeeded in generating germline chimeras using all other cell lines by detecting Venus fluorescence in the fetal gonad (Table 2). The germline chimeras were detected in 2 of 12 fetuses by using long-cultured TgWL2 cells at passage 22 (Table 2 and Fig. S3).

To investigate the pluripotent ability of ES cells, we carried out a single-cell injection into a blastocyst. After injection of the

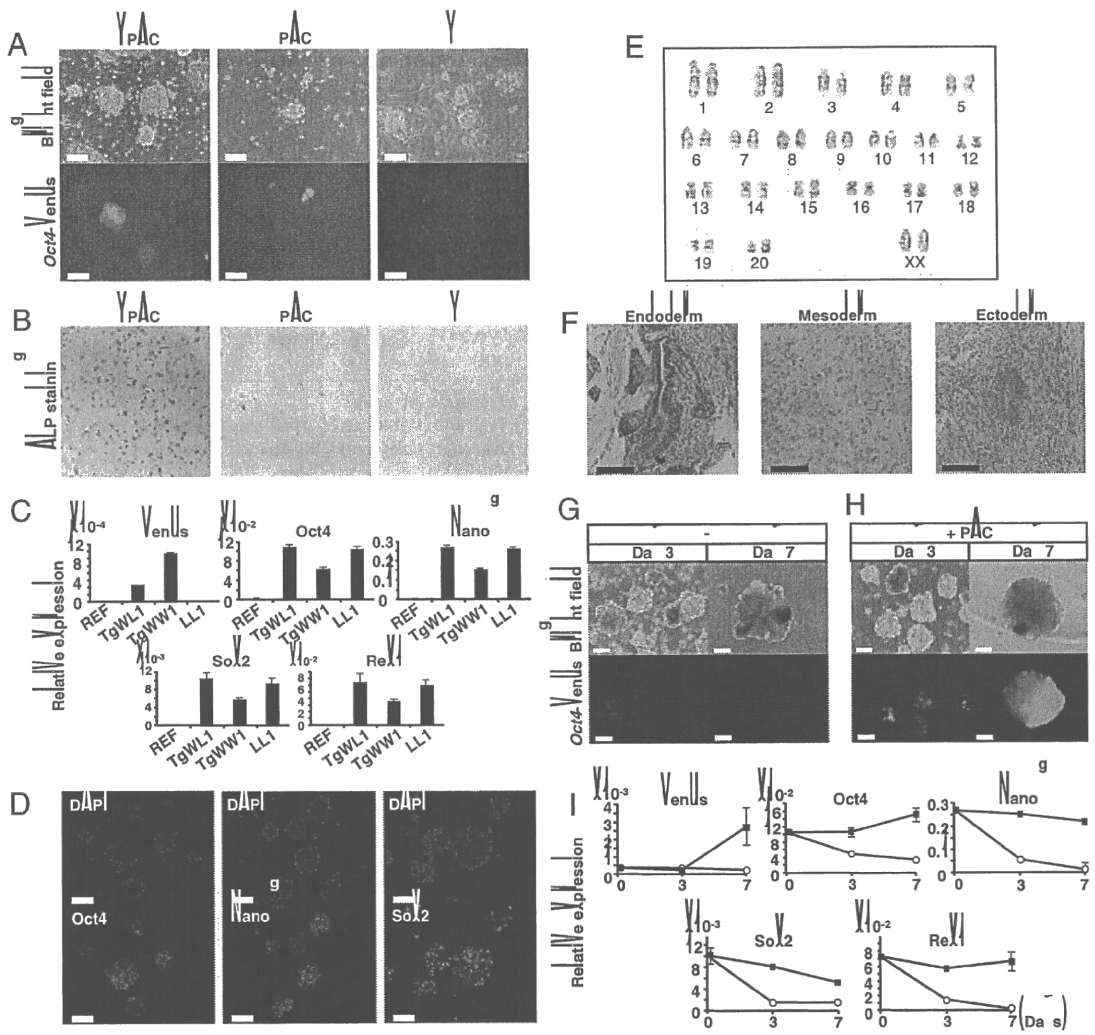

Fig. 2. Characterization of rat ES cells. Effect of Y-27632 (*A*) and ALP (*B*) staining. Dissociated single cells (1×10^5 TgWW1, passage 6) were plated into a well of six-well plates under the condition of MEFs with YPAC (*Left*), PCA (*Center*), or Y (*Right*). (*B*) ALP staining in these cells. (*C*) qPCR analysis of Venus, *Oct4*, *Nanog*, *Sox2*, and *Rex1* in rat ES cell lines. Transcript levels were normalized to *Gapdh* levels. Data are the mean ± SD of one biological sample assayed in three independent experiments and represent the relative expression levels of indicated genes in REF, TgWL1, TgWW1, and LL1. (*D*) Immunofluorescence staining for *Oct4*, *Nanog*, and *Sox2* in rat ES cells. (*E*) Cytogenetic analysis in rat ES cells by G-band staining. Representative data of TgWL2 at passage 7 indicate a chromosomal number of 42, including an XX gender chromosome. (*F*) Histological sections of a teratoma derived from a TgWW1 ES cell line showing three germ layers. Embryoid bodies (TgWL1) were produced in a basic ES cell medium with (*H*) or without (*G*) three PAC inhibitors, excluding Y-27632. A time-course experiment was performed, and the EBs were observed at days 3 and 7. (*I*) qPCR analysis of Venus, *Oct4*, *Nanog*, *Sox2*, and *Rex1* in EBs. Transcript levels were normalized to *Gapdh* levels. Data are the mean ± SD of one biological sample assayed in three independent experiments and represent the relative expression levels of indicated genes in EBs produced without inhibitors (○) or with PAC (■) at days 0, 3, and 7. (Scale bars: 100 μm.)

TgWW1 cell at passage 9, the single cell attached to the internal surface of the blastocyst (Fig. 3*D*). In an embryo day (E) 16.0 fetus gonad, germ cell differentiation was confirmed by the detection of Venus fluorescence (Fig. 3*E*).

To generate coat-color chimeras, TgWL1 cells were injected into Wistar blastocysts using the YPAC injection method. Eight of 23 coat-color chimeras were obtained from the TgWL1 cell line at passage 11 or 12 (Fig. 3*F Right* and Table 3). Without the YPAC injection method, a coat-color chimera was hardly generated despite the fact that the same cell line, TgWL1, was used at earlier passages 6–8 (Fig. 3*F Left* and Table 3). Only 1 male chimera of 44 pups was obtained, but the chimerism was very sparse (Fig. S4). The generation of coat-color chimeras was successful in all six cell lines (Table 3). Those from cell line TgWW1 or LL1 are shown in Fig. S5. After mating with male rats, germline transmission was accomplished in adult female chimeras derived from all six cell lines independent of rat strains (Fig. 3*G* and Table 3). Genotyping analysis indicated that the *Oct4*-Venus transgene of the ES cells (TgWL2) was transmitted to filial (F)1 germline offspring with an agouti coat color (Fig. 3*H*).

Generation of ES Cell-Derived Tg Rats. We proposed to generate ES cell-derived transgenic (esTg) rats harboring the *Oct4*-Venus transgene, which shows a correct Venus expression pattern similar to Oct4 protein (Fig. 2*D*). After introduction of the *Oct4*-Venus transgene containing the same *Oct4* promoter/enhancer region as used in the generation of the conventional transgenic (cvTg) rats, 15 Venus-positive colonies (LL2 line) were picked up. After two passages, silencing of Venus gene expression occurred in 13 of 15 clones, resulting in an apparent heterogeneity in the fluorescence of Venus-positive clones (Fig. 4*A*, arrowheads), whereas only 2 clones kept homogenous Venus fluorescence (Fig. 4*B*). Chimeric

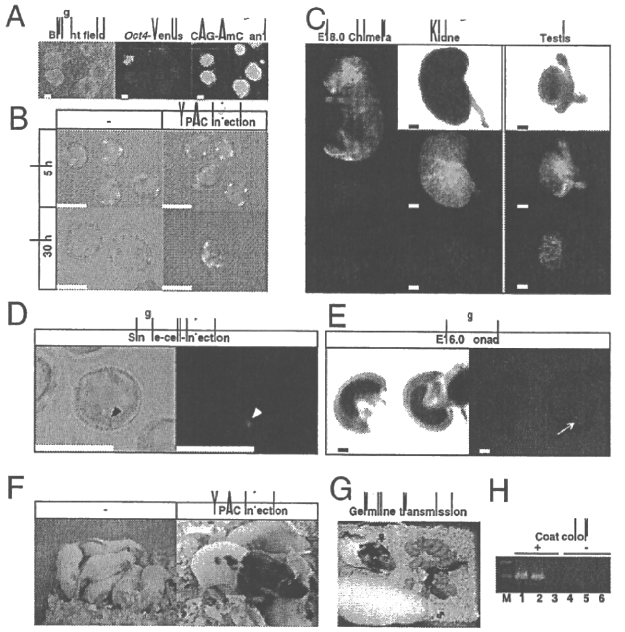

Fig. 3. Generation of germline chimeras by YPAC injection method. (*A*) Expression of AmCyan1 in stable transfectant clones (TgWW1 + C) generated by nucleofection with CAG-AmCyan1 plasmids. (*B*) Effect of YPAC during injection process. The basic ES cell medium with (*Right*) or without (*Left*) YPAC was used during the processes: injection of TgWW1 + C cells into blastocysts and incubation of the blastocysts for 30 h. (*C*) Generation of germline chimeras in embryos. TgWW1 + C cells were injected into Wistar blastocysts. Venus or AmCyan1 fluorescence was observed in E18.0 whole embryo, kidney, and testis. (*D* and *E*) Generation of germline chimeras by single-cell injection. (*D*) A single cell (TgWW1) was injected into a blastocyst. The image shows the injected blastocyst 3 h after incubation. The arrowhead indicates the injected single cell. (*E*) Venus-positive germ cells were detected in gonad at E16.0 (arrow). (*F*) Generation of coat-color chimeras by the YPAC injection. (*G*) Germline transmission in adult chimeras. The chimera (TgWL1) was mated with a Wistar male rat. Germline pups (4 of 16) were confirmed by an agouti coat color. (*H*) Genotyping analysis of F1 offspring of female chimera (TgWL2). The Venus region was amplified by PCR from genomic DNA of tail. M, 100-bp DNA marker; 1, 2, and 3, germline offspring with an agouti coat color; 4, 5, and 6, coat color-negative (albino) offspring. (Scale bars, *A*, *B*, and *D*: 100 μm; *C* and *E*: 300 μm.)

rats were produced via injection of the stable clone into Wistar blastocysts. Furthermore, germline transmission with the *Oct4*-Venus transgene was accomplished in the female chimeras (Fig. 4*C* and Table 3). The esTg embryos at 16.0 days postcoitum (dpc) exhibited Venus fluorescence in the gonads (Fig. 4*D*). The esTg rats were able to mature to adults without apparent abnormalities and had normal reproductive ability. We established ES cell lines from esTg blastocysts to confirm an expression pattern of Venus fluorescence. During outgrowth, their Venus expression pattern (Fig. 4*E*) was similar to that of cvTg blastocysts (Fig. 1*B*). However, long-passaged esTg cell lines (n = 3) maintained stable Venus expression in the ES cells (Fig. 4*F*). This result indicates our success in generating high-quality esTg rats possessing a correct expression pattern of Venus under the *Oct4* promoter/enhancer.

Discussion

Our results demonstrated that the use of a combination of serum and cell signaling inhibitors during outgrowth, cell culture, and blastocyst injection leads to the generation of germline chimeras with extremely high efficiency. Furthermore, we generated genetically modified rats from ES cells, termed esTg rats, growing up healthily and retaining reproductive ability. The advantage of this technology of using rat ES cells is that we can select Tg ES

Table 2. Summary of germline chimeras: Germ cell development in fetal gonad judged by Venus fluorescence

Cell line (gender)	Passage no.	Host blastocyst	Injected embryos	Fetal no.	Germline chimera
TgWL2 (XX)	6	LEA	43	9	1M1F
	22	Wistar	13	3	1F
		Wistar/LEA	23	9	1F
TgWW1 (XX)	6, 7	Wistar	53	9	2M7F
TgWW1 + C (XX)	8	Wistar	46	9	1M1M or F
TgWW1s (XX)	9	Wistar	35	8	1F
TgWW2* (XX)	8	Wistar	28	1	1M

F, female; M, male.
*ES cell line established by using specific serum. TgWW1 + C refers to a stable transfectant possessing CAG-AmCyan1 transgene.

cell clones that possess a correct gene expression pattern of the transgene. Our *Oct4*-Venus esTg rats will be useful for the generation of iPS cells as a pluripotency monitoring system with respect to previous work in mice (9, 18, 19). For further study, addressing the mechanism of the silencing effect on the transgenes should be crucial.

The complete generation of esTg rats might be based on the use of a culture medium containing 20% serum and YPAC, which might provide strong protectection from cell damage during gene introduction with electrical stimuli and maintain pluripotency with a stable karyotype during the cloning and expansion process. To support viability, serum was temporally used in a previous report when rat ES cells were electroporated and cultured overnight in a 2i medium (6). Such efforts are not necessary with our rat ES cells, which are tolerant to the damage induced by gene introduction because of the presence of 20% serum in the YPAC medium. Furthermore, we have confirmed that drug selection through the use of G418 is efficient in rat ES cells for generating genetically modified rats.

Previous work has suggested that the failure in the establishment of authentic rat ES cells over the past 2 decades was attributable to the presence of serum (6, 7). Indeed, serum may contain various kinds of nutrient factors as well as differentiation factors for rat ES cells (20). The reason why we succeeded in the establishment of such significant pluripotent cell lines might be attributable not only to the signaling inhibitors shielding ES cells from differentiation but to the use of the nutrients in the serum. Monitoring serum quality for better ES cell culturing is extremely important. Nevertheless, our combination of YPAC and different serum, which is used for culturing MEFs, allowed stable establishment of rat ES cell lines. Our success is thus partly attributable to the strong potential of YPAC in the maintenance of ES cells regardless of differentiation factors under different culture conditions.

It is noteworthy that leukemia inhibitory factor (LIF) was not necessary in our culture medium, although recent reports have suggested that its addition improved rat ES or rat iPS cell ability to suppress differentiation (6, 7, 10). It has been shown that LIF is the key cytokine secreted by feeders in supporting mouse ES cell self-renewal (21, 22) and that LIF was able to replace the requirement for feeders in propagation (23, 24). Ying et al. (8) demonstrated that a combination of PD0325901 and CHIR99021 enabled mouse ES cells to maintain pluripotency by substituting LIF, feeders, and serum. Considering these reports, the addition of LIF in our culture condition might be dispensable because of the inclusion of serum, MEFs, and the two inhibitors. Actually, the expression level of *Tbx3*, which is involved in mediating LIF signaling (25), was up-regulated in the rat ES cells. Moreover, we found that the expression of a suppressor of cytokine signaling 3 (*SOCS3*), which is one of the STAT3's direct target genes (26),

Table 3. Summary of germline chimeras: Chimeras and germline transmission judged by coat color of F1 pups

Cell line (gender)	Passage no.	Host blastocyst	Injected embryos	Pup no.	Chimera no.	Mating no.	Germline chimera
–YPAC injection							
TgWL1 (XX)	6–8	Wistar	226	44	1M*	0	—
+YPAC injection							
TgWL1 (XX)	11, 12	Wistar	123	23	3M5F	1M3F	1F
TgWL2 (XX)	4, 6	Wistar	70	10	2M3F	1M3F	2F
TgWW1 (XX)	9	Wistar/ LEA	79	19	5M3F	3F	1F
WW1 (XX)	10	LEA	27	7	2M1F	1F	1F
LL1 (XX)	4, 6	Wistar	107	13	3F	2F	1F
LL2 (XX)	9	Wistar	52	6	3F	3F	2F

F, female; M, male.
*Coat-color contribution is sparse (Fig. S5).

was up-regulated after stimulation with rat LIF (27) even in the YPAC medium. Thus, it seems possible to improve the culture condition further by the administration of rat LIF.

The six established ES cell lines in this work were all female. This result does not correspond to mice, because most of the mouse ES cell lines are male. In our present study, we continued to culture rat ES cell lines exhibiting rapid cell proliferation, resulting in the establishment of six female lines. Thus, we speculate that female blastocysts are suitable for the establishment of rat ES cells or that the addition of MEK and GSK3 inhibitors to the culture medium facilitates female-specific rapid cell growth in rat ES cells. A previous study using MEK and GSK3 inhibitors also reported that six of seven rat ES cell lines were female (6).

Although two groups reported the establishment of authentic rat ES cells, only one of several cell lines accomplished germline transmission in each group (6, 7). So far, there is no report of successful generation of knockout/knockin rats from ES cells. Thus, trials to produce more potent cell lines and to find the optimal combination of rat strains for donor ES cells, host blastocysts, and recipient foster female animals remain to be addressed (6, 7). In this study, our YPAC culture and injection method overcame the difficulty of completing germline transmission in all six ES cell lines independent of rat strains. The YPAC condition will enable the selection of preferable rat strains for the generation of genetically modified rats from ES cells, bringing great advantages to research for strain-specific disease models. We believe that the availability of our rat ES cells and the YPAC injection technique will also open up a valuable platform for routinely generating knockout/knockin rats, holding out the promise for generation of previously undescribed disease models.

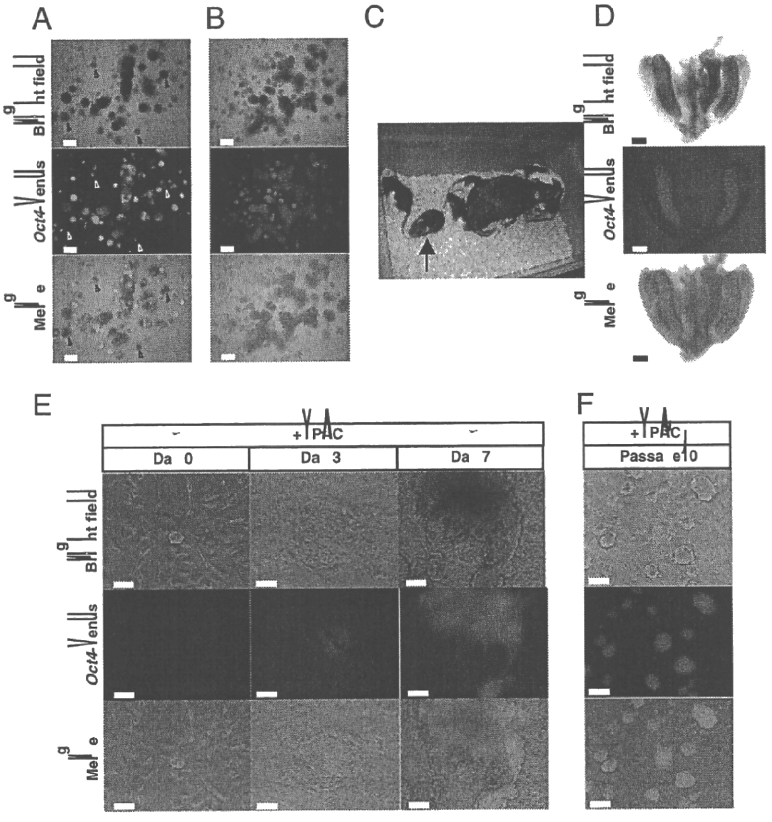

Fig. 4. Generation of Tg rats from ES cells. (*A* and *B*) Cloning and expansion of *Oct4*-Venus transfectants. An *Oct4*-Venus transgene was introduced into ES cells (LL2) at passage 5. Venus-positive clones were passaged without drug selection. (*A*) Arrowheads indicate ES cells with Venus expression silenced. (*B*) ES cells with homogeneous expression of *Oct4*-Venus. (*C*) Generation of Tg rats from ES cell clone displaying homogeneous expression of *Oct4*-Venus. An arrow indicates esTg rats through germline transmission from the chimeric rat. (*D*) Venus fluorescence in gonads of an esTg embryo at 16.0 days postcoitum (dpc). (*E*) Outgrowth of esTg blastocyst in YPAC medium. (*F*) Rat ES cell line derived from an esTg blastocyst. The expression of *Oct4*-Venus did not receive a silencing effect even after 10 passages. (Scale bars, *A*, *B*, and *D*: 300 μm; *E* and *F*: 100 μm.)

Materials and Methods

Media, Feeder, Animals, and Primers. YPAC medium was prepared by the addition of the four respective inhibitors [10 μM Y-27632 (WAKO), 1 μM PD0325901 (Axon Medchem), 0.5 μM A-83-01 (TOCRIS), and 3 μM CHIR99021 (Axon Medchem)] to basic medium. The basic medium is composed of DMEM [including 110 mg/L sodium pyruvate and 200 mM GlutaMAX (GIBCO)], 20% (vol/vol) FBS (ES Cell Qualified FBS, Lot No. 1204059; GIBCO), 0.1 mM 2-mercaptoethanol (SIGMA), 1% nonessential amino acid stock (GIBCO), and 1× antibiotic antimycotic (GIBCO). Mitomycin C-treated MEFs resistant to neomycin (Millipore) were used as feeders and maintained in DMEM/10% (vol/vol) FBS (Lot No. SFB30-1502; EQUITECH-BIO, Inc.) medium with 1× antibiotic antimycotic. Animal experiments were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. The Wistar strain, LEA strain, or a hybrid of the Wistar and LEA strain was used in this work. All the primer sequences are listed in Table S1.

Generation of *Oct4*-Venus Tg Rats via a Conventional Method. The DNA fragment of the *Oct4* promoter region (3.9 kb) was obtained by PCR using KOD Version 2 DNA polymerase (Toyobo) from Wistar rat genomic DNA and was inserted into a pCS2-Venus plasmid (14). The *Oct4* promoter-Venus (*Oct4*-Venus) DNA fragment was injected into pronuclei of fertilized eggs in a Wistar rat strain (Oriental Yeast Co., Ltd.). Six Tg-positive founders were obtained from 222 injected fertilized eggs.

Establishment of Rat ES Cells from Blastocysts. Rat blastocysts were gently flushed out from the uteri of E4.5- or E5.0-timed pregnant rats with basic ES medium. After removal of the zona with acid Tyrode's solution (Ark Resource Co., Ltd.), whole blastocysts were plated onto six-well plates and cultured on MEFs in the basic ES medium with or without YPAC. After around 7 d, the blastocyst outgrowths were cut into pieces and replated under the same YPAC conditions. Emerging ES cell colonies were then dissociated using Accutase and were expanded. Established ES cell lines were routinely maintained under MEF-YPAC conditions and passaged every 3–4 d. Floated colonies were also passaged. Cells were cryopreserved and recovered by conventional procedures using YPAC medium and DMSO as a cryoprotectant. In the cell line of TgWL1 or TgWL2, 1,000 U/mL rat LIF (25) was added to the YPAC medium until passage 4 or 3, respectively.

Quantitative PCR Analysis. Total RNA was isolated using ISOGEN (Nippongene). cDNA was synthesized with 2 μg of the total RNA using Super Script III RT (Invitrogen) and oligo-dT primer (Invitrogen). cDNAs were used for PCR utilizing Platinum SYBR Green qPCR SuperMix UDG (Invitrogen). Optimization of the quantitative (q)RT-PCR was performed according to the manufacturer's instructions (PE Applied Biosystems). All quantitations were normalized to an endogenous control *GAPDH*.

ALP and Immunofluorescent Staining. Cells were fixed in 4% (wt/vol) paraformaldehyde. ALP staining was performed with Vector Blue substrate (Vector Labs) according to the manufacturer's instructions. Primary antibodies used include the following: Oct4 (C-10, 1:20; Santa Cruz), Nanog (1:20; ReproCell), and Sox2 (1:20; BioLegend). Alexa Fluor fluorescent secondary antibodies (Invitrogen) were used at a 1:500 dilution. Nuclei were visualized with DAPI staining.

Teratoma Formation. The 2.6×10^6 TgWW1 cells (passage 5) were injected under the skin of immunodeficient mice. Teratomas were obtained 34 d after the injection. They were embedded in paraffin wax and stained with H&E.

EB Formation. After ES cells were split into single cells using Accutase, they were cultured in a basal ES medium with or without three-inhibitor PAC, excluding Y-27632, on a low-cell-binding dish (NUNC). RNAs were extracted from the EBs at day 3 or 7, followed by qPCR examination.

Blastocyst Injection. The blastocysts from E4.5-timed pregnant rats were placed into 500 μL of injection medium composed of YPAC (or PAC) and basal ES cell medium without antibiotic antimycotic, and they were then incubated for 2–3 h. The well-expanded blastocysts were used for microinjection. For ES cell preparation, 10–20 domed or floated colonies were picked up by hand-made capillary and treated with an Accutase droplet for 5 min, followed by being split into single cells in a droplet of injection medium. The cells were transferred in 500 μL of the injection medium and incubated for 30–60 min at room temperature. After centrifugation, ES cells were transferred into a droplet of the injection medium under mineral oil (SIGMA). Ten to 15 ES cells were injected into each blastocyst and incubated at 37 °C for 3–5 h in the injection medium to allow the recovery of embryos. Ten to 20 embryos were then transferred into the uterine horn of each E3.5-timed pseudopregnant female rat. Chimeric rats were identified by coat color. Germline transmission was confirmed by the F1 rat coat color resulting from mating of chimera or *Oct4*-Venus fluorescence in germ cells in the fetal gonad. Genotyping of animals was carried out by PCR on tail DNA.

Gene Transfection of Rat ES Cells. For nucleofection, 5 μg of CAG-AmCyan1 or 10 μg of *Oct4*-Venus plasmid linearized by SalI was transfected into 3.2×10^6 TgWW1 or 3×10^6 LL2 rat ES cells, respectively, using a Mouse ES Cell Nucleofector Kit (Amaxa, Inc.). The cells were plated on MEFs in the YPAC medium with 2% (vol/vol) matrigel (BD Biosciences). Three combined colonies of CAG-AmCyan1 or a single colony of *Oct4*-Venus transfectant, positive for cyan or green fluorescence, respectively, was picked up by hand-made capillary and expanded without drug selection.

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Review Article

Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis

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In the past several years, the importance of microRNA (miRNA) in cancer cells has been recognized. Proper control of miRNA expression is essential for maintaining a steady state of the cellular machinery. Recently, it was discovered that extracellular miRNAs circulate in the blood of both healthy and diseased patients, although ribonuclease is present in both plasma and serum. Most of the circulating miRNAs are included in lipid or lipoprotein complexes, such as apoptotic bodies, microvesicles, or exosomes, and are, therefore, highly stable. The existence of circulating miRNAs in the blood of cancer patients has raised the possibility that miRNAs may serve as a novel diagnostic marker. However, the secretory mechanism and biological function, as well as the meaning of the existence of extracellular miRNAs, remain largely unclear. In this review, we summarize the usefulness of circulating miRNA for cancer diagnosis, prognosis, and therapeutics. Furthermore, we propose a mechanism for the secretion and incorporation of miRNA into the cells. (*Cancer Sci* 2010; 101: 2087–2092)

In 1993, Ambros and colleagues discovered a gene, *lin-4*, which affected development in *Caenorhabditis elegans* (*C. elegans*); they found that its product was a small non-protein coding RNA, microRNA (miRNA).[1] miRNAs are small regulatory RNA molecules that modulate the expression of their target genes and play important roles in a variety of physiological and pathological processes, such as development, differentiation, cell proliferation, apoptosis, and stress responses.[2] miRNA biogenesis requires several post-transcriptional processing steps to yield the functional mature miRNA.[3] Currently, there are 940 mature human miRNA sequences listed in the miRNA registry (Sanger miRBase release 15; http://www.mirbase.org/). Over the past several years, many miRNAs have been investigated in various human cancers.[4] The deregulation of the expression of miRNAs has been shown to contribute to cancer development through various kinds of mechanisms, including deletions, amplifications, or mutations involving miRNA loci, epigenetic silencing, the dysregulation of transcription factors that target specific miRNAs, or the inhibition of processing. miRNA expression profiling is of increasing importance as a useful diagnostic and prognostic tool, and many studies have indicated that miRNAs act as either an oncogene or a tumor suppressor. Recently, the discovery of miRNAs as novel biomarkers in serum or plasma represented a new approach for diagnostic screening in blood. Since current approaches to cancer screening are invasive and it is difficult to detect cancer in its early stages, it is important to understand the characteristics of secretory miRNAs and their usefulness in cancer detection. In this article, we review and assess the potential usefulness of circulating miRNAs in cancer therapeutics and diagnosis.

Discovery of circulating miRNA in cancer patients

In several studies, miRNA expression profiles have been shown to have signatures related to tumor classification, diagnosis, and disease progression. Since a single miRNA is said to be able to target several mRNAs, aberrant miRNA expression is capable of disrupting the expression of several mRNAs and proteins. For instance, Rosenfeld *et al.*[5] showed that miRNA expression profiles have been useful in detecting the tissue of origin for cancers of unknown primary origin. Furthermore, Lu *et al.*[6] demonstrated that the expression analysis of 217 miRNAs in various human cancers clearly reflects the developmental lineage and differentiation state of the tumors, and they also confirmed a general down-regulation of miRNAs in tumors compared with normal tissues. These findings highlight the potential of miRNA profiling in cancer diagnosis. Most diagnostic expression profiling of miRNAs has been conducted using samples from tumor tissues; however, several studies have shown the diagnostic and prognostic usefulness of circulating miRNAs (see Table 1).[7–20]

One of the first studies measuring miRNA levels in serum was reported by Lawrie *et al.*[7] who demonstrated that the serum levels of miR-21 were associated with relapse-free survival in patients with diffuse large B-cell lymphoma; thus, miR-21 may have potential as a diagnostic biomarker for this disease. Mitchell *et al.*[8] found that, by measuring the serum levels of miR-141, they could distinguish patients with prostate cancer from healthy subjects. In that study, they also demonstrated the presence of circulating tumor-derived miRNAs in blood by using a mouse prostate cancer xenograft model. Furthermore, these circulating miRNAs were also found in the serum of rats, mice, calves, bovine fetuses, and horses, indicating that circulating miRNAs were commonly discovered in mammalian species.[21]

Chen *et al.*[21] showed the miRNA expression profiles for lung cancer, colorectal cancer, and diabetes patients in comparison to those of healthy subjects and found that cancer patients had serum levels of miR-25 and miR-223 that were more elevated than those of healthy subjects. On the other hand, several serum miRNAs were significantly more overexpressed in patients than in healthy subjects in a study of ovarian cancer.[10] In addition, Ng *et al.*[19] showed that miR-92 is more significantly increased in colorectal cancer than in gastric cancer and inflammatory bowel disease as well as normal subjects and can be used as a potential biomarker to detect colorectal cancer in plasma samples. Recently, Hu *et al.*[11] performed a screening to detect serum miRNA to predict the prognosis of non-small-cell lung cancer (NSCLC) using Solexa sequencing followed by an extensively self-validated study in a cohort of 303 patients with stage I to IIIa NSCLC. Eleven serum miRNAs were found to be altered more than 5-fold between longer-survival and

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Table 1. Serum miRNAs as a biomarker

Type of cancer	Biomarker candidate	Reference
Diffuse large B-cell lymphoma (DLBCL)	Expression levels of miR-155, miR-210 and miR-21 were higher in DLBCL patient than control sera	7
	High miR-21 expression was associated with relapse-free survival	
Prostate cancer	Serum levels of miR-141 can distinguish patients with prostate cancer from healthy controls	8
Ovarian cancer	The levels of the 8 specific miRNAs were similar between cellular and exosomal miRNAs. Exosomal miRNA from ovarian cancer patients exhibited similar profiles, which were significantly distinct from profiles observed in benign disease	9
	miR-21, -92, -93, -126 and -29a were significantly overexpressed in the serum from cancer patients compared to controls	10
Non small cell lung cancer	Eleven serum miRNAs were found to be altered more than 5-fold between longer-survival and shorter-survival groups, and levels of four miRNAs were significantly associated with overall survival	11
Acute myeloid leukemia (AML) Acute lymphoblastic leukemia (ALL)	miR-92a decreased in the plasmas of acute leukemia patients	12
Breast cancer	Increased miR-195 levels in patients were reflected in tumors, and circulating levels of miR-195 and let-7a decreased in cancer patients postoperatively, to levels comparable with control subjects	13
	miR-155 was differentially expressed in the serum of women with hormone-sensitive compared to women with hormone-insensitive breast cancer	14
Gastric cancer	The plasma concentrations of miR-17-5p, miR-21, miR-106a, and miR-106b were significantly higher in patients than controls, whereas let-7a was lower in patients	15
Pancreatic cancer	Circulating miR-210 levels are elevated in pancreatic cancer patients	16
Pancreatic ductal adenocarcinoma	The combined analyses of four miRNAs (miR-21, miR-210, miR-155, and miR-196a) in plasma can discriminate patients from normal healthy individuals	17
Squamous cell carcinoma (SCC) of tongue	Plasma miR-184 levels were significantly higher in tongue SCC patients in comparison with normal individuals, and the levels were significantly reduced after surgical removal of the primary tumors	18
Colorectal cancer	Both miR-17-3p and miR-92 were significantly elevated in the patients, and the plasma levels of these miRNAs were reduced after surgery	19
Hepatocellular carcinoma (HCC)	An increased amount of miR-500 was found in the sera of the HCC patients, and its levels in sera returned to normal after the surgical treatment	20

shorter-survival groups, and the levels of four miRNAs (miR-486, miR-30d, miR-1, and miR-499) were significantly associated with overall survival. The four-miRNA signature was also an independent predictor of overall survival for both training and testing samples.

Previously, Yamamoto *et al.*[20] found that miR-500 is an oncofetal miRNA in liver cancer using a global miRNA expression profile in mouse liver development. The expression of miR-500 is high in fetal liver and down-regulated in the developmental process and then up-regulated in the process of liver cirrhosis. miR-500 was abundantly expressed in several human liver cancer cell lines and 45% of human hepatocellular carcinoma (HCC) tissue. Most importantly, an increased amount of miR-500 was found in the sera of HCC patients, which means that liver cancer-specific miRNA, such as miR-500, is circulating in the peripheral blood and can be a novel diagnostic marker. Furthermore, elevated serum levels of miR-500 in HCC patients were significantly reduced after surgery and returned to normal levels. These results reveal that the abundance of miR-500 in the serum of the HCC patients might reflect physiological and/or pathological conditions. Wong *et al.*[18] reported that miR-184 showed significantly higher expression in tongue squamous cell carcinoma (SCC) cells than in the paired normal cells. In addition, the plasma level of miR-184 was much higher in cancer patients with early and advanced tongue SCC than in normal individuals. Moreover, the mean plasma levels of miR-184 were reduced in the patients after the surgical removal of the primary tumor. After the tumor was resected, it was important to use a serum biomarker for patients to monitor any recurrence of the tumor. Using serum miRNAs, such as miR-500 and miR-184, helps determine the next option for treatment in the early stage of cancer and metastasis.

Circulating miRNA carried in particles

Although serum contains ribonuclease, the existence of serum miRNAs suggests that these miRNAs are resistant to RNase digestion. Chen *et al.*[21] showed that serum miRNAs remained stable after being subjected to harsh conditions under which most RNA would be degraded. El-Hefnawy *et al.*[22] showed that plasma RNA is protected from degradation by inclusion in lipid or lipoprotein complexes. Recent studies have revealed the

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