(図 1･C)。肝機能や血清総蛋白は細胞投与による明らかな変化を認めなかった。

D．考察

これまでに腎幹細胞の存在部位として、骨髄に存在するという報告と、腎組織に存在するという報告があり、細胞移植療法として、造血幹細胞や骨髄幹細胞を用いた検討結果が報告されている。今回我々は、腎幹/前駆細胞の形質を有すると考える rKS56 細胞を、急性腎不全モデルラットに投与し、その生着・分化そして一部腎機能の改善効果を観察し得た。

今回腎被膜下への rKS56-LacZ 細胞の投与により、被膜下だけでなく、シスプラチン誘発急性腎不全モデルにおける主要な障害部位である皮髄境界部にも rKS56-LacZ 細胞の生着が確認された。被膜下に投与した rKS56-LacZ 細胞がどのような経路を介して皮髄境界部の障害部位に移動し生着したかという点に関しては今後の検討が必要である。また、今回の検討では rKS56-LacZ 細胞を経腎動脈的に投与したが障害部位への生着が観察されず、投与したｒＫＳ56 細胞は血行性ではなく障害部位まで遊走して生着した可能性が示唆された。今後、被膜下投与した rKS56-LacZ 細胞を連日にわたって追跡観察することによる、障害部位への移動生着の経路の検討が必要である。

また、rKS56-LacZ 細胞腎被膜下投与群にて多くの rKS56-LacZ 細胞が被膜下に集簇する像が観察されたが、主として管腔様構造を呈し、AQP-1 陽性であったことから、腎被膜下部位での尿細管上皮細胞への分化能が示唆された。一方、rKS56-LacZ 細胞腎被膜下投与により腎障害極期における有意な腎機能改善効果を認めたが、今回の検討では腎被膜下に比して腎皮質内に生着が確認された rKS56-LacZ 細胞は少数であり、直接的な傷害尿細管部位への生着による修復機序のみでなく、rKS56-LacZ 細胞が腎再生に関与する液性因子を分泌し腎修復に寄与する可能性が示唆された。これまでに、HGF・BMP-7 等の因子が腎尿細管発生及び障害腎修復に作用することが報告されており、これらの因子や受容体等の rKS56-LacZ 細胞投与による発現変化に関しても検討を進めている。

今後、腎障害の腎幹／前駆細胞投与による再生治療を臨床の現場に応用していくためには、以下の点を解明することが重要と思われる。まず、投与する腎幹／前駆細胞数や投与経路の至適条件の検討が必要である。骨髄由来幹細胞は、経静脈的もしくは経腎動脈的投与にて障害腎に生着することが報告されているが、今回の検討では経腎動脈的投与では腎への生着は認めなかった。上記の至適投与条件の検討は、今後の臨床応用における細胞投与のアプローチ、すなわち腎被膜下に直接投与するか、もしくは腎動脈からカテーテルを用いて投与するか、を検討するうえで必要である。次に、急性腎不全のみならず、慢性腎不全に陥った腎に腎幹／前駆細胞を投与することで、障害腎が機能的・組織学的に改善しうるかどうかを検討する必要がある。しかし、慢性腎不全では腎はすでに間質線維化をきたし細胞外基質・増殖因子濃度勾配等の微小環境(niche)が変化している可能性が高く、尿細管上皮細胞の再生に適していない可能性がある。慢性腎障害モデルにて HGF 投与により腎間質線維化が抑制されたという報告が

あるが、腎再生を促進する増殖因子と腎幹／前駆細胞移植を組み合わせることにより、腎尿細管上皮細胞再生・修復に適した微小環境を整えて幹細胞の生着・分化・ならびに組織修復を促進しうる可能性がある。今回、ラットの腎組織幹／前駆細胞を単離・樹立することが可能であったが、同様にヒトで腎幹／前駆細胞を単離し、かつその未分化な形質を保ったまま、維持培養し、治療応用することが今後の検討課題である。

Ｅ．結論

我々が樹立した成体ラット腎由来の腎幹／前駆細胞様細胞（rKS56 細胞）のラット急性腎不全モデルへの投与による、腎障害の組織学的・機能的修復効果が観察された。

腎不全を対象とした再生医療の実現に向けて未だ克服すべき課題は多いが、今後幹細胞移植の有効性・安全性を検証することにより、将来的に臨床応用が可能となることが期待される。

Ｆ．研究発表

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2) 木野村賢，喜多村真治，山崎康司，菅谷健，前島洋平，杉山斉，槇野博史：腎臓幹/前駆細胞様細胞（rKS56-LacZ 細胞株）による虚血性急性腎不全モデルの障害尿細管修復機序の検討．第 48 回日本腎臓学会総会．横浜．6 月 23-25 日．2005.

Ｇ．知的財産権の出願・登録取得状況（予定を含む）

１．特許取得

特願２００３－０７１０２９：腎臓幹細胞前駆細胞、腎臓幹細胞前駆細胞の分離方法、及び腎疾患の治療法（出願日　平成１５年３月１４日）

２．実用新案特許

なし

３．その他

なし

図説明

図 1-A：シスプラチン誘導急性腎不全ラットモデルにおける腎被膜下投与後の rKS56-LacZ 細胞の分布。細胞投与後 3 日、7 日目の bluo-gal 染色と PAS 染色の二重染色像を示す。

rKS56-LacZ 細胞被膜下投与後 3 日目では、被膜下に LacZ 陽性細胞を認めるのみである (a；×80)。一方 rKS56-LacZ 細胞被膜下投与後 7 日目では、被膜下の領域が肥厚し、LacZ 陽性細胞数は増加、一部は管腔様構造を呈した（b；×80, c;×400)。また、被膜下投与後 7 日目にて、一部皮髄境界部にも LacZ 陽性細胞を認める（d；×80, e;×400)。

図 1・B：rKS56-LacZ 細胞投与腎での、aquaporin-1・Ki-67・IV 型コラーゲンの発現（免疫染色）。rKS56-LacZ 細胞被膜下投与後 7 日目で認めた、管腔様構造を呈する LacZ 陽性細胞は、一部 aquaporin-1 (a;×400)・Ki-67 (b;×400)陽性であった。また、basolateral 側では IV 型コラーゲン陽性尿細管基底膜様構造が観察された (c;×400)。

図 1-C：rKS56-LacZ 細胞治療による Cr の変化。rKS56-LacZ 細胞被膜下投与群にて腎機能障害の有意な改善効果を認めた (day 5)。Day 5：シスプラチン投与 5 日目（rKS56-LacZ 細胞投与後 3 日目）。Day 9：シスプラチン投与 9 日目（rKS56-LacZ 細胞投与後 7 日目）。C：非罹患対照群、T-SC：rKS56 細胞・被膜下投与群、T-IA：rKS56 細胞・左腎動脈投与群、V-SC：生食・被膜下投与群、V-IA：生食・左腎動脈投与群。# $p<0.05$ vs. C、*$p<0.05$ vs. T-SC at day5。各カラムは平均値±SEM。

A

a

b

c

d

e

B

a

b

c

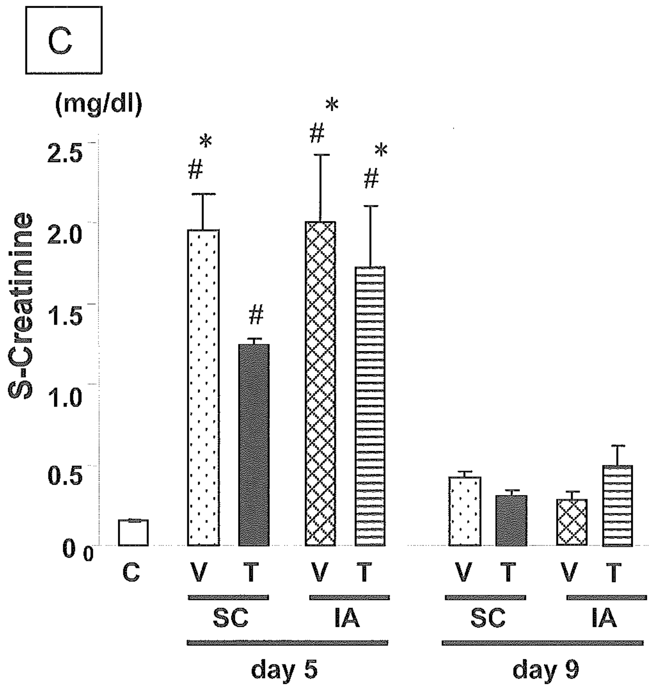

図1

厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）

分担研究報告書

マウス組織特異的幹細胞移植に関する研究

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「動的分子論に立脚したフロンティア基礎化学」　技術員

研究要旨

髄質外層部の尿細管上皮細胞は、急性腎不全や進行性腎障害における間質性腎障害の病態を形成する中心的細胞群である。腎尿細管上皮特異的幹細胞移植による効果検討として、本年度はマウス尿細管上皮特異的幹細胞を用いて、マウス急性腎不全に対して移植実験を実施した。

実施に先立ってマウス尿細管上皮特異的幹細胞の表面抗原を解析し、再生に関わる細胞としての特徴と考えられる CD34, SCa-1, Musashi-1, Stathmine が高発現であることをフローサイトメーターで確認した。シスプラチン(CDDP) 15mg/kg の単回投与は約 5 日の経過の致死的なマウス急性腎不全モデルである。この実験モデルに対して投与 24 時間後にマウス尿細管上皮特異的幹細胞移植を腎被膜下へ行うと、投与 5 日後の血清尿素窒素値は半減し、生存率は 55.6%と上昇した。移植されたマウス尿細管上皮特異的幹細胞のなかには、間質に局在するものや尿細管細胞の一部を構成しているものがあることが確認された。

標準化された cell processing protocol により充分純化されたマウス尿細管上皮特異的幹細胞は、致死的急性腎不全による自然経過（腎死）を改善させる効果がある。

A．研究目的

上皮系幹細胞移植の臨床応用に向けての候補細胞群は、cell processing によって focus を絞り込む作業を繰り返しながら、characterization を順次実施していく必要がある。そしてまた、選別されて得られた上皮系幹細胞に富んだ細胞群は、実際に組織障害修復に効果を発揮しうる可能性があるかどうかを検討することが必須である。

本研究では、Cell Processing Team によって確立されつつある標準的プロトコルで採取した上皮系幹細胞を用いて、本年度はまずマウス由来のものについて、フローサイトメーター(FACS)を用いた解析で、cell processing により変化を受けるマーカーを検出する試みを行なうこととした。その上で、基準に合致する細胞を腎疾患モデルへ移植し、改善効果を血液生化学的、組織学的に評価し、実際の治療効果として結びつくかどうかを検討。今回は特に急性モデルでの検討を中心に行なうこととした。

B．研究方法

（１）移植細胞

C57BL/6J の腎 microdissection により得られた近位尿細管上皮細胞（以下 mProx）を 37℃ 5％CO2 条件下に 10% FBS 添加 DMEM/F-12HAM で培養し、20～30 回継代したものを使用した。

また、神戸理研において mProx の Side Population（SP）分画より single cell sorting で分離された尿細管上皮幹細胞（mProxSP）を樹立。間葉系上皮細胞の培養上清を添加した K-1 medium で培養しながら、single cell sorting を複数回繰り返して純化した細胞群を使用した。細胞注入に際

して、一部蛍光標識を行った。

（２）フローサイトメーター解析(FACS)

表面抗原に関する解析は、trypsine 処理して回収してきた mProx または mProxSP を、CD34 抗体､Kim-1 抗体及び Sca-1 抗体にて、4℃で 30 分間インキュベートした。次に細胞を NaN3 入り PBS で洗浄後､2 次抗体と 4℃で 30 分間インキュベートし、CD34、Kim-1 抗体による陽性細胞の出現率を、FACScan(BD Biosciences)を用いて解析した。

細胞内蛋白発現の FACS 解析では､回収してきた細胞を PBS で洗った後、終濃度 0.5%になるようにホルムアルデヒドを加え 10 分間 37℃でインキュベートした。固定後、90%メタノールを用いて膜を浸透化して抗体染色に用いた。膜の浸透化後のサンプルは 0.5%BSA 入りの PBS で洗浄し 10 分間ブロッキングを行った後に１次抗体を加えて３０分間室温でインキュベートした。次に余分な抗体を洗い落とすために 0.5%BSA 入りの PBS で洗浄した。その後２次抗体を加えて 30 分間室温でインキュベーションし、再び 0.5%BSA 入りの PBS で洗浄し、フローサイトメーターの解析に用いた。

（３）マウス及び培養細胞

本研究では、田辺 RDS より 8～15 週齢雄の C57BL/6J 野生型および EGFP-ヒト L-FABP 遺伝子発現マウスを搬入し､一週間の予備飼育後に実験モデルを適用した。

（４）シスプラチン誘発急性腎不全

予備検討においてシスプラチン 15mg/kg の腹腔内投与により 1 週間以内に致死的な急性腎不全を発症することがわかっている。そこで、C57BL/6J 雄、野生型にシスプラチン 15mg/kg（溶液総量 60ml/kg）を腹腔内投与し、24 時間後に塩酸ケタミン 30mg/kg とキシラジン 8mg/kg の腹腔内投与により麻酔した。左背部を切開し、左腎を露出した。$5.0x10^5$ 個に調整した CMFDA 標識 mProx SP、または non SP を左腎被膜下に投与後（投与総量 100μl）、穿刺部をアロンアルファ A(三共)で塞ぎ、閉腹した。陽性対照群には DMEM/ F-12 HAM を被膜下投与し、陰性対照群には生理食塩水 60ml/kg の腹腔内投与 24 時間後、同様に DMEM/F-12 HAM を左腎に被膜下投与した。シスプラチン投与 48 時間後、72 時間後に生理食塩水 60ml/kg を腹腔内投与した。3 日目(Day3)、5 日目(Day5)に経時的に尾静脈より採血し 7 日目に屠殺して腎機能、組織学的所見を評価した。

（５）腎虚血再灌流障害

C57BL/6 雄、変異型に塩酸ケタミン 30mg/kg とキシラジン 8mg/kg の腹腔内投与により麻酔した。背部正中を切開し、両腎を露出した。両側腎基部に血管鉗子をかけ、37℃に保ったインキュベーターに入れた。30 分後に血管鉗子をはずし､$5.0x10^5$ 個に調整した同様に蛍光標識した mProx SP を両腎被膜下に投与後（投与総量 100μl）、穿刺部をアロンアルファ A(三共)で塞ぎ、虚血腎の再灌流を肉眼的に確認し閉腹した。陽性対照群には DMEM/ F-12 HAM を被膜下投与し、陰性対照群には擬手術後、同様に DMEM/F-12 HAM を両腎に被膜下投与した。24 時間ごとの採尿のほか、虚血再灌流 24 時間後、48 時間後、72 時間後に尾静脈より採血し 5 日目(Day5)に屠殺して腎機能、組織学的所見を評価した。

（６）組織学的検討

腎組織は、10%緩衝ホルマリン溶液により

36時間固定後、パラフィン包埋し、組織切片を3μmに薄切して、蛍光顕微鏡下に移植細胞の局在を直接的に検討した。

（7）血清尿素窒素（BUN）

尿素窒素Bテストワコー（和光純薬）により、プロトコル通りの方法で測定した。

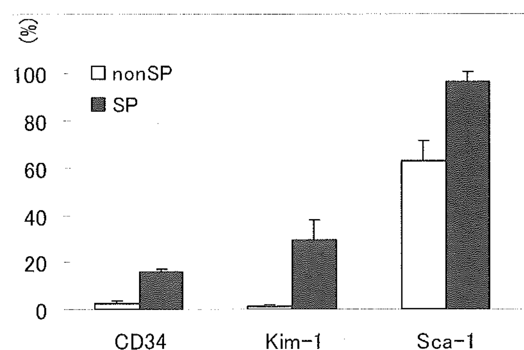
（倫理面への配慮）

動物実験は、全て東京大学医学部・大学院医学系研究科の動物実験に関する倫理規定に準拠し、医学系研究科附属疾患生命工学センター動物次元研究領域当SPFエリアで隔離飼育され、同委員会で承認されたプロトコル（承認番号1520T047）及び遺伝子組み換え生物等の第二種使用等拡散防止措置の承認（承認番号45）をうけて実施されている。

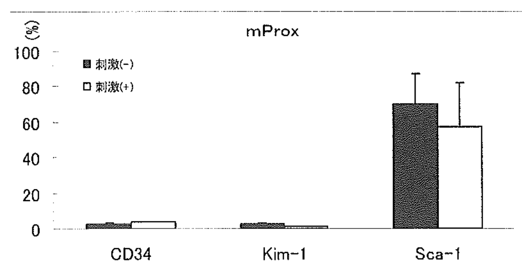
C．研究結果

（1）FACS

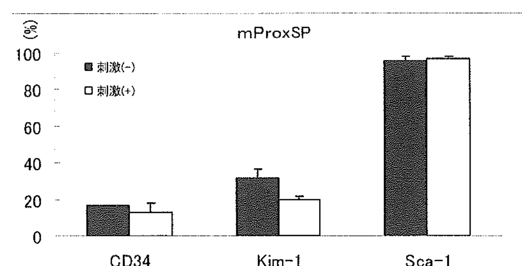
表面抗原の発現をmProxとmProxSPで比較した。下図に示すようにmProxに対してcell processingを受けたmProxSPではCD34, Kim-1, Sca-1陽性細胞数が有意に増加していることが明らかとなった。



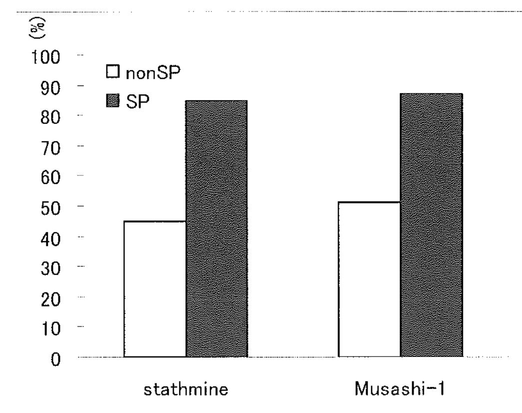
次にATP枯渇による酸化ストレスの実験では（ATP枯渇=刺激(+)）、mProxではCD34とKim-1の発現には大きな変化は認められず、下図のようにSca-1の発現は低下することが示された。



一方、mProxSPではCD34とSca-1の発現にはATP枯渇により変化が認められず、Kim-1の発現が減じることが示された。



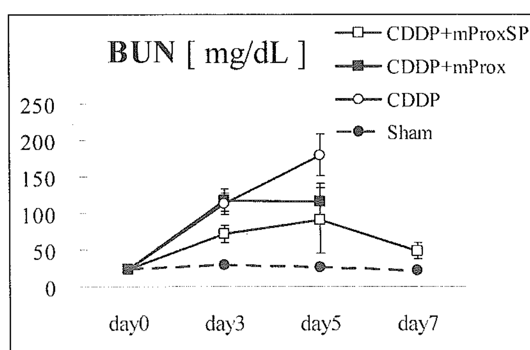
近年、FACSによる細胞質蛋白発現の解析が可能となりつつある。ここでは、Stathmin及びMusashi-1について解析を行ってみたところ、下図のようにmProxに対してmProxSPではStathmin及びMusashi-1の発現が有意に増強していることが明らかとなった。



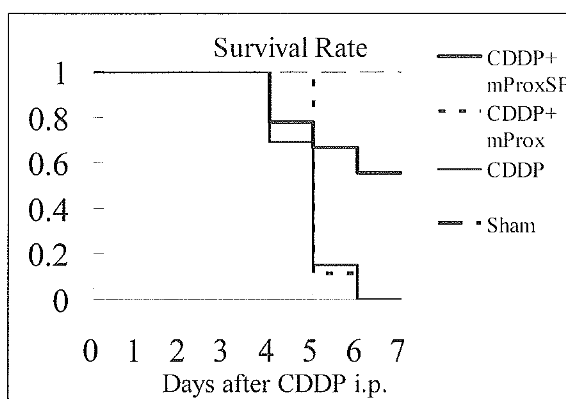
（2）シスプラチン誘発急性腎不全

本検討で用いたシスプラチンの用量では、投与後1,2日の血清尿素窒素(BUN)の上昇

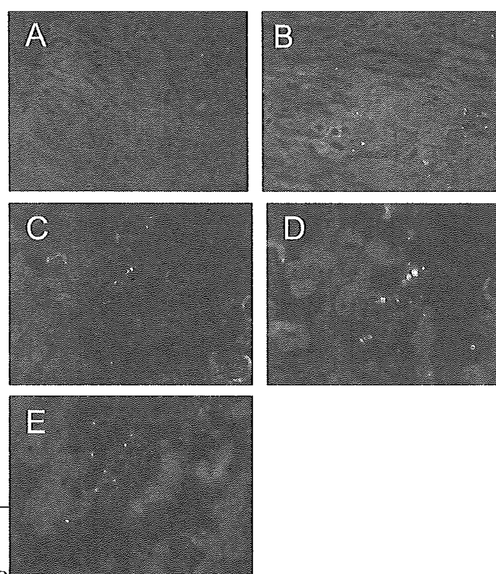
は軽度であるが、投与後 2～3 日目に向けて急激に腎機能が低下し上昇する。3 日目(Day 3) の血清尿素窒素 BUN [mg/dl] は mProxSP (=CDDP+mProxSP, N=9) 、 mProx (=CDDP+mProx, N=9) 、CDDP, (N=13)各群それぞれ 72.1+/-11.4 , 118.4+/-14.8 , 113.4+/-14.5 (mean +/- SE) で 、CDDP+mProxSP では CDDP に対して有意な改善を認めた。



CDDP 投与一週間までの経過で mProxSP 群では day7 まで 5/9 匹が生存。CDDP 群及び CDDP+mProx 群は 5 日目で全例が死亡した。Kaplan-Meier 法におけるログランク検定で CDDP 群に対して CDDP+mProxSP 群の有意な死亡率の逓減が確認された(下図:$p<0.001$)。



腎組織所見の A は CDDP 群より得られた切片で、CDDP 投与 4 日目に瀕死状態で摘出した腎より得られた。近位尿細管の brush border はなく、尿細管腔内に脱落上皮細胞が観察された。B は CDDP+mProx 群の投与後 4 日目で同様の状態の腎組織である。ここでは mProx を蛍光標識しているが、mProx は殆ど認められなかった。C は CDDP+mProxSP 群で、移植細胞が高輝度の蛍光を呈していることが分かる。その高倍率が D である。E も CDDP+mProxSP 群由来の組織であるが、こちらのほうが組織障害の強い場所であり、尿細管細胞が平低下しているためにより多くの蛍光陽性細胞が観察されやすい。標識された細胞が障害された尿細管管腔の一部を構成しており、尿細管再生に関わる所見の可能性が示唆された。



D. 考察

マウス近位尿細管細胞由来の細胞株 mProx は、cell processing によって dorm formation を有し、長期培養にかなり耐える表現型を有するようになった(mProxSP)。これらの細胞では、Sca-1 の発現はほぼ 100%に検出されるようになり、Kim-1、CD34 陽性細胞数も増加することが分かった。更に preliminary な観察で、c-kit 陽性細胞数は少数であるものの、mProxSP では発現

量が cell processing を受けていない野生型（mProx）に比べて高率であることが分かった。急性腎不全の回復期に発現する stathmin や上皮系幹細胞での発現が近年報告されている Musashi-1 の発現陽性細胞が mProxSP に多く発現していることが分かり、cell processing により純化が客観的に証明されたと考えられた。

mProxSP は mProx に比べ、細胞内 ATP 枯渇による酸化ストレス刺激に対してアポトーシスを誘導されにくい傾向があるようで、この点については現在検討中である。Antimycin による ATP 枯渇の実験系では、mProx における Sca-1 の発現は減少するのに対して mProxSP ではその発現は維持され、一方高発現していた Kim-1 が減じることが示唆された。このような一連の変化は、上皮系幹細胞が腎障害環境下においても幹細胞としての機能を温存し、局所の環境要因を整えるうえで重要な能力を示している可能性があると思われた。今後、さらに検討を進めていく予定である。

次に、mProxSP 細胞を障害腎に移植した場合の効果を検討した。CDDP 15 mg/kg の用量設定では投与後 BUN は上昇を開始し、特に 2 日目以降では急上昇し、5 日目までに 200mg/dl に達してほぼ全例死亡する。この実験系で mProxSP を CDDP 投与 24 時間後に被膜下移植した場合、CDDP 投与 5 日目の BUN の上昇が半分に抑えられ、7 日目までには BUN が peak-out して生存する個体が 5/9 に及ぶことが示された。ここで認められた生存効果は統計学的にも有意であることを確認した。一方、mProx 群ではこのような改善効果は検出できなかった。mProxSP 細胞は CDDP 投与 7 日目の腎臓において、被髄境界部の近位尿細管周囲と一部は尿細管細胞間に認められることが分かった。このようにして障害腎に存在する細胞が、FACS 解析で明らかとなりつつある特異性のうち、どのような character を維持しつつ周囲の細胞に影響を与えて局在しているかは、極めて興味深いところで、今後の研究課題である。管腔構造をとるかどうかについては、現在検討をすすめている。

本年度の研究では、cell processing により得られたマウス腎皮質尿細管由来上皮系幹細胞の characterization を一部行ない、確認をとったうえでマウス急性腎障害モデルへの移植実験を実施した。ここで確立しつつある手法をもとにヒト腎皮質尿細管由来上皮系幹細胞での実験を、免疫不全マウスを用いて一部実施したが、次年度では規模を拡大して行う予定である。同時に、マウス慢性腎障害モデルを現在開発中であり[1,2]、一部成果を発表した。これを用いた慢性腎虚血モデルへの移植実験を実施する予定である。

E．結論

マウス急性腎障害の疾患モデルに対する腎尿細管上皮系幹細胞移植治療効果について検討した。移植用細胞の純化プロセス、移植前の確認プロセスを更に詰めることによって、手順の標準化が達成できるレベルになると考えられた。

細胞移植治療は、マウス急性腎不全レベルでは生存率に効果を発揮しうる改善率を示した。ヒト腎尿細管上皮系幹細胞における同様の手順・効果を詳細に検討することが臨床応用に向けて必須と考えられる。

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G．知的財産権の出願・登録取得状況（予定を含む）　なし

厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）

研究成果の刊行物・別刷

研究成果の刊行に関する一覧表

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Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney

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ABSTRACT **Kidney is thought to be a regenerative organ in terms of repair from acute tubular injury. It is unknown whether cell population contributes to repair disordered kidney. We attempted to identify and isolate highly proliferative cells from a single cell. We dissected a single nephron from adult rat kidney. Isolated nephrons were separated into segments and cultured. Outgrowing cells were replated after limiting dilution so that each well contained a single cell. One of cell line which was the most potent to grow was designated as rKS56. rKS56 cells showed cobblestone appearance and expressed immature cell markers relating to kidney development and mature tubular cell markers. rKS56 cells grew exponentially and could be maintained for 300 days without transformation. In different culture conditions, rKS56 cells differentiated into mature tubular cells defined by aquaporin-1, 2 expression, and responsiveness to parathyroid hormone or vasopressin. Engrafted to kidney in rat ischemic reperfusion model, rKS56 cells replaced in injured tubules in part after implantation and improved renal function. These results suggest rKS56 cells possess character such as self-renewal, multi-plasticity and capability of tissue repair. rKS56 may possibly contribute to the future development of cell therapy for renal regeneration.—Kitamura, S., Yamasaki, Y., Kinomura, M., Sugaya, T., Sugiyama, H., Maeshima, Y., Makino, H. Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney. *FASEB J.* 19, 1789–1797 (2005)**

Key Words: S3 segment of proximal tubules · renal tubular epithelial cells · renal progenitor-like cell.

The therapeutic potential of stem/progenitor cells has been the focus of much recent research (1–3). Stem cells possess three characteristic features such as self-renewal, multipotent differentiation (4) and ability to regenerate after organ injury (3, 5). The existence of organ-specific stem/progenitor cells in adult organs has been demonstrated (6). In particular, bone marrow-derived somatic stem/progenitor cells were shown to differentiate into endothelial cells (7), muscles (2, 8), hepatocytes (9, 10), epithelia of gastrointestinal tract (11), neurons (12), and mesangial cells (13, 14). Endothelial progenitor cells present in bone marrow have been used for treating patients with ischemic heart disease and arterial sclerosis obliterans (15).

Meanwhile, in spite of advances in modern clinical nephrology, progressive renal diseases remain incurable disorders lacking specific therapeutic approaches. Patients with ESRD (end-stage renal disease) require renal replacement therapy such as hemodialysis, peritoneal dialysis or kidney transplantation. However, hemodialysis or peritoneal dialysis does not fully compensate for the loss of kidney function. Long-term hemodialysis and peritoneal dialysis often accompany cardiovascular complications and kidney transplantation suffers from the shortage of donor kidneys, and kidney transplants recipient may undergo some complications of immunosuppressive reagents.

The common histological features of ESRD, irrespective of various pathogenic renal disorders, are glomerulosclerosis and tubulointerstitial fibrosis (16–18). Tubulointerstitial fibrosis, rather than a glomerular lesion, is related to exacerbate prognosis of renal function in progressive renal disorders (16), which lead to the requirement for effective therapeutic approaches to regenerate tubular epithelial cells. In contrast to terminally differentiated epithelial cells with limited doubling-cycles, stem/progenitor cells generally can survive and proliferate for a long time period.

A single metanephric mesenchymal cell can differentiate into various segments of nephron except the collecting duct after interaction with the ureteric bud. It is suggested that embryonic kidney contains epithe-

We declare that the establishment and the therapeutic application of rKS56 cells for renal disorders have been filed and submitted to the Japan Patent Office Issue No. 2003-071029 in March, 2003.

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lial stem cells (19). Recently, Dekel et al. (20) reported the usefulness of fetal or embryonic kidney fragments as the source of kidney precursor cells to generate mature kidneys by transplantation. Although there are several reports demonstrating that bone marrow-derived cells can repopulate and differentiate into glomerular mesangial cells (13, 14), renal stem/progenitor cells derived from adult kidney have not been reported yet. Thus, we attempted to establish renal proliferative cells modifying neurosphere methods (21), which could obtain neural stem cells.

In developing kidney, there are two major distinct areas of cell proliferation: the nephrogenic zone in the outer cortex below the renal capsule and the area in the corticomedullary junction corresponding to the primitive S3 segment of the proximal tubule (22). In the present study, we attempted to identify and establish renal highly proliferative cells existing among S3 segments of nephron in the adult rat kidney. The cells may be close to stem/progenitor cell strain and the usage of these cells contributes to the development of therapies for ESRD.

MATERIALS AND METHODS

Microdissection and culture conditions

Single nephrons were obtained by microdissection technique from kidney of male Sprague-Dawley (SD) rats that weighed 100 to 150 g (Clea JAPAN Inc., Tokyo). The experimental protocol was approved by the Animal Ethics Review Committee of Okayama University Graduate School of Medicine and Dentistry. In Brief, animals were anesthetized with pentobarbital 50 mg/kg wt, IP. The abdominal aorta was ligated at two sites, under the right renal artery and 2 cm below the left renal artery. A catheter was then inserted in the abdominal aorta for the perfusion of the left kidney. Perfusion was made first with 10 mL ice-cold solution A, then with 10 mL ice-cold solution B. Solution A contained (mmol/L) NaCl 130, KCl 5.0, NaH_2PO_4 1.0, $MgSO_4$ 1.0, Ca lactate 1.0, Na acetate 2.0, glucose 5.5, HEPES 10, pH 7.4. Solution B was made from solution A by addition of 300 U/mg collagenase type I and 1 g/L BSA. The left kidney was decapsulated, removed, and cut in thin 1 mm transverse sections. The sections were transferred into tubes containing 3 mL of solution B with 10 mmol/L vanadyl ribonucleotide complex (VRC) (Sigma, Saint Louis, MO, USA) and incubated with bubbling oxygen at 37°C under constant agitation. After 20 min of incubation, the slices were rinsed with solution A, then transferred to a microdissection dish containing 10 mL of solution A with 10 mmol/L VRC. Microdissection to nephron segments was performed using dissecting needles under a dissection microscope with a cooling plate at 4°C. Superficial and juxtamedullary glomeruli were extracted separately. Identification of tubule segments was based on previously reported criteria (23). The following segments were microdissected: glomeruli (G), proximal convoluted tubule (S1/PCT), proximal straight tubule (S2, S3), medullary thick ascending limb of Henle's loop (MAL) and collecting duct (CD) (**Fig. 1*A*, *B***). Microdissected segments were transferred to a clean wash dish containing solution A using pipettes coated with 0.1% BSA. After a washing for VRC and binding debris, cleaned microdissected segments were separately transferred into wells of 96-well plates precoated with type IV collagen (BD

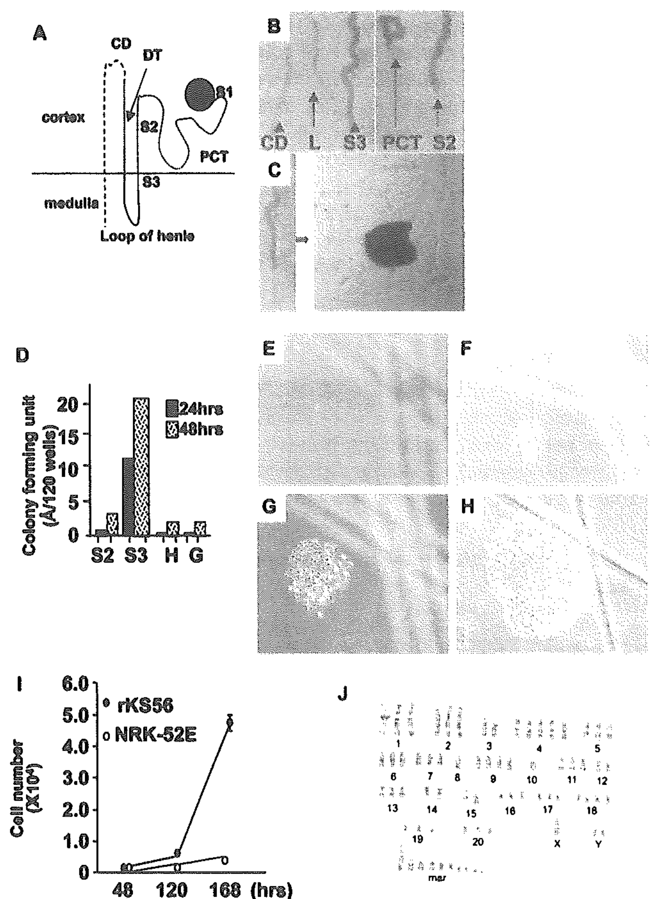

Figure 1. Establishment of rKS56 cell line. *A)* Components of nephron segments. G: glomeruli, S1-S3: proximal tubules, DT: distal tubules, CD: collecting ducts PCT: proximal convoluted tubules. *B)* Segments of nephron. CD: collecting tubule, L:loop of henle, S3:S3 segment, PCT: proximal convoluted tubule, S2:S2 segment. *C)* Outgrowing cells from S3 segment. *D)* Colony forming capacity of cells derived from different nephron segments. The number of colonies (>3 cells/colony)/120 wells (96-well plates) is shown as colony forming units (CFU). Solid bars: 24 h, dotted bars: 48 h. *E–H)* A cell clone derived from the S3 segment possessed potent mitogenic capacity and was designated as rat kidney stem/progenitor (rKS56) cells. After 1 day (*E*), after 3 days (*F*), after 5 days (*G*), after 7 days (*H*). *I)* Cell proliferation curve: rKS56 cells proliferated rapidly with exponential growth, whereas NRK-52E proliferated linearly. *J)* Karyotype of a representative line of rKS56 cells (after 250 days of subculture) detected by G-banding stain. rKS56 cells exhibited near triploid type.

Bioscience, Bedford, MA, USA) and were cultured in a 1:1 mixture of the culture supernatant which are Dulbecco's modified eagle medium [DMEM] (Sigma) containing 10% FCS (Gibco, Grand Island, NY, USA) of mouse mesenchymal cells supernatant (MCS) and modified K1 medium (1:1 mixture of DMEM and Ham's F12 medium (Gibco), supplemented with 10% FCS, 5 μg/mL insulin, 2.75 μg/mL transferrin, 3.35 ng/mL sodium selenious acid (ITS-X: Gibco), 50 nM hydrocortisone (Sigma), 25 ng/mL hepatocyte growth factor (Sigma), 2.5 mM nicotinamide (Sigma) for 24–48 h. Outgrowing cells were observed from all segments under these conditions (Fig. 1*C*). Primary cells were maintained and the cells were replated onto each well of 96-well plates to expand the cell population. After the third passage, the cells

were maintained in a 1:1 mixture of MCS and modified K1 medium, and screened for their mitogenic capacity by plating a single cell into each well of 96-well plates.

For differentiation studies, the cells were cultured in maintaining media without MCS or in the presence of 5 ng/mL of leukemia inhibitory factor (Sigma). NRK-52E cell line was purchased from Riken (Wako, Japan) as a renal epithelial cell line originally harvested from normal rat kidney. The cell line was cultured in DMEM containing 10% FCS. We used the cells between 8 to 10 passages.

Karyotype of rKS56 cells

To check the karyotype of rKS56 cells, we checked the karyotype of rKS56 cells after numerous passages (250 days). The cells were studied in Nihon Gene Research Labs Inc. (Sendai, Japan). Twenty rKS56 cells were analyzed by G-banding stain.

rKS56 cells generated from a single cell and expanded itself

To trace, rKS56 cells were labeled by Vybrant™ DiO cell-labeling solution (Molecular Probes, Eugene, OR, USA). Di-O could mark cell cytoplasm in distinctive fluorescent colors. rKS56 cells were incubated for 10 min with serum free medium containing Di-O solution, and were washed twice with serum free medium. In first culture, these rKS56-Di-O cells were plated. In secondary culture, a single rKS56 cell-Di-O was cloned into each well of 96-plate precoated with type IV collagen by using limiting dilution method. After several cycles of cell division, single rKS56 cell-Di-O was plated in each well of 96-well plate.

Immunofluorescent studies

Cells were cultured on 16-well chamber slides (Nunc-Immuno Plate, Dako, Denmark) precoated with type IV collagen for several days, then fixed with ice-cold acetone for 5 min. The chamber slides or frozen tissue sections were subjected to immunofluorescence staining using primary rabbit antibodies raised to E-cadherin (Santa Cruz, Santa Cruz, CA, USA), c-met (Santa Cruz), AQP-1 (Chemicon, Segundo, CA, USA), AQP-2 (Chemicon), Musashi-1 (Chemicon) and Tamm-horsfall glycoprotein (THP, Biomedical Technologies Inc.) or mouse antibodies raised against pan-cytokeratin (Santa Cruz), Vimentin (Sigma), and secondary antibodies conjugated with FITC (goat IgG; Zymed Laboratories, San Francisco, CA, USA) or rhodamine (sheep IgG; Chemicon) as described previously (18). Images were recorded using confocal fluorescence microscopy. Formalin-fixed and paraffin-embedded tissue sections (4 μm), were processed for immunohistochemistry using an avidin-biotin peroxidase technique (Vectastain; Vector Laboratories, Burlingame, CA, USA) as described (18).

Reverse transcription-PCR

Total RNA was extracted from culture cells by using RNA extraction kit (QIAGEN, Valencia, CA, USA) following the manufacturer's manual. One microgram of total RNA was reverse-transcribed into cDNA and amplified using the One-step RNA PCR kit(AMV, (TAKARA Bio Inc., Shiga, Japan) and specific primer pairs for c-kit, Sca-1, Pax-2, WT-1, GDNF, Wnt-4, AQP-1, ClC, NaCl transporter, AQP-2, and GAPDH, subjected to agarose gel electrophoresis, and visualized as described (18).

Electrophoresis and immunoblotting

Sodium dodecyl sulfate-PAGE (SDS-PAGE) and immunoblotting were performed as described (24). Primary rabbit antibodies raised to Musashi-1 (Chemicon) and actin (Sigma) were used. Secondary goat anti-mouse or anti-rabbit IgG antibody conjugated with horseradish peroxidase was purchased from Sigma.

Measurement of cAMP accumulation in response to PTH or AVP

The amount of cAMP was measured by using cAMP EIA kit (Amersham, Piscataway NJ, USA) following the manufacturer's instruction. Briefly, rKS56 cells grew to confluence in 96-well culture plates precoated with type IV collagen, rinsed twice with DMEM and incubated with media containing 10^{-4} M of IBMX and 10^{-7} M of parathyroid hormone (PTH, Sigma) or 1U of arginine vasopressin (AVP, Sigma) for 10 min at 37°C. Control wells were treated with saline. Then, cellular protein was extracted by the addition of ice-cold ethanol. After incubation for 30 min on ice, the supernatant was obtained, then dried under a vacuum. The dried extracts were dissolved in 500 μL of assay buffer and the concentration of cAMP was measured by enzymeimmunoassay (EIA). The amount of protein was determined by the modified Coomassie blue G dye binding assay with BSA as standard.

Cell implantation study for evaluating the plasticity of rKS56 cells

rKS56 cells were implanted into liver, spleen, kidney, skeletal muscle, or subdermis (5×10^4 cells/organ with black ink to trace the injected site) of anesthesized SD rats (Clea JAPAN Inc.). On day 28 after implantation, the rats were killed and tissues were obtained and processed for formalin fixation followed by paraffin-embedding or frozen section.

Induction of acute renal failure by ischemic reperfusion and cell therapy into injured kidney

Male SD rats (Clea JAPAN Inc.) weighing 100–150 g were used. Acute renal failure was induced by clamp of right renal artery for 40 min after removal of left kidney (ischemic reperfusion: I/R). We preliminary confirmed that acute tubular necrosis occurred mainly in outer medulla at peak of 3–4 days and renal function and morphological changes returned to normal at 7 days. rKS56 cells (1.6×10^7 cells) resuspended in 0.4 mL of PBS with black ink were implanted into subcapsule of each kidney at 4 sites on both abdominal and dorsal side using 20-gauge needle after 40 min of ischemia. Rats of I/R without cell implantation received vehicle buffer (PBS). SD rats were divided into the following subgroups: *1)* 0 day for control (n=6), *2)* I/R treatment group(n=7), *3)* after I/R with rKS56 cells implantation treatment (n=6). At the end of each experimental period, individual 24 h urine sample collections were performed. The SD rats were killed after 4 and 7 days, and kidneys were obtained and processed for formalin fixation followed by paraffin embedding or frozen section. Blood samples were drawn from the vena cava inferior under anesthesia. We measured creatinine, blood urea nitrogen, and urinary N-acetyl-β-D-glucosainidase (U-NAG) as tubulointerstitial injury maker. To trace the mobilization of rKS56 cells, cells were labeled by Vybrant™ DiO cell-labeling solution prior to cell implantation.

Statistical analysis

Data were expressed as mean ± SE. Comparisons between groups were evaluated by Student's *t* test. A *P* value of <0.05 denoted the presence of a statistically significant difference.

RESULTS

Organ culture of dissected nephron segments and establishment of rKS56

Nephron are consisted of several segments like glomeruli (G), proximal convoluted tubule (S1/PCT), proximal straight tubule (S2,S3), medullary thick ascending limb of Henle's loop (MAL) and collecting duct (CD) (Fig. 1*A*). We used an organ culture system for microdissected nephron segments (Fig. 1*B*) to define the origin of proliferating cells. Initial outgrowing cells (Fig. 1*C*) from nephron segments were observed in more than half of the wells in maintaining culture condition and there was no difference among segments on the rate of outgrowth (data not shown). After the limiting dilution, however, only a few cells survived and grew to form cell colony (Fig. 1*D*). Origin of surviving cells was distributed mainly to the S3 segment. Out of 180 single cells derived from S3 segments, we finally obtained 2 clones that survived through limiting dilution repeatedly. One of the clones with more potent growth was designated as rKS56.

Rapid proliferation rate of rKS56

rKS56 proliferated at a rapid rate even when plated at very low cell density (Fig. 1*E–H*). To evaluate the proliferative profile of rKS56, we counted cell number at several time points after replating 100 cells/well in comparison with a renal epithelial cell line, NRK-52E. As shown in Fig. 1*I*, cell number of rKS56 increased dramatically from 120 h to 168 h, and the growth curve indicated exponential growth, while NRK-52E grew steadily and the growth curve indicated linear growth. Based on the growth curve, doubling time of rKS56 was calculated as 16–24 h, which is comparable to those in stem/progenitor cell population (25).

Karyotype of rKS56 cells

One might argue that rKS56 cells are transformed cells similar to tumor cells. In regard to karyotype, we checked the karyotype of rKS56 cells after numerous passages (250 days). rKS56 cells consisted of 66–71 chromosomes and thus considered to be nearly triploid (Fig. 1*J*). However, we could not find out at which point rKS56 cells had converted the karyotype from diploid to triploid. In another sets of experiments, we implanted 1.6×10^7 of rKS56 cells (after 200 days of passages) subcutaneously to nude mice and observed for 13 months. We could not find formation of any tumor like mass in these mice (data not shown). Therefore, we consider that rKS56 cells did not give rise to tumorigenesis and so could not be considered to possess tumor cell phenotype.

rKS56 cells originated from a single cell and expanded itself

In the first culture, rKS56-Di-O cells were detected like mosaicism pattern (**Fig. 2*A*, *B***). In the secondary culture, a single rKS56 cell-Di-O formed colony (Fig. 2*C–E*). After several cycles of cell division, mosaicism of labeled and unlabeled progeny cells could be observed after two passages (Fig. 2*F–H*). Neural stem cells obtained by neurosphere methods could be generated from a single primary sphere. rKS56 cells possess the same characteristics as neural stem cells. These results may suggest that rKS56 cells have the potential for self-renewal.

Immature tubular epithelial-like phenotype of rKS56

Next, we attempted to characterize the rKS56 cells. rKS56 cells exhibited a cobblestone appearance and expressed pancytokeratin (**Fig. 3*A***), indicating epithelial cell phenotype. As for mature tubular epithelial cell markers, water channel aquaporin (AQP)-1 was weakly expressed in cytoplasmic pattern (Fig. 3*B*). rKS56 cells were also positive for vimentin, a mesenchymal cell marker (Fig. 3*C*), and c-met, a receptor for hepatocyte

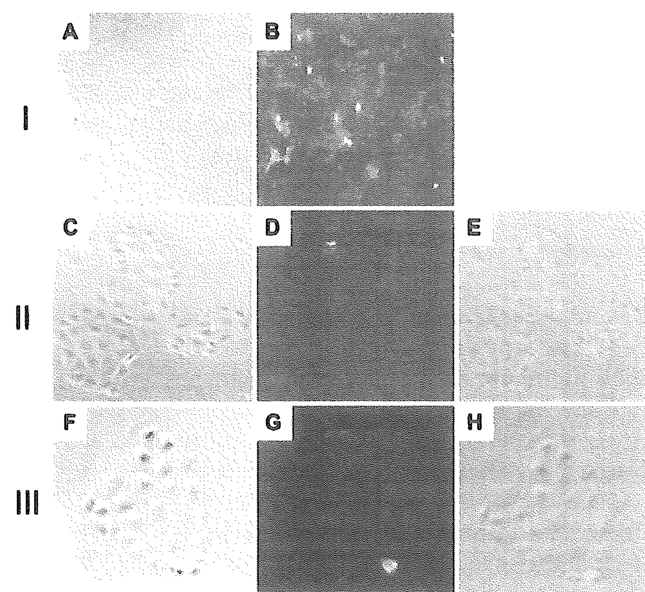

Figure 2. rKS56 cells generated from a single cell. rKS56 Cells were labeled with Di-O and plated. In the first passage, the expanded cell population was composed of Di-O(+) and Di-O(−) cells. Left: light microscopy (*A*), middle: fluorescent microscopy (*B*). Cells were harvested, and a single cell was replated and observed for further two passages. Second passage from a single Di-O positive cell: left: light microscopy (*C*), middle: fluorescent microscopy (*D*), right: merged images (*E*). Third passage from a single Di-O positive cell: left: light microscopy (*F*), middle: fluorescent microscopy (*G*), right: merged images (*H*).

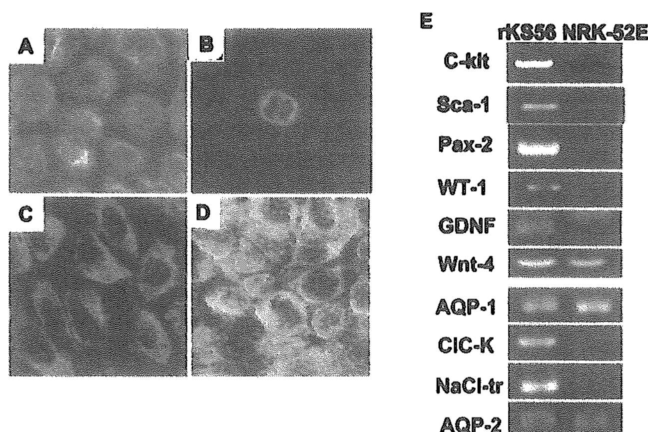

Figure 3. Characterization of rKS56 cells. rKS56 cells expressed pan-cytokeratin (A), aquaporin-1 (*B*), vimentin (*C*), and c-met (*D*) detected by immunocytochemistry (x400). *E)* Expression of development-associated genes and renal-specific genes in rKS56 cells was observed by RT-PCR. ClC-K: chloride channel-K, NaCl tr: NaCl transporter.

growth factor (Fig. 3*D*). RT-PCR revealed that rKS56 expressed various molecules relating to progenitor cell marker, nephrogenesis and mature tubular cell functions (Fig. 3*E*). rKS56 cells also expressed mRNAs for Sca-1 and c-kit, markers of progenitor cells, and expressed mRNAs for Pax-2, GDNF, WT-1 and Wnt-4 which are essential molecules involved in nephrogenesis, while NRK-52E showed limited expression. rKS56 also expressed mRNAs for AQP-1, chloride channel, NaCl transporter, AQP-2 which are functional molecules in mature tubular epithelial cells, while NRK-52E showed limited expression. These results suggest that rKS56 cells are derived from renal epithelial cells and possess relatively immature phenotype as compared with NRK-52E.

Musashi-1 expression in rKS56

Specific markers for immature renal stem/progenitor cells have not been identified yet. We, therefore, examined the expression of Musashi-1, which is a well known marker for neural stem cells. Interestingly, rKS56 expressed protein in a cytoplasmic pattern (**Fig. 4*A***) and Musashi-1 mRNA detected by RT-PCR (Fig. 4*B*). The expression of Musashi-1 in rKS56 was further confirmed by immunoblot using cell lysates. rKS56 contained Musashi-1 protein 2.5-fold higher than NRK-52E (Fig. 4*C*). These results suggest that rKS56 cells may have an immature character such as neural stem cells.

Ability of rKS56 to differentiate into mature renal tubular epithelial cells

Because rKS56 cells appear to possess immature renal epithelial phenotype, we explored whether rKS56 cells could differentiate into mature tubular epithelial cells under various culture conditions. In general, AQP-1 is expressed on renal proximal tubular epithelial cells and cells in the descending loop of Henle. AQP-2 is expressed mainly on collecting duct epithelial cells and weakly on cells in ascending loop of Henle and distal tubular epithelial cells. THP is expressed mainly in epithelial cells in the loop of Henle. When rKS56 cells were cultured with MCS, AQP-1 and AQP-2 were observed as cytosolic pattern (**Fig. 5*A*, *C***) and only a single cell was positive for THP (Fig. 5*B*). However, when rKS56 cells were cultured without MCS, AQP-1 was observed as membrane pattern (Fig. 5*D*), and AQP-2 was observed as membrane pattern (Fig. 5*F*). Numerous THP positive cells were observed under culture condition without MCS in maintenance culture media (Fig. 5*E*). These findings suggest that rKS56 cells may differentiate to renal component cells such as AQP-1 positive cells, AQP-2 positive cells and Tamm-Horsfall positive cells.

The phenotypic changes into more mature tubular epithelial cells were further confirmed by responses to parathyroid hormone (PTH) and arginine vasopressin (AVP). Generally, proximal tubular epithelial cells respond to PTH and distal tubular epithelial cells and collecting duct cells respond to AVP. The response to these hormones was evaluated by accumulation of cAMP. Addition of LIF to maintenance culture condition enhanced cAMP accumulation in response to PTH (**Fig. 6*A***). Furthermore, maintenance culture condition with LIF and without MCS dramatically increased cAMP accumulation in response to PTH and AVP (Fig. 6*B*). These findings suggest that rKS56 may have the capacity to differentiate into mature tubular epithelial cells.

Ectopic implantation of rKS56 cells results in tubular differentiation

To examine the plasticity of rKS56 cells in vivo, cells were transplanted into various organs including liver,

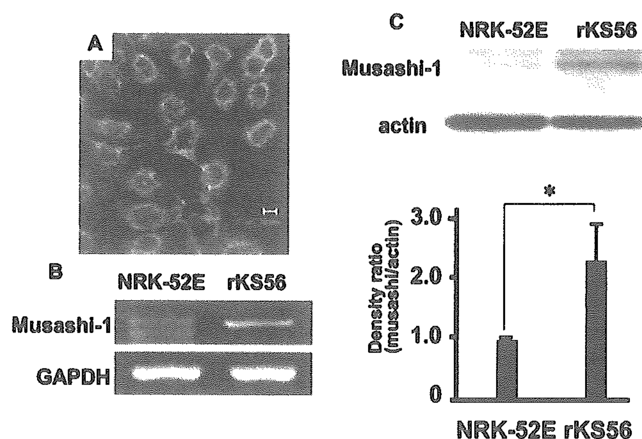

Figure 4. Musashi-1 expression in rKS56 cells. rKS56 cells expressed Musashi-1 protein detected by immunocytochemistry (×400, *A)* Similar results were observed by RT-PCR (*B*) and immunoblot (*C*). (*C*, upper panel) Each band was scanned and subjected to densitometry. Intensities of Musashi-1 protein relative to actin are shown (C, lower panel). The expression of Musashi-1 in rKS56 cells was 2.5-fold higher than NRK-52E cells. *$P < 0.05$ vs. NRK-52E. Each column consists of mean ± SE.

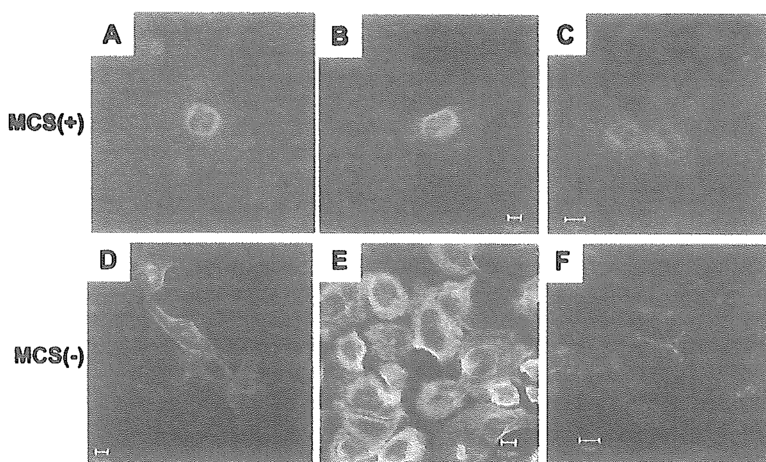

Figure 5. Differentiation of rKS56 cells. rKS56 cells exhibited weak expression of aquaporin-1 (AQP-1, *A*), Tamm-Horsfall protein (THP, *B*), and aquaporin-2 (AQP-2, *C*) under maintenance culture conditions detected by immunocytochemistry (x400). However, under maintenance culture condition without MCS, rKS56 cells exhibited differentiated renal tubular epithelial phenotype highly expressing kidney-specific proteins such as AQP-1 (*D*), THP (*E*), and AQP-2 (*F*), and intracellular localization of these proteins were altered. AQP-1 and AQP-2 were detected as plasma membrane pattern and not as cytoplasmic pattern under culture condition without MCS.

spleen, kidney, skeletal muscle and skin of SD rats. On day 28 after implantation, we could not identify any cell clusters in the liver or spleen (data not shown), whereas a small mass composed of numerous tubule-like structure was observed at the intramuscular injection sites identified by retention of black ink (**Fig. 7*A*, *B***). These tubule-like structures were positive for AQP-1 and AQP-2 (Fig. 7*C*, *D*).

Engraftment and differentiation of rKS56 cells in acute tubular injury model

We next examined the capacity of rKS56 cells to repair injured tubules. rKS56 cells relabeled with Di-O were injected subcapsularly near corticomedullary lesion in the kidney after 40 min of ischemia. On the 7th day after cell implantation, localization of implanted rKS56 cells was evaluated. Most of the Di-O positive cells were observed in tubules in the corticomedullary region and were positive for cytokeratin (**Fig. 8*A–C***). These cells coexpressed AQP-1 in apical and basolateral side (Fig.

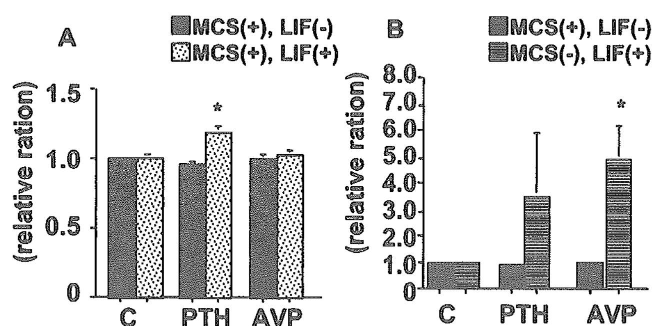

Figure 6. Hormone response of rKS56 cells. Under maintenance condition, rKS56 cells did not respond to PTH and AVP (*A, B*: filled bar). However, in culture condition containing leukemia inhibitory factor (LIF), the rKS56 cells responded only to PTH (*A*, dotted bar). In culture condition without supplementation of condition medium of mesenchymal cells (MCS) and LIF, the rKS56 cells responded to AVP more markedly than stimulation with PTH (*B*, hatched bar). *C)* Saline control, PTH: parathyroid hormone, AVP: arginine vasopressin, $*P < 0.05$ vs. saline control. Each column consists of mean ± SE.

8*D–F*). Di-O positive cells were observed not only at the sites of injection but also at distinct sites such as cortex and medulla (Fig. 8*G–I*).

We could not observe any significant differences in the level of serum creatinine (Cr) and blood urea nitrogen (BUN) between I/R rats treated with rKS56 cells and untreated animals (Cr of I/R rats 4 days 0.370 ± 0.017; Cr of I/R rats 7 days 0.370 ± 0.015; Cr of cell therapy and I/R rats 4 days 0.407 ± 0.052 [P=0.4866 vs. Cr of I/R rats 4 days]; Cr of cell therapy and I/R rats 7 days 0.400 ± 0.020 mg/dl [P=0.3223 vs. Cr of I/R rats 7 days], BUN of I/R rats 4 days 19.3 ± 0.704; BUN of I/R rats 7 days 18.7 ± 2.08; BUN of cell therapy rats 4 days 19.97 ± 0.977 [P=0.5924 vs. BUN of I/R rats 4 days]; BUN of cell therapy rats 7 days 21.4 ± 2.514 mg/dl [P=0.4957 vs. BUN of I/R rats 7 days]). Although we could find increase of U-NAG levels after 7 days in I/R induced/untreated group, rats receiving implantation of rKS56 cells showed a significant suppression in U-NAG levels (Fig. 8*J*). The significant suppression of U-NAG elevation suggested that damage of tubulointerstitial lesion was protected. Implanted rKS56 cells could replace injured tubular cells and differentiate into mature tubular epithelial cells, at least proximal tubular epithelial cells, in acute tubular necrosis model.

DISCUSSION

In the present study, we purified a cell population exhibiting a high proliferation rate from the S3 segment in adult rat kidney. From these cells, we established an epithelial-like cell line:rKS56, which showed three characteristic features of stem/progenitor cells: self-renewal, multiple plasticity restricted to renal epithelial cells and regenerative ability by replacing injured tubular epithelial cells in the ATN model.

Although kidney is an organ with less mitosis under normal conditions as compared with other organs such as intestine or skin, the kidney has the capacity to regenerate itself once injured or partially ablated. Acute tubular necrosis, for example, occurs by expo-