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非免疫性溶血性貧血を対象とした診断システムの構築

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非免疫性溶血性貧血を対象とした診断システムの構築

Recent progress in laboratory diagnosis of non-immune hemolytic anemia

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〔臨床検査　58：327-335，2014〕

Point

- 直接抗グロブリン試験(DAT)，赤血球表面マーカー検査(CD55/CD59)，赤血球表面 IgG 分子数測定検査による免疫性溶血性貧血を否定する．
- 家族歴の聴取，赤血球像の観察を実施する．
- 溶血性貧血の診断基準に基づき，貧血と黄疸を伴うが溶血を主因としないほかの疾患として，特に骨髄異形成症候群(MDS)と先天性赤血球異形成貧血(CDA)については骨髄像を検討する．
- 赤血球 EMA 結合能検査は，赤血球膜異常症の診断に必須の項目である．
- 今後は網羅的で迅速な遺伝子検査の導入が不可欠となる．

Keywords

赤血球酵素異常症，不安定ヘモグロビン症(UHD)，遺伝性球状赤血球症(HS)

はじめに

筆者らは，一般臨床検査にて診断を確定することが困難な溶血性貧血症例を対象とした特殊検査，特に赤血球酵素異常症に関する遺伝学的検査を実施している．赤血球酵素異常症は解糖系，ペントースリン酸経路，グルタチオン生合成・還元系，ヌクレオチド代謝系などの酵素遺伝子変異によって生じる先天性溶血性貧血の一型である．

筆者らが主として対象としている赤血球酵素異常症は，赤血球膜骨格蛋白の異常によって生じる遺伝性球状赤血球症(hereditary spherocytosis；HS)などの膜異常症とは異なり，通常赤血球形態には大小不同などの非特異的異常以外認められないことから，先天性非球状赤血球性溶血性貧血(congenital non-spherocytic hemolytic anemia；CNSHA)と称されている．この CNSHA のなかには赤血球酵素異常症以外に不安定ヘモグロビン症(unstable hemoglobin disease；UHD)が含まれ，さらに溶血性貧血以外の鑑別疾患として骨髄異形成症候群(myelodysplastic syndrome；MDS)や先天性赤血球異形成貧血(congenital dyserythropoietic anemia；CDA)などが挙げられる．

本稿では，依頼元の各医療機関や臨床検査センターで赤血球形態異常を指摘されず，“非球状性”

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溶血性貧血として筆者らの検査室に検査の依頼がある赤血球膜異常症症例が増えていることなど，現在実施しているCNSHAのスクリーニング検査について概説する．

先天性溶血性貧血の診断チャート

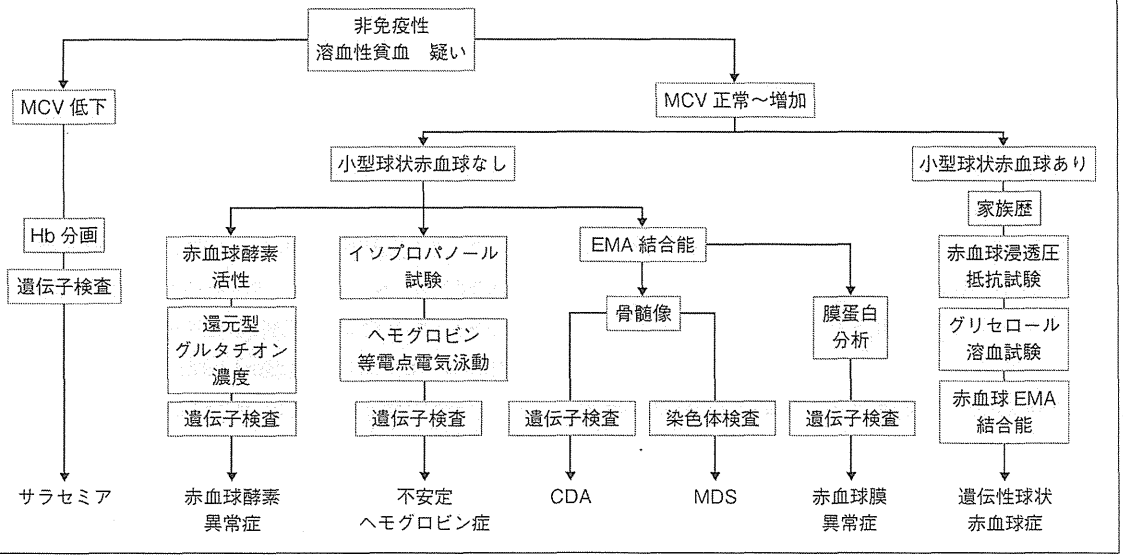
表1[1]に溶血性貧血の診断基準を示す．筆者らの検査室では，直接抗グロブリン試験(direct antiglobulin test；DAT)陰性で赤血球表面IgG(immunoglobulin G)分子数の増加も認めず，CD55/CD59二重陰性赤血球数増加が認められない非免疫性溶血性貧血を対象に，図1のチャートで示す

表1　溶血性貧血の診断基準(厚生労働省研究班による2004年度改訂版)

1. 臨床所見として，通常，貧血と黄疸を認め，しばしば脾腫を触知する．ヘモグロビン尿や胆石を伴うことがある．
2. 以下の検査所見がみられる．
   1) ヘモグロビン濃度低下
   2) 網赤血球増加
   3) 血清間接ビリルビン値上昇
   4) 尿中・便中ウロビリン体増加
   5) 血清ハプトグロビン値低下
   6) 骨髄赤芽球増加
3. 貧血と黄疸を伴うが，溶血を主因としない他の疾患(巨赤芽球性貧血，骨髄異形成症候群，赤白血病，congenital dyserythropoietic anemia，肝胆道疾患，体質性黄疸など)を除外する．
4. 1.，2.によって溶血性貧血を疑い，3.によって他疾患を除外し，診断の確実性を増す．しかし，溶血性貧血の診断だけでは不十分であり，特異性の高い検査によって病型を確定する．

〔特発性造血障害に関する調査研究班：自己免疫性溶血性貧血．特発性造血障害疾患の診療の参照ガイド，p144，2010(http://zoketsushogaihan.com/file/AIHA.pdf)より転載〕

図1　非免疫性溶血性貧血診断フローチャート



直接抗グロブリン試験，赤血球表面IgG分子数，CD55/CD59二重陰性赤血球検出などの検査項目により非免疫性溶血性貧血がほぼ否定された症例について，上記のようなフローチャートで診断を進めていく．
MCV：mean corpuscular volume(平均赤血球容積)，Hb：hemoglobin，EMA：eosin 5′-maleimide，CDA：congenital dyserythropoietic anemia(先天性赤血球異形成貧血)，MDS：myelodysplastic syndrome(骨髄異形成症候群)．

ような諸検査を実施している．

溶血性貧血では，骨髄における赤芽球過形成を反映して網赤血球増加を認めるため，通常平均赤血球容積(mean corpuscular volume；MCV)は正球性～大球性貧血を示す．申込時には，網赤血球数を含む血算，血液生化学検査としてAST(aspartate aminotransferase)，ALT(alanine aminotransferase)，LDH(lactate dehydrogenase)，ビリルビン(総・間接)，Fe，TIBC(total iron-binding capacity)，フェリチン，ハプトグロビンなどを参考にして，慢性溶血の有無を確認する．グルコース-6-リン酸脱水素酵素(glucose-6-phosphate dehydrogenase；G6PD)異常症など急性溶血発作で発症する症例では，ヘモグロビン尿や尿中ヘモジデリンの検出が重要である．

末梢血塗抹標本による赤血球形態の確認

溶血性貧血に共通した赤血球形態として，大小不同(anisocytosis)，多染性(polychromatophilia)がある．小型球状赤血球はHSや自己免疫性溶血性貧血(autoimmune hemolytic anemia；AIHA)で観察される．不安定ヘモグロビンが赤血球内で変性して沈殿すると，Heinz小体と呼ばれる構造が赤血球内に出現する．UHDの重症例では，Heinz小体の出現により赤血球は変形能が障害され，網内系で赤血球膜の一部がHeinz小体とともにちぎり取られる．機械的機序で生じる溶血性貧血で認められるような奇形赤血球，破砕赤血球(schistocyte)が観察される．奇形赤血球，破砕赤血球は赤血球膜異常症の重症型でも認められる[2]．

赤血球酵素異常症では，特徴的な赤血球形態を示すことが少ない．ピリミジン5′-ヌクレオチダーゼ(pyrimidine-5′-nucleotidase；P5N)異常症では，赤血球中にピリミジンヌクレオチドが蓄積し，Wright染色で赤血球の細胞質に好塩基性斑点(basophilic stippling)と呼ばれる微細な封入体を観察できる．P5Nは鉛により阻害を受けるため，後天性疾患としては鉛中毒の際にも観察される．摘脾後のピルビン酸キナーゼ(pyruvate kinase；PK)異常症では，ウニ状ないし金平糖状の赤血球(echinocyte，spiculated erythrocyte)が観察される．

CNSHA病型診断のための検体採取から検査までの流れ—血液検体の処理

1．採血量・方法

抗凝固剤(無菌ヘパリン)存在下で発端者(5～10 mL)および健康成人対照(5 mL，血縁者は不可，年齢と性別を明記してもらう)から採取した静脈血をすぐに冷所(4℃)に移す．患者と同時に必ず正常対照から採血し，サンプル調製までの保存・輸送条件による測定値の解釈における内的標準とすることで，人為的な検査値の異常の発生をチェックすることができる．120日という赤血球の寿命を考えて，被検者は最低1カ月以上赤血球輸血を受けていないことが必要である．輸血を受けている場合，測定した赤血球酵素活性は輸血された正常赤血球の酵素活性の影響を受け，見掛け上正常活性を呈する可能性があるからである．

2．輸送

測定場所が遠方の場合には，翌日必着の宅配便を利用して蓄冷材とともに発泡スチロールの箱に検体を入れてもらう．昨今，宅配便のクール指定が必ずしも確かな温度管理をされていない事例が散見されることから，“4℃指定”のクール便よりも上記の方法が確実である．患者が測定医療機関に直接来院できる際には，受診を促すことも必要である．

検体が蓄冷材に直接触れると凍結する恐れがあるので，ガーゼなどの緩衝材を入れてもらう．全血のまま4℃の冷蔵保存では，採血後5日間は酵素活性の低下は認められない．到着後すぐに検体の状態を確認する．夏場など発泡スチロールにドライアイスを入れてしまう例もあり，その場合には赤血球が溶血してしまい，測定不能となるので注意が必要である．

1．イソプロパノール試験

1）測定意義

異常ヘモグロビン症は，グロビン鎖におけるアミノ酸配列異常の結果，ヘモグロビンの質的変化をきたして発症する一連の疾患群を示す．このなかには，熱あるいはイソプロパノールに対する感受性の亢進を示す例が含まれ，その異常ヘモグロビンを不安定ヘモグロビンと呼ぶ．異常ヘモグロビンのうち，赤血球内での安定性が非常に低く，変性して沈殿することにより溶血を促進させる病態をUHDといい，過去にはHeinz小体溶血性貧血と表現されていた．

従来，自己溶血試験で48時間後に血清が茶褐色になり，孵置後の赤血球にHeinz小体を多数観察する場合，不安定ヘモグロビンの存在が強く疑われるが，現在CNSHAのスクリーニング検査として全例にイソプロパノール試験を行い，不安定ヘモグロビンの有無を確認している．

2）測定の実際

ヘパリン血からセルロースカラムで白血球・血小板を除去した赤血球沈渣を用いる．

（1）赤血球沈渣500 μLをガラス製試験管に移し，蒸留水250 μLを加えてソニケーションした後，トルエンを250 μL加え，1分間vortexする．
（2）5分間室温に放置し，さらにもう1回1分間vortexする．
（3）3,500回転，20分間室温で遠心する．
（4）上層のトルエン，中間層の赤血球膜成分を混入しないように溶血液をパスツールピペットで別の試験管に移す．
（5）イソプロパノール試験用バッファー（イソプロパノール17 mL，1Mトリス-塩酸バッファー，pH 7.4，83 mLを混合したもの）2 mLを分注し，37℃で5分間以上保温したガラス製試験管に溶血液200 μLを加え，転倒混和する．
（6）37℃で孵置し，5分後，15分後，30分後に沈殿の有無を観察する．
（7）5分後に沈殿を認める場合を（＋），15分後以降に沈殿の析出が観察されたら（±），30分で沈殿を認めなかったら（－）と判定する．

3）結果の解釈

1歳未満の乳児やサラセミア症例など，ヘモグロビンF（hemoglobin F；Hb F）濃度の高いサンプルでは15分で沈殿を形成することがあり，偽陽性となることがある．東南アジアで高頻度に観察される．

UHDの病態は，代償性溶血，慢性溶血性貧血あるいは急性溶血発作例など多彩な症状をきたす．両親由来の対立遺伝子のどちらか一方に変異がある場合に溶血を起こすため，常染色体優性遺伝形式をとるが，新生変異による発症例では家族歴で溶血性貧血は認められない．

イソプロパノール試験で陽性の場合には，等電点電気泳動（isoelectric focusing；IEF）や遺伝子検査による確定診断が必要である．

2．還元型グルタチオン定量

1）測定意義

グルタチオンは，赤血球で最も重要なアンチオキシダントである．**図2**のように初段階でシステイン，グルタミン酸からグルタミルシステイン，次の段階でグリシンが加わり，グルタチオンが形成される．この2段階の反応を触媒する酵素が，γグルタミルシステイン合成酵素およびグルタチオン合成酵素である．この2つの生合成系酵素に異常がある場合には，還元型グルタチオン（glutathione；GSH）定量で著減が認められる．また，グルタチオン還元酵素（glutathione reductase；GR）反応の補酵素であるニコチンアミドアデニンジヌクレオチドリン酸（nicotinamide adenine dinucleotide phosphate；NADP）の産生障害を生じるG6PD異常症でもGSH濃度は低下する．

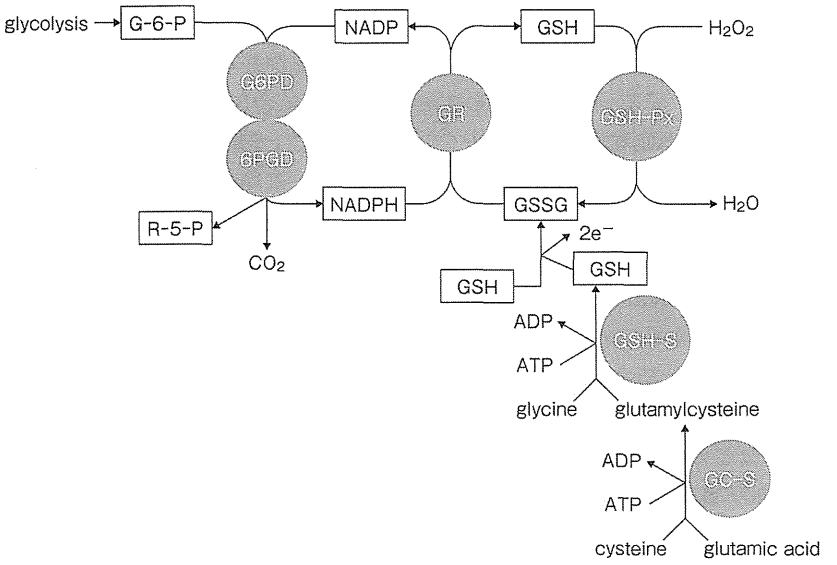
2）測定原理

SH（sulfhydryl）基を有する化合物にDTNB〔5,5′-dithiobis（2-nitrobenzoic acid）〕を加えるとDTNBの定量的還元反応が起こり，412 nmに吸収を有する黄色を呈することを利用する．GSHは過剰のDTNBにより酸化型グルタチオン（glutathione disulfide；GSSG）となる．

3）結果の解釈

筆者らの検査室でのGSH正常値は65.9～88.5

図2 活性低下が先天性溶血性貧血の原因となるグルタチオン生合成・還元系酵素



青地白抜きの円で示す6種の酵素活性低下が先天性溶血性貧血の原因となることが知られている.
G-6-P：glucose-6-phosphate, NADP：nicotinamide adenine dinucleotide phosphate, NADPH：reduced nicotinamide adenine dinucleotide phosphate, GSH：glutathione, GSSG：glutathione disulfide, R-5-P：ribose 5-phosphate, G6PD：glucose-6-phosphate dehydrogenase, 6PGD：6-phosphogluconate dehydrogenase, GR：glutathione reductase, GSH-Px：glutathione peroxidase, GSH-S：glutathione synthetase, GC-S：γ-glutamylcysteine synthetase.

(M±SD)である．前述の通り，グルタチオン生合成系，還元系障害で低下する以外にも，赤血球内酸化ストレスの増大をきたすさまざまな病態で低下するため，特異性は低い．一方，P5N異常症，一部のMDSではその赤血球内含量が増加することが知られている．また，GSH還元系の機能が正常な赤血球では，アセチルフェニルヒドラジンのような酸化剤を加えてもGSH量はほとんど変化しないが，G6PDおよびGR異常症では酸化刺激によりGSH量の低下をきたす．

3．赤血球EMA結合能

1）測定意義

わが国の先天性溶血性貧血症例の約70％を占めるHSは，α・βスペクトリン(*SPTA1*, *SPTB*)，バンド3(*SLC4A1*)，アンキリン(*ANK1*)およびバンド4.2(*EPB42*)遺伝子の変異により，これら膜骨格を形成する蛋白の質的・量的な異常をきたし，発症する．赤血球浸透圧抵抗試験は手技が簡単である反面，その感度および特異性の低さから非定型的HSや軽症HSを見逃すことが臨床的に問題となっている．実際摘脾前のHSにおいては，約1/3の症例で浸透圧抵抗の低下が認められない．また，妊娠や腎不全患者，免疫性溶血性貧血例などで偽陽性を呈することもある．

現在，HSのスクリーニング検査として最も有用なのは，赤血球EMA(eosin 5′-maleimide)結合能測定検査である[3]．EMAは赤血球膜貫通蛋白の1つ，バンド3の細胞外ループにあるLys430残基と結合する蛍光色素であり，HSにおける赤血球表面積の減少に伴い，1つの赤血球当たりのバンド3分子数が減少することを検出する．

2）測定の実際

血漿を除き，生理的食塩水で洗浄した赤血球5 μLをリン酸緩衝生理的食塩水(phosphate buffered saline；PBS)で溶解した0.5 mg/mL EMA液に懸濁して暗所で1時間孵置，時々攪拌する．その後，遠心操作により非結合色素を除き，PBS/BSA(phosphate buffered saline/bovine serum albumin，0.5％牛血清アルブミンを含むPBS)で3回洗浄後，500 μLのPBS/BSAで再懸濁する．そのうちの100 μLにPBS/BSAを1,400 μL加えたものをサンプルとし，FL-1チャネル(緑色

蛍光)を用いてフローサイトメトリーを行い，平均チャネル蛍光強度(mean channel fluorescence：MCF)を測定する．

3）結果の解釈

HSにおいては，その分子異常がアンキリン，スペクトリン(α・β)，バンド3，4.2蛋白のいずれであっても，結果として赤血球表面積の減少をきたすため，バンド3に結合するEMA量が減少する．したがって，赤血球表面積の減少を起こすほかの疾患，HPP(hereditary pyropoikilocytosis)，SAO(Southeast Asia ovalocytosis)，CDA2(congenital dyserythropoietic anemia type Ⅱ)などの病態においてもEMA結合能の低下を認める．自験例において，HS以外の溶血性貧血，G6PDやPK異常症などの赤血球酵素異常症，サラセミア，鎌状赤血球貧血，UHD，発作性夜間ヘモグロビン尿症(paroxysmal nocturnal hemoglobinuria；PNH)などの病態では，EMA結合能の低下は認められなかった．正常対照とHS症例との比較ではMCFは80%以下に低下することが多く，軽度のEMA低下例では，臨床所見やほかの検査データを参考にしながら慎重に病型診断する必要がある．

EMA結合能が極端に低下している症例で，赤血球形態上HPP[4]様の奇形赤血球，破砕赤血球が観察される場合は，αスペクトリン遺伝子変異の複合ヘテロ接合体であるHPP，あるいはスペクトリン・アンキリンの複合異常を考慮する．

最近のレビュー[5]によるHS診断におけるEMA結合能の感度は93%，特異性は98%であり，酸性化グリセロール溶血試験(acidified glycerol lysis test；AGLT)では感度が95%，特異性が91%とされ，感度面ではややAGLTのほうが上回る結果となっている．いずれの方法も浸透圧抵抗試験の68%(新鮮血)，81%(孵置血)を大きく上回っていた．

溶血液で検討する項目—赤血球酵素活性測定

1．測定意義

赤血球代謝異常によって赤血球寿命が短縮する一連の先天性溶血性貧血では，赤血球酵素活性を測定することで診断が可能である[6,7]．解糖系酵素異常症(図3)の場合，活性低下を示した酵素より上位の中間代謝産物が赤血球内に蓄積していることを証明する．あるいは表3に示す遺伝様式を参考にして両親の酵素活性も測定することが望ましい．さらに構造遺伝子の変異を確認することが確定診断となる．

2．測定の実際

1）白血球，血小板の除去による赤血球精製

ほとんどの酵素は赤血球より白血球・血小板のほうが1細胞当たりの酵素活性が高く，それらの細胞を分離除去してから赤血球溶血液を作製しないと，赤血球酵素活性は正確に測定することはできない．筆者らはmicrocrystalline cellulose/α-cellulose のカラムを通すことで白血球，血小板などほかの血球を除いている．

①生理食塩水にSigmacell cellulose type 50 (Sigma-Aldrich® 社)，α-Cellulose powder (Sigma-Aldrich® 社)を等量混合し，生理的食塩水に懸濁する．上澄みに残って沈殿しない細かいセルロース粒子は丁寧に取り除いておき，4℃に保存する．10 mLディスポシリンジの外筒に定性濾紙No.2(ADVANTEC® 社)を敷き，約1.5～2 cmの高さまで用意したセルロース懸濁液を詰める．

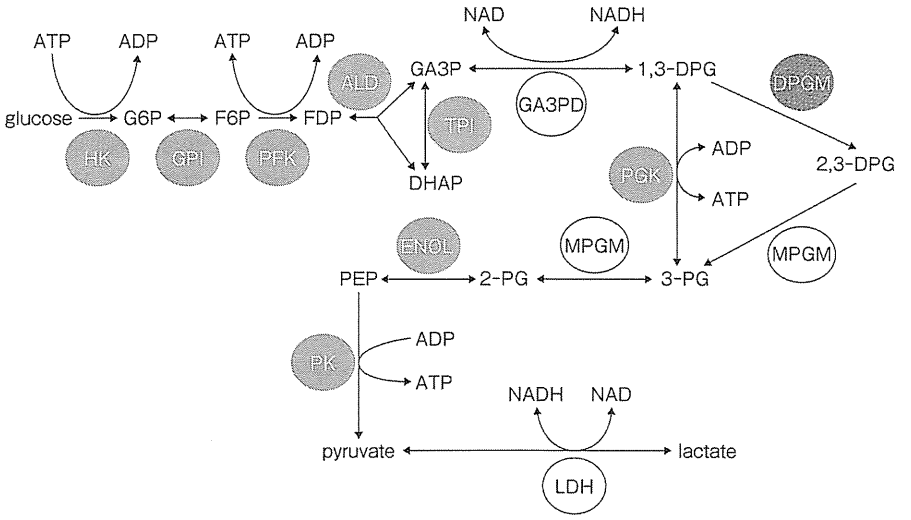
②ヘパリン採血して得た全血を1,500 g，4℃，10分間遠心して，血漿・バッフィーコートを除いた後，赤血球層に等量の生理食塩水を加えて赤血球浮遊液とする．これを①で用意したセルロースカラムに通し，その上から冷生理食塩水を加えて赤血球を濾過する．

③濾過した赤血球浮遊液を冷生理食塩水で3回洗浄後，赤血球層を生理食塩水で約2倍に希釈し，これを測定用血球浮遊液とする．当日すぐに測定しない場合は，赤血球層にグリセロール/ソルビトールの1：1混合液を等量加え，よく混合してから液体窒素または−80℃に保存する．

2）溶血液作製

血漿，白血球，血小板を除いた後，低張液中で超音波破砕して得られる赤血球溶血液を用いて，

図3 活性低下が先天性溶血性貧血の原因となる解糖系酵素



青地白抜きの円で示す8種の酵素活性低下が先天性溶血性貧血の原因となることが知られている．DPGM異常症は赤血球2,3-DPG濃度低下により，ヘモグロビンの酸素親和性が高くなり，赤血球増加症の原因となる．
NAD：nicotinamide adenine dinucleotide，NADH：reduced nicotinamide adenine dinucleotide，2,3-DPG：2,3-diphosphoglyceric acid，G6P：glucose-6-phosphate，F6P：fructose-6-phosphate，FDP：fructose 1,6-bisphosphate，GA3P：glyceraldehyde-3-phosphate，DHAP：dihydroxyacetone phosphate，1,3-DPG：1,3-diphosphoglycerate，3-PG；glycerate 3-phosphate，2-PG：glycerate 2-phosphate，PEP：phosphoenolpyruvate，HK：hexokinase，GPI：glucose phosphate isomerase，PFK：phosphofructokinase，ALD：aldolase，TPI：triosephosphate isomerase，GA3PD：glyceraldehyde-3-phosphate dehydrogenase，PGK：phosphoglycerate kinase，DPGM：diphosphoglycerate mutase，MPGM：monophosphoglycerate mutase，ENOL：enolase，PK：pyruvate kinase，LDH：lactate dehydrogenase．

酵素活性を測定する．

測定用血球浮遊液0.2 mLに安定化液1.8 mLを加え混和した後，約5秒間超音波破砕器にてソニケーションし，1：20溶血液とする．これをさらに10倍あるいは100倍希釈することにより，1：200，1：2,000倍溶血液を作製する．

3）代表的な赤血球酵素活性の測定法

①グルコース-6-リン酸脱水素酵素(G6PD)

キュベットA：0.1M Tris-HCl/0.5 mM EDTA pH 8.0，10 mM $MgCl_2$，0.2 mM NADP，0.6 mM G-6-P(glucose-6-phosphate)，0.6 mM 6-PGA(6-phosphogluconic acid)．

キュベットB：0.1M Tris-HCl/0.5 mM EDTA pH 8.0，10 mM $MgCl_2$，0.2 mM NADP，0.6 mM 6-PGA，1：20溶血液20 μL，ブランク・キュベットAで溶血液なし．

340 nm，37℃，10分間スキャンする．キュベットAとキュベットBの吸光度変化の差がG6PD活性となる．

②ピルビン酸キナーゼ(PK)

0.1M Tris-HCl/0.5 mM EDTA pH 8.0，10 mM $MgCl_2$，100 mM KCl，0.2 mM NADH，1.5 mM ADP(adenosine 5′-diphosphate)，5 mM PEP(phosphoenolpyruvate)，6U LDH(lactate dehydrogenase)，1：20溶血液20 μL，ブランク・溶血液なし．

340 nm，37℃，10分間スキャンする．ブランクとの吸光度変化の差がPK活性となる．

3．結果の解釈

溶血性貧血では網赤血球増多を伴うことが多く，結果の解釈には十分な注意が必要である．すなわち，赤血球寿命に伴い活性が徐々に低下する酵素(age-dependent enzyme)については，網赤血球における酵素活性が成熟赤血球の数倍から10倍以上を示すために，溶血性貧血では酵素活性低下がマスクされてしまうことがある．**表2**には健康成人対照と赤血球酵素異常症以外の網赤血球増加例における酵素活性基準値の比較を示す．

表2　正常対照と網赤血球増加例の赤血球酵素活性比較

酵素名	基準値 (IU/gHb)	網赤血球増加例 (IU/gHb)
HK	1.08～1.46	2.86～5.86
PK	13.0～19.8	23.6～39.0
G6PD	7.61～9.81	12.8～22.6
AK	165～307	187～305

HK：hexokinase，PK：pyruvate kinase，G6PD：glucose-6-phosphate dehydrogenase，AK：adenylate kinase.

赤血球酵素異常症で頻度の高いPK異常症，G6PD異常症はPK，G6PDがage-dependent enzymeであることから，網赤血球増加例で活性が高いこと，一方アデニル酸キナーゼ(adenylate kinase；AK)活性は赤血球寿命に伴い活性低下がないことから，正常対照と網赤血球増加例で基準値がほぼ重なることが分かる．

酵素の高次構造や発現機構，蛋白の構造と機能の関連がすでに判明した酵素では，遺伝子DNAや網赤血球RNAを用いた遺伝子解析(**表3**)が生化学的手法に代わる新たな診断法になりつつある．

表3　活性低下が先天性溶血性貧血の原因となる赤血球酵素の構造遺伝子と遺伝形式

	酵素名	遺伝子(OMIM)	遺伝形式
1	ヘキソキナーゼ	*HK1*(*142600)	AR
2	グルコースリン酸イソメラーゼ	*GPI*(*172400)	AR
3	ホスホフルクトキナーゼ	*PFKM*(*610681)	AR
4	アルドラーゼ	*ALDOA*(*103850)	AR
5	三炭糖リン酸イソメラーゼ	*TPI1*(*190450)	AR
6	ホスホグリセリン酸キナーゼ	*PGK1*(*311800)	XR
7	エノラーゼ	*ENO1*(*172430)	AR
8	ピルビン酸キナーゼ	*PKLR*(*609712)	AR
9	グルコース-6-リン酸脱水素酵素	*G6PD*(*305900)	XR
10	6-ホスホグルコン酸脱水素酵素	*PGD*(*172200)	AR
11	アデニル酸キナーゼ	*AK1*(# 612631)	AR
12	アデノシンデアミナーゼ	*ADA*(102730)	AD
13	ピリミジン5′-ヌクレオチダーゼ	*NT5C3*(*606224)	AR
14	γグルタミルシステイン合成酵素	*GCLC*(# 230450)	AR
15	グルタチオン合成酵素	*GSS*(# 231900)	AR
16	グルタチオン還元酵素	*GSR*(+138300)	AR
17	グルタチオンペルオキシダーゼ	*GPX1*(*138320)	AR

1～8：解糖系，9・10：ペントースリン酸経路，11～13：プリン・ピリミジン代謝，14～17：グルタチオン合成・代謝系の酵素．
OMIM：Online Mendelian Inheritance in Man(http://omim.org/search/advanced/entry)，AR：autosomal recessive(常染色体劣性)，AD：autosomal dominant(常染色体優性)，XR：X-linked recessive(X染色体劣性)．

CNSHA検査精度の向上

非免疫性溶血性貧血という仮診断のもと，筆者らが2005～2010年に検索した244例の最終診断は，G6PD異常症62例，HS 41例，サラセミア14例，PK異常症11例，その他のまれな赤血球酵素異常症11例，UHD 5例，その他PNH，AIHAなど8例であり，全体の約60％を診断し得た．米国のBeutlerら[8]によると1982～1988年，722例を対象にした結果が診断率28.1％，2000～2004年の自験212例に対しては24.5％であり，過去のスクリーニング結果と比較して，赤血球膜異常

症のスクリーニング検査として赤血球EMA結合能を導入したことにより，CNSHAとして検索を依頼される症例に赤血球膜異常症を診断することができるようになり，結果として診断率が向上している．

おわりに

先天性溶血性貧血症例の多くは，新生児期の重症黄疸や遷延する溶血性貧血により気付かれるが，成人発症例も散見される．赤血球形態に典型的な異常を認めない非球状性溶血性貧血症例のなかにも赤血球EMA結合能の低下例があり，赤血球膜異常症のスクリーニング検査としての有用性が確認できた．一方，解析した溶血性貧血症例の約40％はいまだ原因不明であり，溶血性貧血を惹起する新規病因遺伝子の変異が示唆されるため，今後は，網羅的遺伝子検査を含めた診断システムの構築が急務と考えられる．

筆者らは，先天性溶血性貧血に関する診療サポートを目的としたNPO法人を設立し，2013年12月からこのNPOを窓口とした検査受託を開始している(血液難病診療サポート：http://anemia-support.org/index.html)．DAT陰性AIHAの検査を実施している自治医科大学地域医療学センター亀崎豊実先生，ヘモグロビン異常症の解析を受託している福山臨床検査センター社と連携して，溶血性貧血症例の担当医からの症例相談や検査依頼を受け付け，治療方針や日常生活の注意点など患者や家族への情報提供も進めている．

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Ribosomal protein L11- and retinol dehydrogenase 11-induced erythroid proliferation without erythropoietin in UT-7/Epo erythroleukemic cells

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Erythropoiesis is the process of proliferation, differentiation, and maturation of erythroid cells. Understanding these steps will help to elucidate the basis of specific diseases associated with abnormal production of red blood cells. In this study, we continued our efforts to identify genes involved in erythroid proliferation. Lentivirally transduced UT-7/Epo erythroleukemic cells expressing ribosomal protein L11 (RPL11) or retinol dehydrogenase 11 (RDH11) could proliferate in the absence of erythropoietin, and their cell-cycle profiles revealed G_0/G_1 prolongation and low percentages of apoptosis. RPL11-expressing cells proliferated more rapidly than the RDH11-expressing cells. The antiapoptotic proteins BCL-XL and BCL-2 were expressed in both cell lines. Unlike the parental UT-7/Epo cells, the expression of hemoglobins (Hbs) in the transduced cells had switched from adult to fetal type. Several signal transduction pathways, including STAT5, were highly activated in transduced cells; furthermore, expression of the downstream target genes of STAT5, such as *CCND1*, was upregulated in the transduced cells. Taken together, the data indicate that RPL11 and RDH11 accelerate erythroid cell proliferation by upregulating the STAT5 signaling pathway with phosphorylation of Lyn and cyclic AMP response element-binding protein (CREB).

Erythropoiesis, the process of production of red blood cells, consists of several stages that depend on various specific cytokines; these factors promote the differentiation and proliferation of hematopoietic stem cells into mature erythrocytes. The maturation process of erythrocytes involves many steps, including chromatin condensation, hemoglobinization, enucleation, and expulsion of certain organelles. Erythropoietin (Epo), the major growth factor in erythropoiesis, plays an essential role in proliferation and prevention of apoptosis, starting at the stage of the initial erythroid precursor.

Understanding erythroid proliferation and maturation will help to clarify the pathogenesis and prognosis of several hematologic diseases that are accompanied by anemia resulting from the abnormal production of erythroid cells. Such insights should lead to improvements in therapeutic approaches for these conditions. Most of these diseases, which include myelodysplastic syndrome and acute erythroleukemia, are still too difficult to manage, and specific treatments remain to be developed. This situation prompted us to elucidate the pivotal genes that control the growth and proliferation of erythroid cells.

To determine novel essential genes involved in this process, we performed studies using UT-7/Epo, an erythropoietin-dependent human erythroleukemic cell line [1]. Based on our previous research, we examined candidate genes with potential roles in erythroid growth and maturation by delivering genes from a human fetal

TK and TI contributed equally to this work.

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liver–derived Entry complementary DNA (cDNA) library into UT-7/Epo cells, using a lentiviral system [2,3]. After identifying eight candidate genes in a colony-forming assay, we focused on two potential candidate genes, ribosomal protein L11 (*RPL11*) and retinol dehydrogenase 11 (*RDH11*), in subsequent experiments. Here, we demonstrated that these lentivirally transduced cells could proliferate and produce fetal Hb (γ-globin) and adult Hb (β-globin) in a culture medium that lacked Epo. Moreover, during the proliferation of these erythropoietin-independent transduced cells, the STAT5 signaling pathway was significantly upregulated relative to the levels in parental UT-7/Epo cells.

Materials and methods

Cell culture conditions

The UT-7/Epo cell line [1] was cultured in IMDM (Gibco Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 1 U/mL human recombinant Epo (R&D Systems, Minneapolis, MN) at 37°C in 5% CO_2.

Screening for candidate genes in erythropoiesis

The process of screening candidate genes involved in erythropoiesis was performed as previously described [2]. In brief, 8 candidate genes with full-length insertions in transduced cells were selected from our previous report. cDNA from each gene was cloned into the pCSII-EF-RfA-IRES2-Venus lentiviral vector (kindly provided by H. Miyoshi, RIKEN, Tsukuba, Japan) using Gateway Clonase Enzyme Mix (Invitrogen, Carlsbad, CA). All constructs were verified by DNA sequencing. Specific lentiviral supernatant was produced from 293T cells and used to transduce UT-7/Epo cells. Cells transduced with each of the 8 lentiviruses were cultured in methylcellulose (Nacalai Tesque, Kyoto, Japan) without Epo for 1 month before analysis.

Hematopoietic colony formation assay

A total of 1×10^4 colony-derived cells were collected and seeded into 1 mL of methylcellulose using a 2.5-mL syringe and an 18G needle. The mixture of cells and methylcellulose was dispensed into 35 × 10 mm tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ) at 1 mL per dish. Dishes were gently tilted and rotated to distribute the methylcellulose evenly, and then 3 mL of sterile water were added into an extra uncovered dish before incubation for 1 month at 37°C and 5% CO_2. Colonies in each dish were counted at day 30 and then picked, cytospun onto glass slides, and stained with May–Grunwald Giemsa solution (Nacalai Tesque) for microscopic observation. Photographs of colonies were taken using a microscope equipped with the AxioVision software (Zeiss, Oberkochen, Germany).

Western blotting

Transduced cells, including UT-7/Epo cells, were collected at 24, 48, and 72 hours. Cells were lysed with lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 1.0% NP-40. The protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Whole-cell extracts (5 μg/lane) were subjected to 12.5% sodium dodecyl sulphate (SDS) -polyacrylamide gels, and protein was transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The immunoreaction was performed by incubating the membrane for 1 hour at room temperature (RT) with primary antibodies as follows: mouse antihuman BCL-XL (Santa Cruz Biotechnology, Santa Cruz, CA; dilution, 1:200), mouse antihuman BCL-2 (Santa Cruz Biotechnology; dilution, 1:200), or mouse anti–β-actin (C4, sc-47778, Santa Cruz Biotechnology; dilution, 1:1,000). Membranes were incubated at RT for 1 hour with horseradish peroxidase (HRP) -conjugated secondary antibody: antimouse immunoglobulin G antibody (IgG Ab) (sc-2005, Santa Cruz Biotechnology; dilution of 1:10,000). Antigen–antibody reactions were detected using the enhanced chemiluminescence assay (Amersham Biosciences, Piscataway, NJ). Western blots were analyzed on an LAS3000 (Fuji Film Co., Tokyo, Japan).

Gene expression analysis by quantitative reverse transcription polymerase chain reaction (RT-PCR)

To determine the expression of STAT5 regulated genes, RNAs were extracted from UT-7/Epo and RPL11- and RDH11-transduced cells at day 3 using the RNeasy Mini kit QIAGEN (QIAGEN, Hilden, Germany). Concentration of RNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) before proceeding to cDNA synthesis with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Expression of *PIM2* and *CCND1* was analyzed using the Applied Biosystems StepOne Plus Real-Time PCR system (Life Technologies, Grand Island, NY). For detection of *PIM2*, the forward primer was 5′-TGGGCATCCTCCTCTAT GAC-3′, and the reverse primer was 5′-GTACATCCTCGGCTGG TGTT-3′. For *CCND1*, the forward primer was 5′-GATCAA GTGTGACCCGGACT-3′, and the reverse primer was 5′-TCC TCCTCTTCCTCCTCCTC-3′. The PCR mixture was as follows: 10 μL Fast SYBR Green master mix (Life Technologies), 0.2 μL forward primer (10 μmol/L), 0.2 μL reverse primer (10 μmol/L), 1.0 μL cDNA, and 8.6 μL dH_2O. The PCR conditions were as follows: 95°C for 20 sec (holding stage); 40 cycles of 95°C for 3 sec and 60°C for 30 sec (cycling stage); and 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec (melting curve stage).

To confirm *Bcl-xL* gene expression in RPL11- and RDH11-transduced cells, quantitative RT-PCR was performed using the following primers: h*Bcl-xL* forward: 5′-CTGCCTCACTTCCTAC AAGAGC-3′ and h*Bcl-xL* reverse: 5′-CTGAGGTAGGGAAG ACCCTG-3′. In brief, RNAs were extracted from RPL11- and RDH11-transduced cells and UT7/Epo cells at 24, 48, and 72 hours before converting to cDNA using SuperScript III First-Strand Synthesis System (Invitrogen). PCR mixture was: 5 μL Fast SYBR Green master mix (Life Technologies), 0.1 μL *Bcl-xL* forward and reverse primers (10 μmol/L), or 0.1 μL *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* forward and reverse primers (5 μmol/L), 1.0 μL cDNA, and 3.8 μL dH_2O. The PCR was performed as above.

Cell proliferation assay

To determine the growth and proliferation of UT-7/Epo and RPL11- and RDH11-transduced cells, proliferation assays were performed using Cell Count Reagent SF (Nacalai Tesque). Briefly, each cell line was seeded into 96-well flat-bottom plates at 1×10^3 cells/well in 100 μL culture medium, with or without Epo. After growth for 2, 4, and 6 days, 10 μL of Cell Count Reagent SF was added to each well and incubated for 1 hour at 37°C in 5% CO_2. Absorbance at 450 nm (ref. 650 nm) was recorded using a

microplate reader (Thermo Scientific). The experiments were performed in triplicate, and data were analyzed by plotting the corrected absorbance at 450 nm on the y axis and time points on the x axis.

For detection of growth factors produced in an autocrine manner, culture media from RPL11- and RDH11-transduced cells at 48 hours were collected and filtered through 0.22 μm syringe filter before used. The erythropoietin levels of these collected culture media were measured by LSI Medience Corporation (Tokyo, Japan). The UT7/Epo cells deprived of Epo were cultured with medium collected from RPL11- or RDH11-transduced cells for 2, 4, and 6 days before assessment of cell proliferation using Cell Count Reagent SF, as described above.

Determination of STAT5 signaling pathway involving in cell proliferation using STAT5 inhibitor

To determine whether STAT5 signaling pathway was involved in cell proliferation of RPL11- and RDH11-transduced cells, STAT5 inhibitor (573108, Merck Millipore, Darmstadt, Germany) was added in culture medium for inhibition of cell growth [4]. Drug was dissolved with dimethyl sulfoxide (DMSO, Nacalai, Japan), diluted with medium, and used at the final concentrations of 100 and 200 μmol/L with 0.1% DMSO in cell proliferation assay.

UT7/Epo with Epo and RPL11- and RDH11-transduced cells were cultured in medium with STAT5 inhibitor at the final concentrations of 100 and 200 μmol/L for 12 hours. After washing the treated cells with phosphate buffer saline (PBS), cells were seeded into 96-well flat-bottom plates at 1×10^3 cells/well in 100 μL drug-free medium. Untreated cells were used as the control group. Cells were cultured until days 2, 4, and 6 before analysis using Cell Count Reagent SF as mentioned earlier.

Flow-cytometry analysis for intracellular Hb expression

UT-7/Epo and RPL11- and RDH11-transduced cells were cultured in medium with or without Epo for 2 days before analysis of intracellular Hb expression. Cells were collected and fixed with cold 0.05% glutaraldehyde for 10 min at RT. After washing with PBS-0.1% bovine serum albumin (BSA), cells were permeabilized for 5 min at RT with 0.1% Triton X-100 (Nacalai Tesque) and then blocked with PBS-BSA. Cells were incubated at RT for 15 min with diluted primary antibody in 0.1% BSA in PBS: fetal hemoglobin-allophycocyanin (F-APC)–conjugated mouse anti-Hb (Invitrogen; dilution, 1:17) or β-PerCP-Cy5.5–conjugated mouse anti-Hb (Santa Cruz Biotechnology; dilution, 1:200). Antibody-stained cells were analyzed on a FACSCalibur (BD Biosciences, Seattle, WA) using the CellQuest software.

Cell-cycle analysis

UT-7/Epo and RPL11- and RDH11-transduced cells were seeded in 12-well plates at 2×10^5 cells/well and incubated at 37°C for 24, 48, or 72 hours in medium with or without Epo. At each time point, cells were collected, washed with PBS, and fixed with cold 70% ethanol for 10–14 hours. Cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-bromodeoxyuridine (BrdU; BD Biosciences) for 30 min, and then treated with RNase A (Nacalai Tesque) and 7-AAD (BioLegend, San Diego, CA) to exclude nonviable cells. The cell-cycle profile (i.e., the proportions of cells at G_0/G_1, S, and G_2/M phases, as well as apoptotic cells) was analyzed on a FACSCalibur with the CellQuest software.

Phosphokinase array for analysis of signaling pathways in transduced cells

To identify the signal transduction pathways activated in transduced cells, samples were analyzed using the Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN). In brief, cells were cultured with and without Epo for 12 hours, and cell lysates were prepared using the lysis buffer provided in the kit. Then, the provided membranes were blocked with Array Buffer 1 prior to incubation with cell lysates. After overnight incubation at 2–8°C, membranes were washed, and specific kinases were detected using Detection Antibody Cocktail A and B, provided in the kit. Membranes were washed and probed with Streptavidin-HRP (BD Biosciences) before being analyzed using an LAS3000 (Fuji Film Co., Tokyo, Japan). Pixel densities were measured using a transmission-mode scanner and image analysis software.

To focus particularly on STAT5 signaling pathway involved in the growth and proliferation of RPL11- and RDH11-transduced cells, STAT5 inhibitor at final concentration of 100 μmol/L was added into culture medium of all cell lines. After 12 hours, samples were prepared and assayed as above.

Immunocytochemical detection for CREB, Lyn, and JAK2 phosphorylation

To determine the phosphorylation of CREB, Lyn, and JAK2, cells were cultured with or without Epo. After 12 hours, RPL11- and RDH11-transduced cells and UT7/Epo cells were harvested, cytospun at 450 rpm for 5 min, and let dry for 2 hours at RT. Cells were fixed with 1% paraformaldehyde in PBS for 10 min at RT. After washing with ice cold PBS for 3 times, cells were permeabilized and blocked using 0.05 % Triton X-100 in 1% BSA/PBS for 30 min. Cells were then incubated with diluted primary antibody: mouse antihuman phospho-CREB (dilution 1:25, R&D Systems, Abingdon, UK), rabbit antihuman phospho-JAK2 (dilution 1:50, abcam, Cambridge, UK), mouse antihuman phospho-Lyn (dilution 1:25, R&D Systems, UK) in blocking buffer at 4°C overnight. After washing 3 times with PBS, cells were incubated with secondary antibody: Alexa Fluor 647 donkey antimouse (dilution 1:500; Life Technologies), Alexa Fluor 647 donkey antirabbit (dilution 1:500, Invitrogen) for 30 min at RT in the dark. Mounting and fixing were performed using VECTASHIELD with DAPI (Vector Laboratories, Inc., CA) before analysis, followed by the observation using fluorescence imaging with Olympus Ix81 Inverted Microscope (Olympus America, Center Valley, PA).

In addition, Hela cells treated with 200 nmol/L PMA (phorbol 12-myristate 13-acetate; Sigma, St. Louis, MO) for 2 hours were used as positive control to detect CREB phosphorylation. One mmol/L of Pervanadate was prepared from Sodium orthovanadate (Sigma) and hydrogen peroxide (Nacalai Tesque) diluted with PBS as previously described [5]. Jurkat cells and Hela cells treated with 1 mmol/L Pervanadate were respectively used as positive control for JAK2 phosphorylation and Lyn phosphorylation.

Statistical analysis

Data are shown as means ± SEM. A p value <0.05 was considered to represent statistical significance.

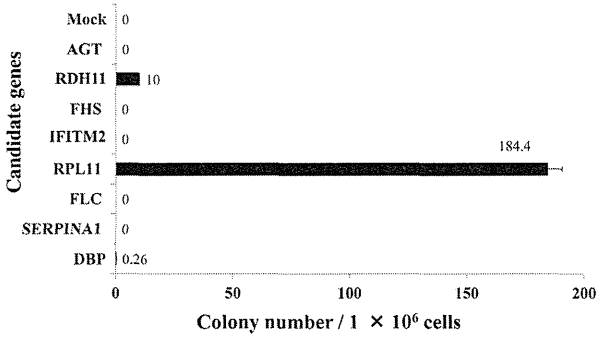

Figure 1. Identification of eight candidate genes involved in erythroid proliferation. From our screening, eight candidate genes with full-length insertions were detected. They were angiotensinogen (*AGT*), retinol dehydrogenase 11 (*RDH11*), ferritin heavy chain subunit (*FHS*), interferon-induced transmembrane protein 2 (*IFITM2*), ribosomal protein L11 (*RPL11*), ferritin light chain (*FLC*), serpin peptidase inhibitor clade A (*SERPINA1*), and D-site binding protein (*DBP*). In colony formation assays, RPL11-transduced cells yielded the highest average number of colonies (about 184). All colonies were cultured for 1 month in semisolid medium without Epo.

Results

Determination of candidate genes, and mechanisms involving in erythroid proliferation of RPL11- and RDH11-transduced cells

To identify candidate genes involved in human erythropoiesis, we first prepared lentiviruses expressing eight candidate genes, and used these viruses to transduce UT-7/Epo cells. These genes encoded angiotensinogen (AGT), ferritin heavy chain subunit (FHS), interferon-induced transmembrane protein 2 (IFITM2), ferritin light chain (FLC), ribosomal protein L11 (RPL11), retinol dehydrogenase 11 (RDH11), serpin peptidase inhibitor clade A (SERPINA1), and D-site (DBP) binding protein. After culture in semisolid medium without Epo for 1 month, we found that two of these candidate factors, RPL11 and RDH11, resulted in formation of a larger number of colonies than the other genes (RPL11, 184.4 ± 6.2; RDH11, 10.0 ± 0; Fig. 1). Colonies were positive for Venus expression (data not shown).

To further investigate cell proliferation, we next transferred the colonies derived from UT-7/Epo and RPL11- and RDH11-transduced cells into liquid culture and subjected them to proliferation assays at various time points. In the assay we used, higher absorbance at 450 nm reflected higher cell proliferation. UT-7/Epo cells incubated with Epo (■) proliferated most rapidly, whereas no proliferating cells could be detected in UT-7/Epo cells incubated without Epo (×), particularly on days 4 and 6 (Fig. 2A). In contrast to nontransduced cells, both of the RPL11- (▲) and RDH11- (●) transduced cells cultured in the absence of Epo increased cell proliferation. Compared to RDH11- (●) transduced cells, RPL11- (▲) transduced cells proliferated 2.35-, 2.67-, and 6.64-fold faster on days 2, 4, and 6, respectively; these differences were statistically significant. In addition, on day 6, RPL11- (▲) transduced cells exceeded the proliferation of UT-7/Epo cells (■) cultured in the presence of Epo. Even under the Epo-free condition, both RPL11- and RDH11-transduced cells maintained their proliferation, suggesting that the products of the transduced genes could substitute for Epo signaling in UT-7/Epo erythroleukemic cells.

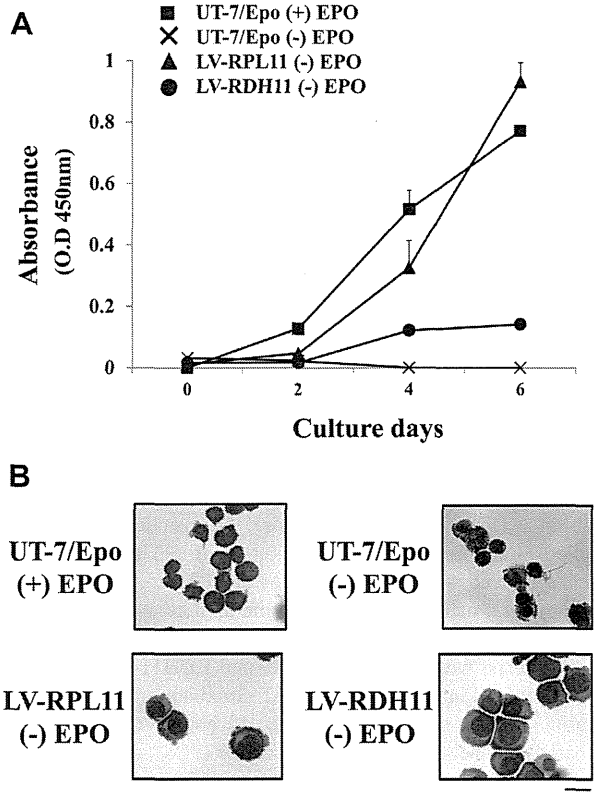

Figure 2. (**A**) Erythroid proliferation of transduced cells cultured without Epo. Cell proliferation assay of UT-7/Epo and RPL11- and RDH11-transduced cells in liquid culture. Without Epo, UT-7/Epo cells could not proliferate, whereas in the presence of Epo, these cells could proliferate very well, especially at days 2 and 4, with average ODs of 0.12 and 0.51, respectively. At day 6, RPL11-transduced cells without Epo yielded the highest cell number among these three groups, with an average OD of 0.93. (**B**) Cell morphology. UT-7/Epo cells in the presence of Epo (Upper left). UT-7/Epo cells, RPL11- and RDH11-transduced cells by lentiviruses (LV-RPL11, LV-RDH11), were cultured in the absence of Epo for 72 hours (upper right, lower left, and lower right, respectively). Cells were cytospun and subjected to May–Grunwald Giemsa staining. Scale bar = 10 um.

Morphological observation by May–Grunwald Giemsa staining indicated that by 72 hours, UT-7/Epo cells cultured without Epo had condensed nuclei and exhibited apoptotic features (Fig. 2B). On the other hand, relatively larger cells with less condensed nuclei were observed in both RPL11- and RDH11-transduced samples, compared with nontransduced cells, irrespective of the presence of Epo. This

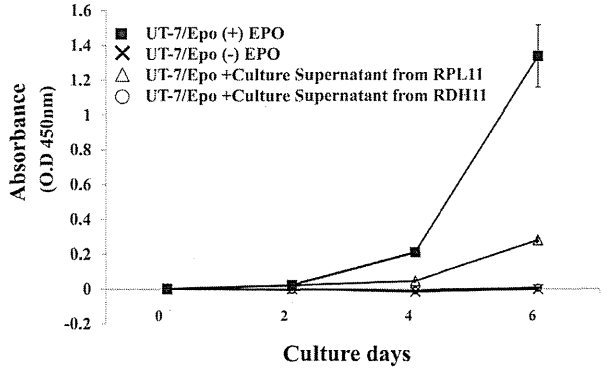

Figure 3. The proliferation of UT-7/Epo cells in the supernatant of RPL11- and RDH11-transduced cells. In order to investigate whether RPL11- and RDH11-transduced cells proliferated in an autocrine manner, UT-7/Epo cells were cultured in the absence of Epo with the supernatant of RPL11- and RDH11-transduced cells. The proliferation of UT-7/Epo cells cultured in the supernatant of RPL11- and RDH11-transduced cells was significantly decreased.

observation implies that RPL11- and RDH11-transduced cells proliferated in an immature state.

To investigate whether RPL11- and RDH11-transduced cells proliferated in autocrine manner, culture medium from respective transduced cells was used to culture UT-7/Epo without Epo. At days 4 and 6, the proliferation of UT-7/Epo cells was moderately suppressed by the culture medium from RPL11-transduced cells but completely suppressed by that from RDH11-transduced cells (Fig. 3). The Epo levels of culture supernatant of respective transduced cells were measured and were not detected, as observed with nontransduced UT-7/Epo without Epo (data not shown).

To evaluate differentiation stage, we used intracellular staining to assess Hb expression in transduced UT-7/Epo cells after 2 days of culture. Based on flow-cytometric analysis, 94.0% of UT-7/Epo cells cultured with Epo expressed β-globin, whereas only 1.2% of them expressed γ-globin. Similarly, UT-7/Epo cells cultured without Epo predominantly expressed β-globin. By contrast, both RPL11- and RDH11-transduced cells cultured without Epo expressed γ-globin (41.5% and 38.3% of cells, respectively), whereas ~30% of both types of transduced cells expressed β-globin (Supplementary Figure 1, online only, available at www.exphem.org). Taken together, these data indicate that transduction of *RPL11* and *RDH11* into UT-7/Epo cells induced and maintained their proliferation in an immature state.

Change of cell-cycle status in RPL11- and RDH11-transduced cells

To investigate the mechanisms underlying proliferation, we performed cell-cycle analyses by BrdU and 7-AAD staining, followed by flow cytometry (Supplementary Figure 2, online only, available at www.exphem.org). UT-7/Epo cells cultured with Epo exhibited a prolonged S phase after 24, 48, and 72 hours of culture. On the other hand, UT-7/Epo cells cultured without Epo exhibited a reduction in the number of S-phase cells (35.0%, 17.7%, 8.2%), in accordance with increasing the number of apoptotic cells (0.5%, 5.9%, 14.8%). By contrast, both RPL11- and RDH11-transduced cells cultured without Epo exhibited a lower percentage of apoptotic cells at every time point than nontransduced cells did. UT-7/Epo cells cultured with Epo had the lowest percentage of apoptotic cells among these cell lines, whereas UT-7/Epo cultured without Epo had the highest percentage of apoptotic cells and G_2/M arrest, especially after 72 hours of culture (Fig. 4).

To clarify the mechanisms of inhibition of apoptosis in RPL11- and RDH11-transduced cells cultured without Epo, we evaluated the expression of two antiapoptotic proteins, BCL-XL and BCL-2. We found that both types of transduced cells expressed these proteins. By contrast, UT-7/Epo cultured without Epo did not express either antiapoptotic protein, reflecting the higher percentage of apoptotic cells in this group. As previously reported [6], prominent BCL-XL expression and slight BCL-2 expression were detected in UT-7/Epo cells cultured in the presence of Epo (Fig. 5). Quantitative RT-PCR to detect BCL-XL expression also showed the same results (Fig. 6).

Signaling pathways of two transduced cell lines

To elucidate the signal transduction pathways involved in RPL11- and RDH11-driven proliferation, we performed phosphokinase array analysis after 12 hours of culture in the absence of Epo (Fig. 7A). The phosphorylation statuses of p53 (S392), Akt (T308), and AMPKa1 were almost the same among the four samples tested: UT-7/Epo cells cultured with or without Epo and RPL11- and RDH11-transduced cells cultured without Epo. The phosphorylation of p38 was the highest in UT-7/Epo cells cultured with Epo, and phosphorylation of p53 (S46) was the highest in RDH11-transduced cells. On the other hand, phosphorylation levels of both CREB and Lyn were higher in RPL11- and RDH11-transduced cells, and phosphorylated Chk-2 and AMPKa2 were upregulated in the Epo-free condition, regardless of gene transduction. Phosphorylated STAT5a (Y699) and HSP27 were downregulated in UT-7/Epo cells cultured without Epo relative to UT-7/Epo cells cultured with Epo; these phosphoproteins were upregulated in RPL11- and RDH11-transduced cells to the same level as in UT-7/Epo with Epo (Fig. 7B).

To ascertain that STAT5 signaling pathway was involved in the proliferation of RPL11- and RDH11-transduced cells, we conducted phosphokinase array and proliferation assay using these cells in the presence of STAT5 inhibitor. Our results from phosphokinase array confirmed that STAT5 phosphorylation was dramatically decreased in the presence of STAT5 inhibitor (Fig. 8A). Importantly, proliferation assay revealed that RDH11-transduced cells showed significantly decreased proliferation at any observed points in the presence of 100 and

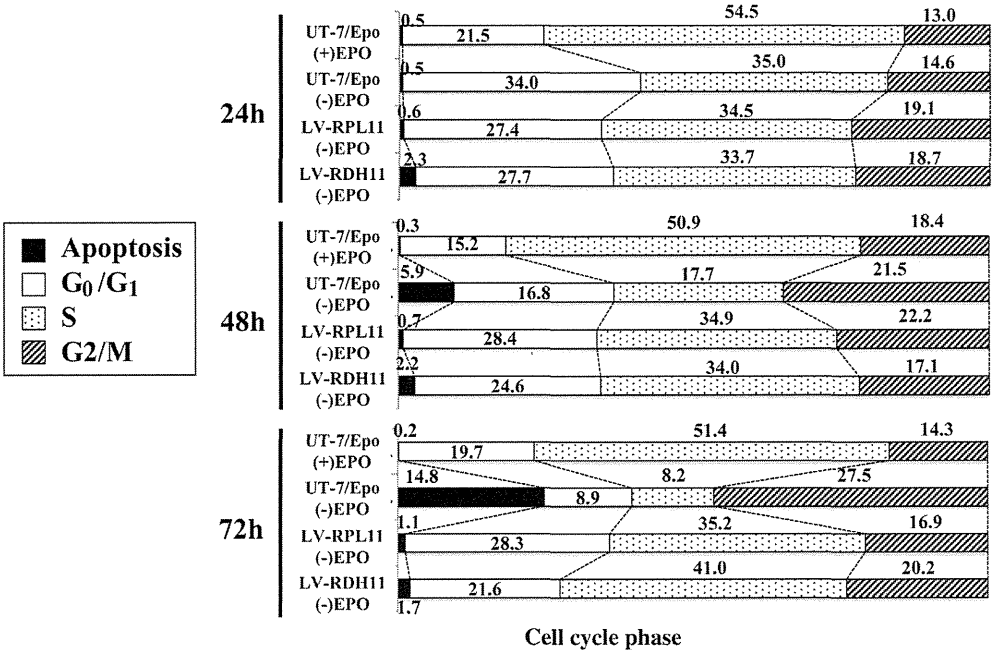

Figure 4. Cell-cycle determination of three cell lines. At 24, 48, and 72 hours after cultured, cells were collected and analyzed with flow cytometry. UT-7/Epo cells without Epo exhibited the highest apoptosis and G_2/M arrest at 72 hours (14.8% and 27.5% of cells, respectively). The lowest percentage of apoptosis and the highest percentage of S phase arrest at every time point were observed in UT-7/Epo cultured with Epo. Between the 2 types of transduced cells, RPL11-transduced cells exhibited the lower percentage of apoptosis than RDH11-transduced cells, especially at 24 and 48 hours.

200 μmol/L STAT5 inhibitor, whereas RPL11-transduced cells showed significantly decreased proliferation only at day 2 in the presence of 200 μmol/L STAT5 inhibitor (Fig. 8B). CREB, Lyn, and JAK2 phosphorylation were also studied using immunocytochemistry, and the phosphorylation of both CREB and Lyn were observed (Fig. 8C). Of note, the phosphorylation of JAK2 could not be demonstrated in our study (data not shown).

To further examine STAT-5 regulated genes, we observed the expression of *PIM2* and *CCND1* by real-time PCR analysis [7,8]. The results showed that *PIM2* expressions were not different among the samples, but *CCND1* expression was elevated by 43.4-fold in RDH11-transduced cells and 2.5-fold in RPL11-transduced cells compared with those in the UT-7/Epo control (Fig. 9).

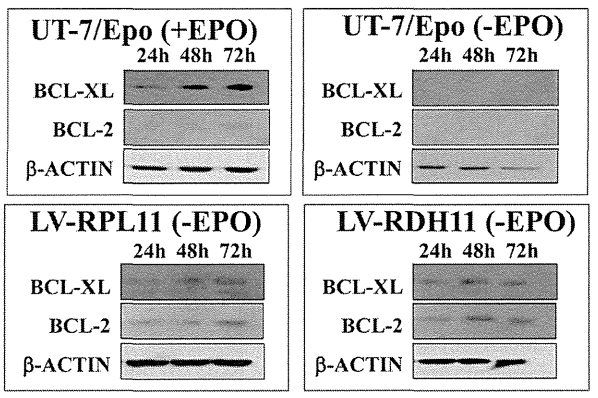

Figure 5. Expression of antiapoptotic proteins was demonstrated by Western blotting. Neither BCL-XL nor BCL-2 was detected in UT-7/Epo cultured without Epo, whereas the expression level of BCL-XL was higher than that of BCL-2 in UT-7/Epo cultured with Epo. Both types of transduced cells also expressed BCL-XL and BCL-2 at every time point. β-ACTIN was used as internal control.

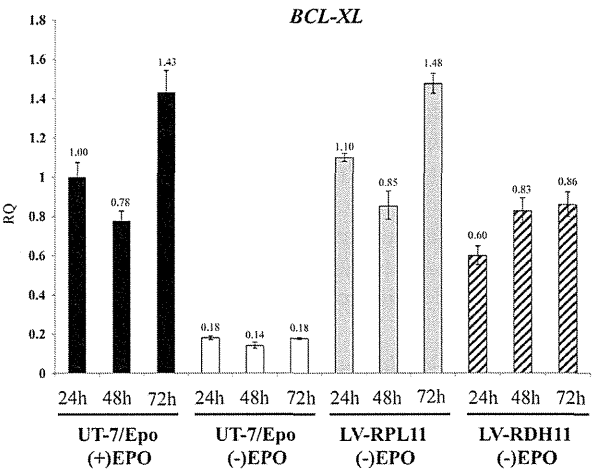

Figure 6. Quantitative RT-PCR of *Bcl-xL* gene. The expression of *Bcl-xL* gene of RPL11- and RDH11-transduced cells was demonstrated. The highest expression was detected in all cell lines at 72 hours. *RQ* = relative quantitation.

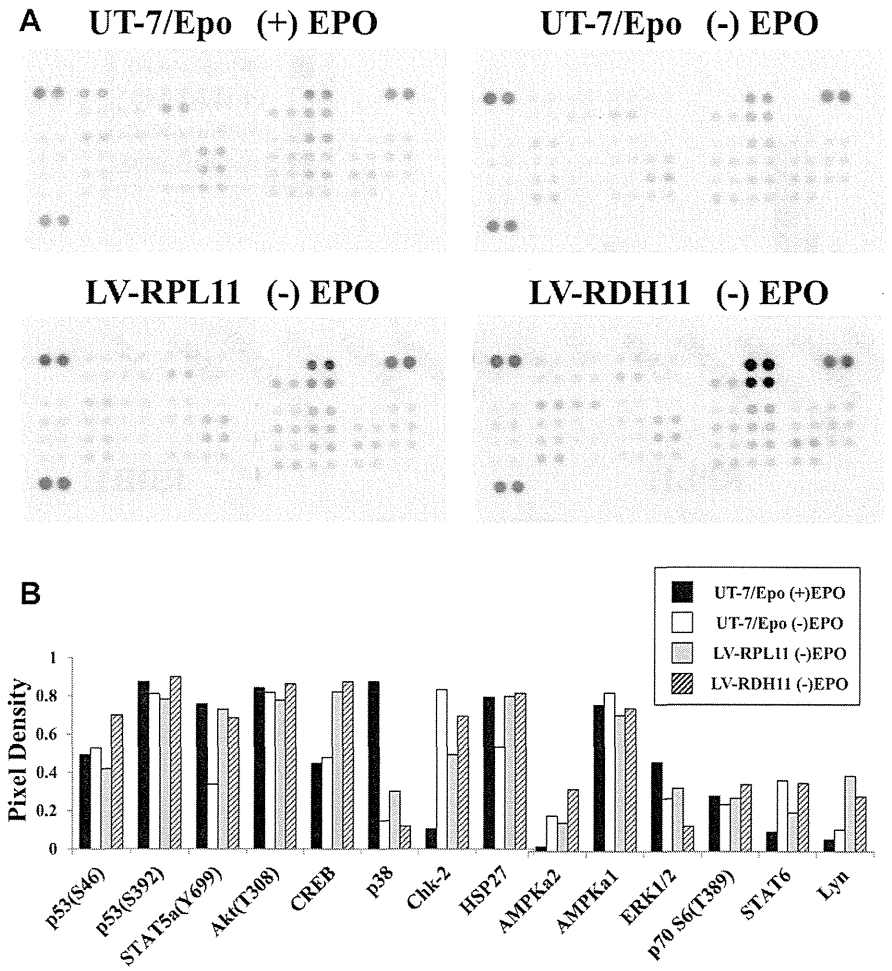

Figure 7. (**A**) Pixel densities of phosphokinase arrays performed on three cell lines. After 12 hours, cells were lysed with lysis buffer and further processed for detection of kinase activation. Pixel densities were evaluated and analyzed using LAS3000 (Fuji Film Co., Tokyo, Japan). (**B**) Determination of signaling pathways in UT-7/Epo and both types of transduced cells. Phosphokinase arrays performed on three cell lines demonstrated that STAT5a (Y699) activation was markedly reduced in UT-7/Epo cultured without Epo, with a pixel density of ~0.4. In UT-7/Epo cultured with Epo, the level of STAT5a (Y699) phosphorylation was almost at the same as in both types of transduced cells cultured with Epo, with a pixel density of ~0.8. The Akt (T308) and AMPKa1 pathways were also activated at almost the same level in all cell lines, with pixel densities of ~0.8. CREB, and Lyn kinases were predominantly activated only in transduced cells, with pixel density ratios (transduced cells vs. UT-7/Epo cells) of ~2.

Discussion

Our findings indicate that the overexpression of RPL11 and RDH11 can maintain the growth and proliferation of UT-7/Epo cells in culture conditions in the absence of Epo. Interestingly, the proliferation of both RPL11- and RDH11-transduced cells was not due to autocrine manner as shown in Figure 3. Gene transfer of *RPL11* to UT-7/Epo cells resulted in more increased number of cells and colonies than that of *RDH11*. In addition, the percentage of apoptotic cells in RPL11-transduced cells was much lower than that in RDH11-transduced cells. Therefore, it is possible that *RPL11* has greater potential than *RDH11* to induce the proliferation of UT-7/Epo cells. RPL11 has been recently demonstrated to be essential for normal cell proliferation by supporting ribosomal biogenesis and translation capacity [9]. In the special context of erythroid proliferation, RPL11 has been previously reported to increase the translation of a specific set of transcripts, such as Bag1, which encodes an Hsp70 cochaperone, and Csde1, which encodes an RNA-binding protein, and both were expressed at increased levels in erythroblasts [10]. A recent report using zebrafish embryos also showed that RPL11 could support hematopoietic iron metabolism and Hb synthesis, whereas the promotion of erythroid proliferation by RDH11 is due to all-*trans*-retinoic acid, an active metabolite of this enzyme's catalytic process [11–13]. As demonstrated in this study, these effects result in promotion of erythroid proliferation by RPL11 and RDH11. Notably, increased expression level of *RDH11* gene in UT-7/Epo cells might not significantly increase the level of retinoic acids produced in these cells, because the substrate for the enzymatic reaction is limited. Moreover, the apoptosis

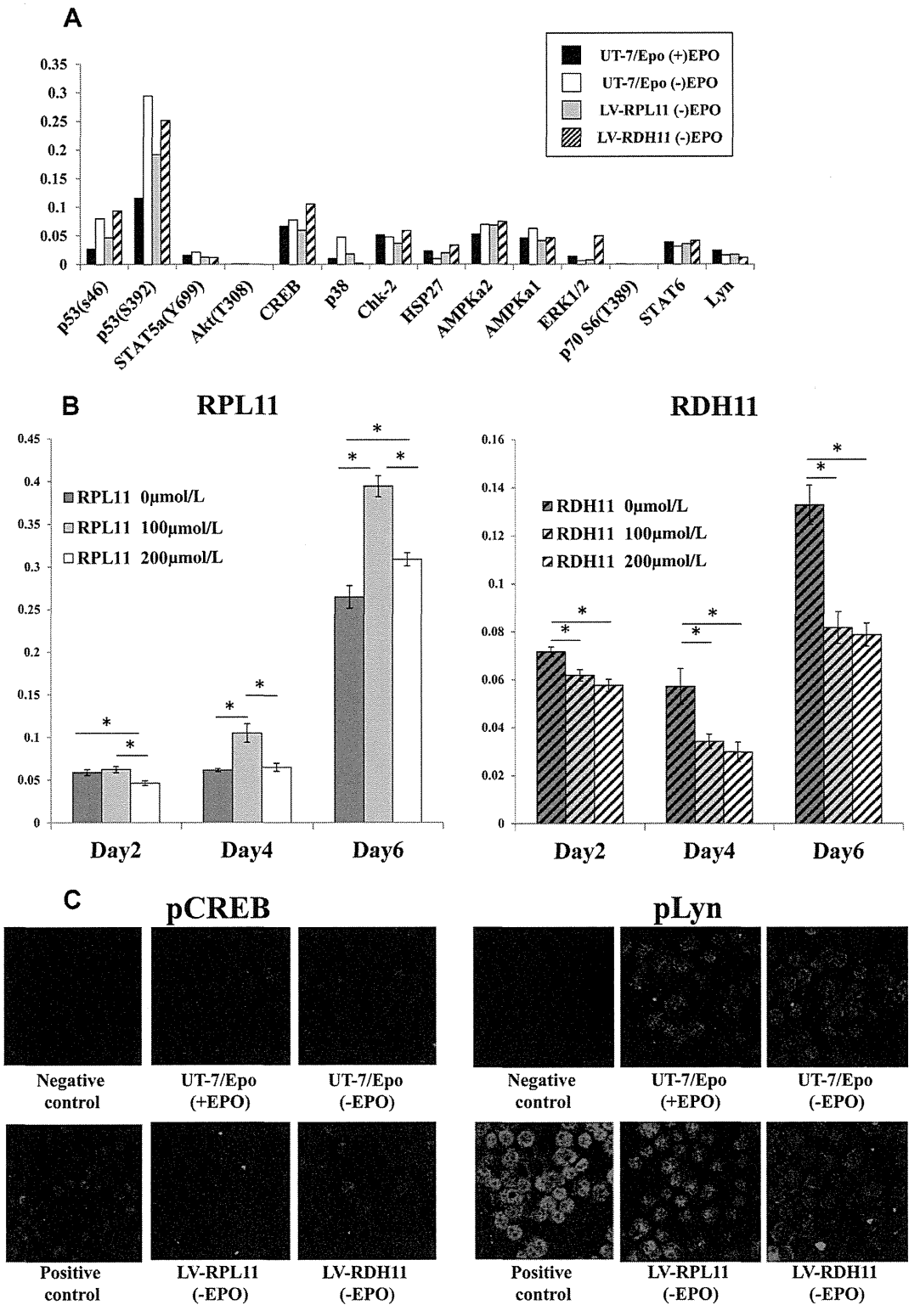

Figure 8. (**A**) Phospho-kinase array with STAT5 inhibitor at a final concentration of 100 μmol/L for 12 hours. To ascertain the STAT5 signaling pathway involved in RPL11- and RDH11-transduced cells, STAT5 inhibitor was added in the culture medium. STAT5a (Y699) was demonstrated to be significantly decreased by phosphokinase array. (**B**) Cell proliferation assays of RPL11- and RDH11-transduced cells with STAT5 inhibitor. RPL11- and RDH11-transduced cells were cultured for 2, 4, and 6 days in the presence of STAT5 inhibitor at final concentrations of 100 and 200 μmol/L. Cells were harvested and processed for proliferation assay. At day 2, the proliferations of RPL11- and RDH11-transduced cells were significantly inhibited at 200 μmol/L of STAT5 inhibitor. *statistical significance. (**C**) The phosphorylation of CREB and Lyn using immunocytochemistry. UT-7/Epo with or without Epo, and RPL11- and RDH11-transduced cells were harvested and processed for immunocytochemistry. The phosphorylation of CREB and Lyn was demonstrated.