マイコプラズマ検査法に関する研究

研究分担者　氏名　　原澤　亮

所属　　　岩手大学　農学部

役職　　　教授

研究要旨：住血マイコプラズマはヘモプラズマ（hemoplasma）とも呼ばれ，宿主動物の赤血球で増殖し，溶血性貧血を引き起こすことが知られている。前年度の報告で示したとおり，ウシにおけるヘモプラズマ感染はさして稀なことではない。したがって，培養動物細胞の培養液に広く用いられているウシ血清の原料となる血液におけるヘモプラズマ汚染は警戒を要するものと考えられる。これまでに，２菌種のヘモプラズマがウシに感染することが判明している。そこで本研究ではウシのヘモプラズマ菌種を特異的に同定・鑑別するために 16S-23S rRNA 遺伝子間スペーサー領域の一次構造と想定される二次構造の解析を試みた。

A．研究目的

ヘモプラズマは哺乳動物の赤血球表面に寄生する無細胞壁原核生物の総称で，これにはかつてリケッチア目アナプラズマ科のヘモバルトネラ属あるいはエペリスロゾーン属に属していた菌種のほか，新たに発見された菌種が含まれる。これらヘモプラズマ菌種はいずれも，16S rRNA 遺伝子の塩基配列相同性，宿主細胞外での増殖，および細胞壁ペプチドグリカンの欠如などの性状からマイコプラズマ属の菌種として認定されたものであるが，試験管内での人工培養が成功しないため性状解析ならびに分類学的な検討が遅れている。本研究では動物細胞培養にウシ血清が用いられることに着目し，とくにウシを宿主とするヘモプラズマ菌種について，その 16S-23S rRNA 遺伝子間スペーサー領域の構造解析を目的とした。ウシには *Mycoplasma wenyonii* および 'Candidatus Mycoplasma haemobos' の２菌種が感染することが知られているので，これらの２菌種について当該領域を PCR により増幅させ，その産物の塩基配列を決定し，さらにその二次構造を推定した。

B．研究方法

岩手県内で飼育されている乳牛から採取した全血（EDTA 添加）を被検サンプルとして，ヘモプラズマの 16S-23S RRNA 遺伝子間スペーサー領域の増幅をエンドポイント PCR により試みた。マイコプラズマに共通のプライマー配列（ forward 5'-GTTCCCAGGTCTTGTACACA-3' および reverse

5'-CAGTACTTGTTCACTATCGGTA-3'）を
設計し，サーマルサイクラーを用いて 94℃
30
秒、55℃２分、72℃２分の条件で３０サイ
クルの PCR を行った。

boxB
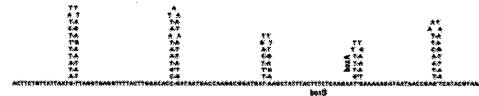
C．研究結果

今回設計したプライマー配列を用いてエン
ドポイント PCR を行うことにより，ウシに
感染する２種類のヘモプラズマのうち、
Mycoplasma wenyonii からは増幅産物が得
られなかったが，‘*Candidatus* Mycoplasma
haemobos’ の 16S-23S rRNA 遺伝子間スペー
サー領域を増幅させることに成功した。さ
らに PCR 産物の塩基配列を決定し，この領
域にスペーサーtRNA 遺伝子が欠如している
ことを明らかにした。一次構造の比較から
ヘモプラズマが *Mycoplasma fastidiosum* と
同じ分類群に属することが示唆された。
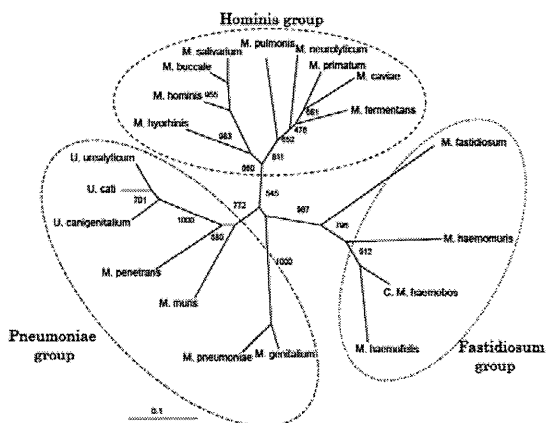
また，想定される二次構造を調べたところ，
boxA および boxB に相当する配列が保存し
ていることが判明した。

D．考察

ウシの２種類のヘモプラズマのうち１種類
について，その 16S-23S rRNA 遺伝子間スペ
ーサー領域の構造を決定することに初めて
成功した。この領域は近縁の菌種間の系統
的な違いを見い出すために微生物分類学で
汎用されていることから，これを用いて既
知のマイコプラズマ菌種との系統関係を調
べたところ，ヘモプラズマが *M.
fastidiosum* と同じ系統に含まれることが
明らかになった。また，二次構造を調べた
ところ，ヘモプラズマの 16S-23S rRNA 遺伝
子間スペーサー領域は従来から知られてい
る一般のマイコプラズマでの相当領域と極
めて類似の構造上の特徴を備えていること
が判明し，アンチターミネーターとしての
機能を備えていることが示唆された。

E．結論

ウシを宿主とする住血液マイコプラズマ
‘*Candidatus* Mycoplasma haemobos’ の
16S-23S rRNA 遺伝子間スペーサー領域をエ
ンドポイント PCR により増幅させ，その塩
基配列を初めて明らかにした。その配列に
基づき，ヘモプラズマの系統分類上の位置

を推定することができた。

F．健康危険情報
ウシのヘモプラズマについては報告がない
が，ヒツジおよびブタのヘモプラズマが人
へ感染することが海外で報告されていて，
人獣共通病原体として認識されるようにな
ってきた。

G．研究発表
1．論文発表
Ohtake, Y., Nishizawa, I., Sato, M., Watanabe, Y., Nishimura, T., Matsubara, K., Nagai, K., and Harasawa, R. (2011) *Mycoplasma ovis* detected in free-living Japanese serows, *Capricirnis crispus*. J. Vet. Med. Sci. 73: 371-373.

Obara, H., Fujihara, M., Watanabe, Y., Ono, H.K., and Harasawa, R. (2011) A feline hemoplasma, '*Candidatus* Mycoplasma haematominutum', detected in dog in Japan. J. Vet. Med. Sci. 73: 841-843.

Giangaspero, M., and Harasawa, R. (2011) Species characterization in the genus *Pestivirus* according to palindromic nucleotide substitutions in the 5'-untranslated region. J. Virol. Methods 174: 166-172.

Suzuki, J., Sasaoka, F., Fujihara, M., Watanabe, Y., Tasaki, T., Oda, S., Kobayashi, S., Sato, R., Nagai, K., and Harasawa, R. (2011) Molecular identification of '*Candidatus* Mycoplasma haemovis' in sheep with hemolytic anemia. J. Vet. Med. Sci. 73: 1113-1115.

Fujihara, M., Obara, H., Watanabe, Y., Ono, H.K., Sasaki, J., Goryo, M., and Harasawa, R. (2011) Acidic environments induce differentiation of *Proteus mirabilis* into swarmer morphotypes. Microbiol. Immunol. 55: 489-493.

Sasaoka, F., Suzuki, J., Watanabe, Y., Fujihara, M., and Harasawa, R. (2011) Rapid identification of hemoplasma species by palindromic nucleotide substitutions at the GAAA tetraloop helix in the specificity domain of ribonuclease P RNA. J. Vet. Med. Sci. 73: 1517-1520.

Giangaspero, M., Ibata, G., Savini, G., Osawa, T., Tatami, S., Takagi, E., Moriya, H., Okura, N., Kimura, A., and Harasawa, R. (2011) Epidemiological survey of *Border disease virus* among sheep from northern districts of Japan. J. Vet. Med. Sci. 73: 1629-1633.

Watanabe, Y., Fujihara, M., Obara, H., Nagai, K., and Harasawa, R. (2011) Two genetic clusters in swine hemoplasmas revealed by analyses of the 16S rRNA and RNase P RNA genes. J. Vet. Med. Sci. 73: 1657-1661.

Giangaspero, M., Osawa, T., Orusa, R., Frossard, J.-P., Naidu, B., Robetto, S., Tatami, S., Takagi, E., Moriya, H., Okura, N., Kato, K., Kimura, A., and Harasawa, R. (2011) Epidemiological survey for visna-maedi among sheep in northern prefectures of Japan. Vet. Ital. 47: 437-451.

Fujihara, Y., Sasaoka, F., Suzuki, J., Watanabe, Y., Fujihara, M., Ooshita, K., Ano, H., and Harasawa, R. (2011) Prevalence of hemoplasma infection among cattle in the western part of Japan. J. Vet. Med. Sci. 73: 1653-1655.

2．学会発表

笹岡文菜, 鈴木　尋, 渡邉祐策, 藤原正俊, 原澤　亮 (2011) ヘモプラズマの RNase P RNA 遺伝子および 16S-23S rRNA 遺伝子間領域の解析. 第 65 回日本細菌学会東北支部総会（山形市）8月 18 日

藤原正俊, 原澤　亮 (2011) 尿素添加液体培地における菌体長の変化について. 第 65 回日本細菌学会東北支部総会（山形市）8 月 18 日

小田伸一, 赤川彰崇, 田崎智子, 小林沙織, 鈴木　尋, 原澤　亮 (2011) ヒツジおよびヤギにおける重度貧血とヘモプラズマ感染. 第 114 回日本畜産学会（十和田市）8 月 26 日

上坂友香理, 西村貴志, 西部進矢, 出口善隆, 藤原正俊, 原澤　亮, 松原和衛 (2011) 盛岡市乙部地区周辺のニホンカモシカ（*Capricornis Crispus*）の生息状況調査. 第 17 回日本野生動物医学会大会講演要旨集: 139.（東京）9 月 30 日

Suzuki, J., Sasaoka, F., Fujihara, M., Watanabe, Y., Tasaki, T., Oda, S., Kobayashi, S., Sato, R., Nagai, K., and Harasawa, R. (2011) Molecular identification of 'Candidatus Mycoplasma haemovis' in sheep with hemolytic anemia. IUMS2011, Sapporo, Sept. 8.

Giangaspero, M., Bonfini, B., Orusa, R., Osawa, T., Tatami, S., Takagi, E., Moriya, H., Okura, N., Kato, K.,

Kimura, A., and Harasawa, R. (2011) Serological evidence of *Coxiella burnetii* (Q Fever) in sheep flocks in northern prefectures of Japan. IUMS2011, Sapporo, Sept. 8.

Fujihara, M., Nosaka, Y., Aizawa, S.-I., and Harasawa, R. (2011) Swarming motility of *Paenibacillus alvei* was suppressed by Tn*916* insertion into FliD operon or sensor histidine kinase gene. IUMS2011, Sapporo, Sept. 8.

Giangaspero, M., Harasawa, R., Bonfini, B., Orusa, R., Osawa, T., Tatami, S., Takagi, E., Moriya, H., Okura, N., Kato, K., Kimura, A. (2011) Bacterial diseases with zoonotic potential in sheep flocks in northern Japan. IUMS2011, Sapporo, Sept. 9.

Watanabe, Y., Fujihara, M., Nagai, K., and Harasawa, R. (2011) Two clusters in hemoplasmas detected from a commercial pig farm in Japan. IUMS2011, Sapporo, Sept. 9.

Giangaspero, M., Ibata, G., Savini, G., Osawa, T., Tatami, S., Takagi, E., Moriya, H., Okura, N., Kimura, A., and Harasawa, R. (2011) Epidemiological survey for *Border disease virus* among sheep from northern districts of Japan. IUMS2011, Sapporo, Sept. 15.

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（創薬基盤推進研究事業）

分担研究報告書

疾患研究の為の細胞コレクションの資源化ならびに品質評価法・特性解析法開発に関する研究

研究協力者： 平田みつひ

研究要旨

品質評価法・特性解析法開発による細胞情報付加の分野に関する研究について、本年度は、ヒト幹細胞の染色体異常株の特性解析を実施するため、染色体異常株からの染色体正常細胞のクローニングと染色体正常株と異常株における遺伝子発現解析を行い、ゲノムワイドな遺伝子発現解析がヒト iPS 細胞の資源化の際の品質検査として必要かどうかを検討した。

A.： 研究目的

近年、創薬研究・細胞治療研究において、機能保持細胞や多分化能を有する幹細胞の利用が期待されている。培養資源研究室 JCRB 細胞バンクでは、ヒト間葉系幹細胞（Mesenchymal Stem Cell,MSC）だけでなく、ヒト人工多能性幹（induced pluripotent stem：iPS）細胞も 16 株の寄託を受け、資源化を行いつつある。ヒト iPS 細胞株は、株間やクローン間により特性が異なり、分化指向性があることが明らかにされつつある。しかし、研究者が研究に利用するための十分な細胞情報が整備されておらず、どの細胞株を選択するべきか、その基準となる情報がほとんどない。近年、これらの細胞は、再プログラム化や継続的な培養維持によって、染色体レベル、染色体領域内レベルおよび一塩基レベルで異常が蓄積し、ゲノムレベルでの異常が引き起こされうることが報告されている。特に、細胞増殖に関わる遺伝子が集中する20番染色体で変化が多いことが明らかにされた。このような異常は、創薬応用の際に影響が懸念される。現在、当研究室では資源化を行う際に、細胞の染色体レベル、染色体領域内レベルおよび一塩基レベルの異常を、高解像度 CGH アレイや核型により解析を進めているが、ヒト iPS 細胞の資源化に際してゲノムワイドな遺伝子発現解析は行っていない。そこで本研究では、京都大学 iPS 細胞研究所より供与されたヒト皮膚線維芽細胞由来 iPS 細胞で染色体異常株と、それをクローニングする事により得た染色体正常株をモデルとして、ゲノムワイドな遺伝子解析を行う。具体的には、

①染色体異常株からの染色体正常細胞のクローニング

京都大学 iPS 細胞研究所より供与されたヒト皮膚線維芽細胞由来 iPS 細胞のうち1株が染色体異常株であったため、その細胞集団から、染色体正常株をクローニングする。

②染色体正常株と異常株における遺伝子発現の差を解析する

染色体正常クローンと異常クローンの DNA マイクロアレイ解析を用いて、ゲノムワイドな遺伝子発現の解析を行う。

上記の解析を行って、ゲノムワイドな遺伝子発現解析がヒト iPS 細胞の資源化の際の品質検査として必要かどうかを検討することを目的とする。

B：研究方法

①染色体異常株からの染色体正常細胞の
クローニング

京都大学 iPS 細胞研究所より供与されたヒト
皮膚線維芽細胞由来 iPS 細胞の維持培養
は、80 ％ Dulubecco's modified Eagle's
medium（DMEM, GIBCO, GrandIsland, NY,
USA）/Ham's F-12 medium（F12, GIBCO）に、
20 ％ knockout serum replacement（Invitrogen,
Carlsbad, CA, USA）、1 mM L-glutamine
（Sigma, St.
Louis, MO, USA ）、 0.1 mM
ᵝ-mercaptoethanol （ GIBCO ）、 1 ％
nonessential amino acids （GIBCO）、5 ng/ml
human recombinant FGF-2 （ Katayama

Kagaku Kogyo L.T.D., Okayama, Japan）を添
加した KSR-based 培地を使用し、マイトマイ
シン C により不活性化された CF-1 マウス胚
線維芽フィーダー細胞（MEF, Millipore,
Billerica, MA, USA）上で、37 度、5 ％ CO$_2$気相
下で培養した。細胞は 1 mg/ml dispase
（Roche, Mannheim, Germany）によって剥離
し、5 日毎に継代した。京都大学 iPS 細胞研
究所より供与されたヒト iPS 細胞1株は、染
色体異常株であったため、その細胞集団か
ら、コロニーをピックアップし、染色体正常株
をクローニングした（図1）。
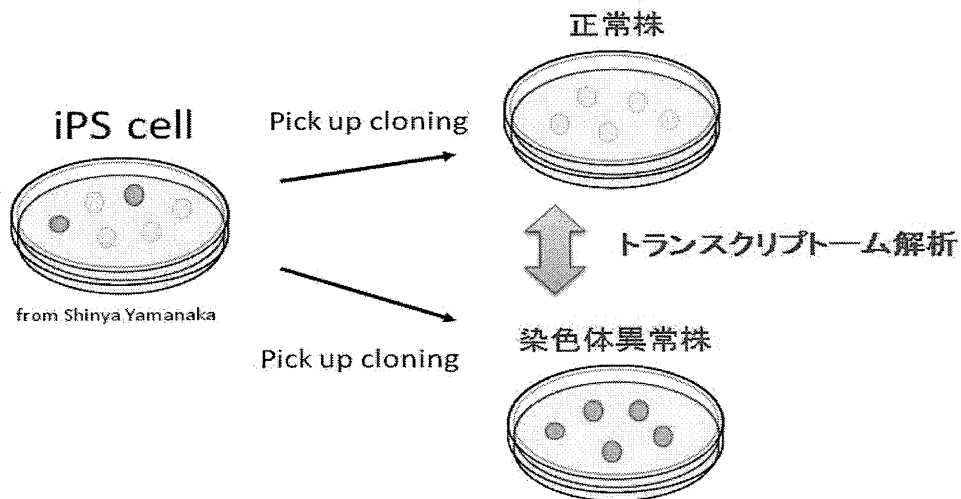
正常iPS細胞と染色体異常iPS細胞のクローニング

図1

クローニングした細胞は、核型解析および
CGH 解析により染色体が正常であることを確
認した。また、京都大学 iPS 細胞研究所より
染色体異常株の供与を受け、核型解析によ
り確認を行った。

②染色体正常株と異常株における遺伝子発
現の差を解析する

　未分化ヒト iPS 細胞株の total RNA は、
RNeasy Mini kit を用いて抽出した。抽出した

total RNA は、アジレントバイオアナライザー2100 により RNA 分解されていないことを確認した。mRNA の Cy3 標識（一色法）は、メーカーの指示書にしたがって Low input quick amp labeling kit（Agilent）用いて行った。

標識した mRNA は、4x44k v2 Whole Human Genome DNA Microarray chip（Agilent）と 65 度で 17 時間ハイブリダイゼーションさせた。チップ上の各スポット値のシグナル強度は、G2505C Microarray Scanner System を使用して測定し、各遺伝子の発現比は Partek Genomics Suite software program を使用して算出した。

C：結果

その染色体異常株と正常株のトランスクリプトームを比較したとき、121 プローブ（115 遺伝子）が共通して 1.5 倍以上の発現変動を示した（図2A）。

それら共通して発現変動を示した遺伝子群に中には、染色体異常株において顕著に発現が高い遺伝子群が見つかった（図2B）
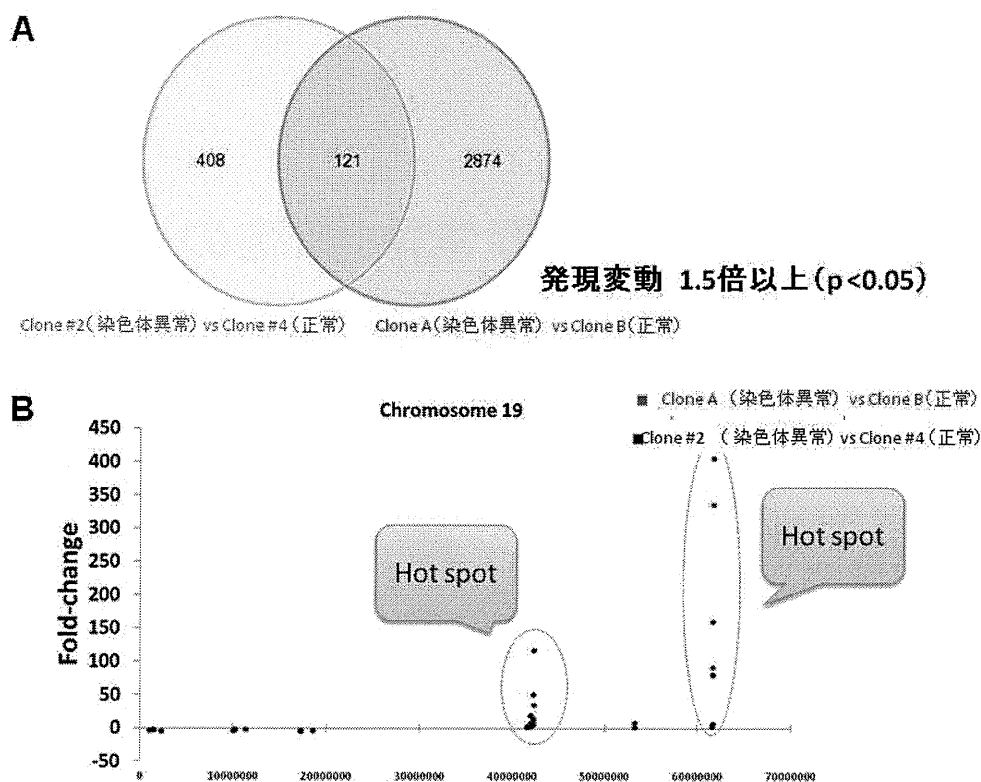

図2 染色体異常ヒトiPS株と正常株のトランスクリプトーム比較解析

それらの遺伝子群は、染色体異常株の染色体 19 番の中で近接する遺伝子群であった。これらの結果は、染色体異常株において「ホットスポット」として高発現する遺伝子群が存在することを意味し、染色体異常のバイオマーカーとして有用な遺伝子群候補であると考えられる。なお、まだ、論文発表を行ってないため、細胞名や遺伝子名なのどの詳細な記載は差し控える。

D: 考察

　ヒト ES/iPS 細胞の培養は、ゲノムが不安定であることが知られており、品質維持を確保するため、できるだけ不特定物質および異種動物由来因子を排除し、安定して培養を行うことができ、ロット管理できる組成の明らかな条件が望まれている。しかし、一般にはウシ血清やウシ血清代替品としてのKnockout Serum Replacetnt (KSR)とフィーダー細胞が頻用されている。この培養法が比較的安定であることから、再生医療や創薬応用を行う際にも使用を検討されているのが現状である。KSR は組成は一般には公開されていないが、複数の動物由来成分を含み、原材料の入手経路が複雑であることが推測され、トレーサビリティーの管理が難しく、また、ロット差がある。ウィルスなどの病原体の混入の確認をロット毎に確認する必要があり、品質管理にコストがかかる。また、動物由来糖鎖成分が含まれ、これを細胞が取り込むため、移植の際に拒否反応の原因となる可能性がある。フィーダー細胞はマウス胎児由来線維芽細胞が頻用されているが、これもウィルスなどの病原体の混入の確認をロット毎に確認する必要があり、安全性の確認にコストがかかる。ヒト胎児組織由来細胞をフィーダー細胞の使用も試みられているが、倫理問題などの解決が難しい。これらの問題を解決するべく、フィーダー細胞を用いない無血清培養条件が報告されているが、すべての組成を既知の成分を用いた培養条件は、我々と Thomoson らのグループだけである。しかし、だれもが簡単にすべての未分化ヒトES/iPS 細胞を安定に培養するのは難しい。未分化ヒト ES/iPS 細胞の維持は、FGF-2 とTGF-ʲ/activin/Nodal シグナルなどが必要であると言われている。しかし、それらシグナルの下流経路は、複雑にクロストークして未分化維持だけでなく分化にも関与し、未分化な状態の不安化を引き起こしていると推測される。より安定した培養法の開発が望まれるが、一方でその品質管理法開発が望まれている。ゲノムワイドな解析を行うことにより、品質評価をすることは可能ではあるが、その解析にかかる時間やコストは、応用を促進する上において、考慮するべきである。今回解析を行った遺伝子群の中から、今後、染色体異常のバイオマーカーが同定できれば、効率的に品質管理を行うことが可能となる。本研究は将来有望なヒト多能性幹細胞を培養資源とするための品質管理法開発の一助となると考える。

E: 結論

　ゲノムワイドな遺伝子発現解析がヒト iPS 細胞の資源化の際の品質検査に有用であると考えられた。

F: 健康危険情報

　なし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Saito S, Morita K, Kohara A, Masui T, Sasao M, Ohgushi H, Hirano T.	Use of BAC array CGH for evaluation of chromosomal stability of clinically used human mesenchymal stem cells and of cancer cell lines.	Hum Cell	24(1)	2-8	2011
Mimura S, Kimura N, Hirata M, Tateyama D, Hayashida M, Umezawa A, Kohara A, Nikawa H, Okamoto T, Furue MK.	Growth factor-defined culture medium for human mesenchymal stem cells.	Int J Dev Biol.	55(2)	181-187	2011
菅三佳，高田圭，小原有弘，末盛博文，青井貴之，中村幸夫，古江-楠田美保	ヒト多能性幹細胞の命名法の国際統一規格案について.	再生医療	vol.11	2-8	2011
Sugita S, Shimizu N, Watanabe K, Katayama M, Horie S, Ogawa M, Sugimoto Y and Mochizuki M.	Diagnosis of bacterial endophthalmitis by broad-range quantitative PCR.	Br J Haematol	95(3)	345-349	2011
Ng SB, Selvarajan V, Huang G, Zhou J, Feldman AL, Law M, Kwong YS, Shimizu N, Nagami Y, Aozasa K, Salto-Tellez M and Chng WJ.	Activated oncogenic pathways and therapeutic targets in extranodal nasal-type NK/T cell lymphoma revealed by gene expression profiling.	J Phatol.	223	496-510	2011
Abe T, Segawa Y, Watanabe H, Yotoriyama T, Kai Y, Yasuda A, Shimizu N and Tojo N	Point-of-Care Testing System Enabling 30-min Detection of Influenza Genes.	LAB CHIP	11	1166-1167	2011
Yagasaki H, Kato M, Shimizu N, Shichino H, Chin M and Mugishima H.	Autoimmune hemolytic anemia and autoimmune neutropenia in a child with erythroblastopenia of childhood (TEC) caused by human herpesvirus-6 (HHV6).	Ann Hematol	90 (7)	851-852	2011
Watanabe A, Tagawa H, Yamashita J, Teshima K, Nara M, Iwamoto K, Kume M, Kameoka Y, Takahashi N, Nakagawa T, Shimizu N and Sawada K.	The role of microRNA-150 as a tumor suppressor in malignant lymphoma.	Leukemia	25 (8)	1324-1334	2011
Sugita S, Ogawa M, Inoue S, Shimizu N and Mochizuki M.	Diagnosis of ocular toxoplasmosis by two polymerase chain reaction (PCR) examinations: qualitative multiples and quantitative real-time.	Jpn J Ophthalmol.	55	495-501	2011
Sugita S, Shimizu N, Watanabe K, Katayama M, Horie S, Ogawa M, Sugimoto Y and Mochizuki M.	Detection of Candida & Aspergillus species DNA using broad-range real-time PCR for fungal endophthalmitis.	Graefe Arch Clin Exp	250 (3)	391-398	2011
Ng SB, Yan J, Huang G, Selvarajan V, Tay J, Lin B, Bi C, Tan J, Kwong YL, Shimizu N, Aozasa K, Chng W.	Dysregulated MicroRNAs Affect Pathways and Targets of Biological Relevance in Nasal-type Natural Killer / T-cell Lymphoma.	Blood	118(18)	4919-4929	2011
Imadome K, Yajima M, Arai A, Nakazawa A, Kawano F, Ichikawa S, Shimizu N, Yamamoto N, Morio T, Ohga S, Nakamura H, Ito M, Miura O, Komano J, and Fujiwara S.	Novel Mouse Xenograft Models Reveal a Critical Role of CD4+ T Cells in the Proliferation of EBV-Infected T and NK Cells.	PLoS Pathogens	7(10)	e1002326	2011
Kuwana Y, Takei M, Yajima M, Imadome K, Inomata H, Shiozaki M, Ikumi N, Nozaki T, Shiraiwa H, Kitamura N, Takeuchi J, Sawada S, Yamamoto N, Shimizu N, Ito M and Fujiwara S	Epstein-Barr Virus Induces Erosive Arthritis in Humanized Mice.	PloS ONE	6(10)	e26630	2011

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ramakrishnan R, Donahue H, Garcia D, Tan J, <u>Shimizu N</u>, Rice AP and Ling P.	Epstein-Barr virus BART9 miRNA modulates LMP1 levels and affects growth rate of nasal NK T cell lymphomas.	PloS ONE	6(11)	e27271	2011
Ohtake, Y., Nishizawa, I., Sato, M., Watanabe, Y., Nishimura, T., Matsubara, K., Nagai, K., and <u>Harasawa, R.</u>	Mycoplasma ovis detected in free-living Japanese serows, Capricornis crispus.	J. Vet. Med. Sci.	73	371-373	2011
Obara, H., Fujihara, M., Watanabe, Y., Ono, H.K., and <u>Harasawa, R.</u>	A feline hemoplasma, 'Candidatus Mycoplasma haemominutum', detected in dog in Japan.	J. Vet. Med. Sci.	73	841-843	2011
Giangaspero, M., and <u>Harasawa, R.</u>	Species characterization in the genus Pestivirus according to palindromic nucleotide substitutions in the 5'-untranslated region.	J. Virol. Methods	174	166-172	2011
Suzuki, J., Sasaoka, F., Fujihara, M., Watanabe, Y., Tasaki, T., Oda, S., Kobayashi, S., Sato, R., Nagai, K., and <u>Harasawa, R.</u>	Molecular identification of 'Candidatus Mycoplasma haemovis' in sheep with hemolytic anemia.	J. Vet. Med. Sci.	73	1113-1115	2011
Fujihara, M., Obara, H., Watanabe, Y., Ono, H.K., Sasaki, J., Goryo, M., and <u>Harasawa, R.</u>	Acidic environments induce differentiation of Proteus mirabilis into swarmer morphotypes.	Microbiol. Immunol.	55	489-493	2011
Sasaoka, F., Suzuki, J., Watanabe, Y., Fujihara, M., and <u>Harasawa, R.</u>	Rapid identification of hemoplasma species by palindromic nucleotide substitutions at the GAAA tetraloop helix in the specificity domain of ribonuclease P RNA	J. Vet. Med. Sci.	73	1517-1520	2011
Giangaspero, M., Ibata, G., Savini, G., Osawa, T., Tatami, S., Takagi, E., Moriya, H., Okura, N., Kimura, A., and·<u>Harasawa, R.</u>	Epidemiological survey of Border disease virus among sheep from northern districts of Japan.	J. Vet. Med. Sci.	73	1629-1633	2011
Watanabe, Y., Fujihara, M., Obara, H., Nagai, K., and <u>Harasawa, R.</u>	Two genetic clusters in swine hemoplasmas revealed by analyses of the 16S rRNA and RNase P RNA genes.	J. Vet. Med. Sci.	73	1657-1661	2011
Giangaspero, M., Osawa, T., Orusa, R., Frossard, J.-P., Naidu, B., Robetto, S., Tatami, S., Takagi, E., Moriya, H., Okura, N., Kato, K., Kimura, A., and <u>Harasawa, R.</u>	Epidemiological survey for visna-maedi among sheep in northern prefectures of Japan	Vet. Ital.	47	437-451	2011
Fujihara, Y., Sasaoka, F., Suzuki, J., Watanabe, Y., Fujihara, M., Ooshita, K., Ano, H., and <u>Harasawa, R.</u>	Prevalence of hemoplasma infection among cattle in the western part of Japan.	J. Vet. Med. Sci.	73	1653-1655	2011

RESEARCH ARTICLE

Use of BAC array CGH for evaluation of chromosomal stability of clinically used human mesenchymal stem cells and of cancer cell lines

Soichiro Saito · Keiko Morita · Arihiro Kohara ·
Tohru Masui · Mari Sasao · Hajime Ohgushi ·
Takashi Hirano

Abstract Array-based comparative genomic hybridization (aCGH) using bacterial artificial chromosomes (BAC) is a powerful method to analyze DNA copy number aberrations of the entire human genome. In fact, CGH and aCGH have revealed various DNA copy number aberrations in numerous cancer cells and cancer cell lines examined so far. In this report, BAC aCGH was applied to evaluate the stability or instability of cell lines. Established cell lines have greatly contributed to advancements in not only biology but also medical science. However, cell lines have serious problems, such as alteration of biological properties during long-term cultivation. Firstly, we investigated two cancer cell lines, HeLa and Caco-2. HeLa cells, established from a cervical cancer, showed significantly increased DNA copy number alterations with passage time. Caco-2 cells, established from a colon cancer, showed no remarkable differences under various culture conditions. These results indicate that BAC aCGH can be used for the evaluation and validation of genomic stability of cultured cells. Secondly, BAC aCGH was applied to evaluate and validate the genomic stabilities of three patient's mesenchymal stem cells (MSCs), which were already used for their treatments. These three MSCs showed no significant differences in DNA copy number aberrations over their entire chromosomal regions. Therefore, BAC aCGH is highly recommended for use for a quality check of various cells before using them for any kind of biological investigation or clinical application.

Keywords Validation of cell line · BAC ·
BAC array CGH

Introduction

Comparative genomic hybridization (CGH) and array-based CGH (aCGH) can detect DNA copy number aberrations in the entire human genome [1, 2]. In fact, to detect DNA copy number aberrations, aCGH has been used to examine many cancers and cancer cell lines for diagnosis and prognosis [3–7]. Moreover, in Korea an aCGH chip was approved for use to diagnose hereditary diseases and inherent chromosomal disorders, such as Down syndrome and Turner's syndrome, which are caused by chromosomal aberrations [Korean Food and Drug Administration (KFDA; http://www.macrogen.com/eng/macrogen/press_list.jsp)]. Bacterial artificial chromosome (BAC) aCGH has attracted attention as a superior method for genome-wide analysis not only to detect DNA copy number aberrations, but also to evaluate hereditary chromosomal disorders.

In recent years, regenerative medicine using mesenchymal stem cells (MSCs) has received much attention

S. Saito · K. Morita
Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan

A. Kohara · T. Masui
Division of Bioresources, National Institute of Biomedical Innovation, Osaka, Japan

M. Sasao · H. Ohgushi
Health Research Institute, AIST, Tsukuba, Japan

T. Hirano (✉)
Collaboration Promotion Department, AIST, Central 6,
1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan
e-mail: hirano-takashi@aist.go.jp

T. Hirano
Okinawa Science and Technology Promotion Center,
Uruma, Japan

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[8, 9]. However, safety issues concerning the MSC applications, especially with respect to tumorigenesis, remain to be solved [9–11]. The BAC aCGH method would be useful for the evaluation of chromosomal stability and instability, which are closely related to tumorigenesis.

In this study, we performed BAC aCGH to evaluate chromosomal stability of HeLa cells, Caco-2 cells, and MSCs. The HeLa cell line was established as the first human cancer cell line derived from a cervical cancer and is one of the most widely used cell lines in the world [12, 13]. However, numerous other established cell lines are now used as a substitute for HeLa cells [13]. The Caco-2 cell line was established from a human colon cancer [14]. Even though the Caco-2 cell was derived from a colon cancer, it has been available for use as a convenient reference model for theoretical predictions of intestinal drug absorption in drug discovery [15]. Therefore, the stability of Caco-2 cells should be established for such a screening purpose. MSCs are expected to be applied for regenerative medicine, and they are already used clinically for the treatment of various diseases [16, 17]. The safety issue regarding the chromosomal stability of these cells thus becomes increasingly important for future clinical applications.

Materials and methods

Cell lines and DNA extraction

HeLa cells (human cervical cancer cell line) of three different numbers of passage times were used for this study. HeLa-A was purchased from the American Type Culture Collection (ATCC, Manassas, VA), and DNA was directly extracted without cultivation. The number of passage times of HeLa-A was approximately 100 according to an attached product information sheet from ATCC. HeLa-B and HeLa-C were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan), and the number of their passage times was 122 for HeLa-B and 150 for HeLa-C.

Three different types of Caco-2 cells (human colon cancer cell line) were also used for this study. Briefly, Caco-2 was purchased from ATCC and designated as Caco-2-a, and its DNA was directly extracted without any cultivation because this DNA was regarded as a control. Caco-2-b was maintained by a commercial institution and analyzed after 63 passage times. Caco-2-c was maintained by the same commercial institution, cultured on microporous membranes (0.4 μm diameter), and analyzed after 58 passage times.

The ACBRI-519 cell line, which was derived from normal human intestinal epithelial cells, was used as a counterpart of Caco-2 cells in this study. ACBRI-519 cells were purchased from Cell System Corp. (Kirkland, WA).

Three MSCs were derived from individual bone marrow samples that were actually used clinically [16]. MSC-1 was derived from a 69-year-old female and analyzed at passage number 3 after primary culture using bone marrow. In a similar manner, MSC-2 was derived from a 16-year-old female and analyzed at passage number 3. MSC-3 was derived from a 34-year-old male and analyzed at passage number 4. MSC-4 was derived from the same individual as MSC-3, but analyzed at passage number 7.

Extraction of genomic DNA was carried out by using SepaGene (Sanko Junyaku, Tokyo, Japan) except MSCs, and genomic DNA of MSCs was extracted by using the Gentra Puregene Cell Kit (Qiagen, Hilden, Germany). Each procedure of DNA extraction was according to the manufacturer's respective protocols.

BAC aCGH

BAC aCGH analysis was carried out as described previously [7]. Briefly, 500 ng of genomic DNA from a given cell line as the test sample and 500 ng of gender-matched reference genomic DNA (Promega Corporation, Madison, WI) were labeled with cyanine3-dCTP (Perkin Elmer Inc., Waltham, MA) for reference DNA or cyanine5-dCTP (Perkin Elmer) for test DNA by random priming in 50-μl reaction volumes by using the Bioprime DNA Labeling System (Life Technologies Corporation, Carlsbad, CA) and Array Kit (Macrogen, Seoul, Korea, http://www.macrogen. com). After labeling, unincorporated fluorescent nucleotides were removed by using a QIAquick polymerase chain reaction (PCR) purification kit (Qiagen). Labeled test and reference DNAs were mixed and dissolved in hybridization solution (Macrogen) containing 100 μl Cot-1 DNA solution and 4 μl yeast tRNA solution (Macrogen). The array CGH was provided by Macrogen MAC Array KARYO 4000. This array slide had 4030 BAC clone DNAs in duplicate and covered the entire human genome with 1-Mbp resolution. The hybridization-to-wash procedure was carried out by using a Hybristation (Digilab Inc., Holliston, MA). Hybridization was carried out at 37°C for 48–72 h on the Hybristation with continuous agitation. The wash procedure was as follows: 50% formamide/2× standard saline citrate (SSC) at 46°C for 15 min, followed by 0.1% SDS/2× SSC at 46°C for 30 min, PN buffer (0.1 M Na$_2$PO$_4$/0.1% NonDiet P-40, Nakarai Tesque, Kyoto, Japan) at 37°C for 15 min, and 2× SSC at 37°C for 5 min. The array slides were scanned at 532 and 635 nm by using a GenePix4000A (Molecular Devices, Sunnyvale, CA) and analyzed by Mac Viewer software (Macrogen). The Mac Viewer software analyzed the results as follows: (1) averaged the fluorescence ratios of the replicates and calculated

the standard deviation (SD), (2) rejected individual spot data based on several criteria including weak fluorescent signals, (3) adjusted Cy5/Cy3 ratios such that ratios of the normal genomic regions were always equal to 0, despite variations in dye labeling efficiency, and (4) plotted data relative to the position of the clones on the human genome, according to July 2003, University of California, Santa Cruz cartography. In this study, all BAC aCGH analyses were confirmed to calibrate by the hybridization of the normal male DNA versus normal female DNA. The entire SD value of the $\log_2$ ratio calculated for chromosomes 1–22 was 0.07. Accordingly, DNA copy number abnormalities were defined as more than three times higher than the SD in order to account for experimental errors. For this research, a $\log_2$ ratio of 0.3 was employed to indicate abnormal differences, with the normalized $\log_2$ ratio of fluorescence intensity of over 0.3 being taken as gain and one of below −0.3 as loss.

Results and discussions

Evaluation of HeLa cells

To evaluate the chromosomal stability and instability of HeLa cells, we analyzed three different HeLa cells by BAC aCGH, as shown in Fig. 1. In the case of HeLa-A, DNA was directly extracted from ATCC HeLa cells without cultivation. HeLa-B and HeLa-C were cultured for different periods of time prior to DNA extraction (HeLa-B for 122 passages and HeLa-C for 150 passages). Novel DNA copy number loss occurred at chromosomes 3 and 13 in HeLa-C [Fig. 1a, b(i), (iii), respectively]. Moreover, at 9p13.1–p24.3, on the short arm of chromosome 9, CGH profiles showed a tendency of the DNA copy number to decrease with increased passage time [Fig. 1a, b(ii)]. Similar results were obtained for the entire regions of chromosome 1 [Fig. 1a(*)]. In contrast, the CGH profiles showed a tendency for the DNA copy number for the entire regions of both chromosomes 21 and 22 to increase with increased passage time [Fig. 1a(*)]. Additionally, Table 1 summarizes the average of $\log_2$ ratios for the above-mentioned regions obtained from BAC aCGH analysis. These results indicate that chromosomal instability including DNA copy number alterations was generated by long-term culture of HeLa cells. HeLa-C, in comparison to HeLa-A, would be distinguished as a variant of HeLa cells or might be a different cell. To summarize our analysis using BAC aCGH, continuous cultivation of HeLa cells caused a significant change at the chromosomal level. Until now, chromosomal changes in cultured cells have been recognized only empirically. If a chromosomal change occurs, it will result in a significant change at the expression level.

For scientific research using cultured cells, such a change is extremely critical. Based on our present findings, we stress the importance of validation of experimental cultured cells even at the chromosomal level.

Evaluation of Caco-2 cells

This colon cancer cell line is well known to be a heterogeneous cell line and to differentiate spontaneously into small intestinal epithelial cells after its cultures have reached confluence [18–20]. Such differentiated Caco-2 cells can be cultured as monolayers on permeable filters and correlate well with the absorption system of normal intestinal cells. Therefore, Caco-2 cells are used industrially as a simulation model of intestinal drug absorption in drug discovery [15]. As described above, HeLa cells displayed chromosomal instabilities including DNA copy number alterations in a passage time-dependent manner. To evaluate the chromosomal stability including DNA copy number aberrations of Caco-2 cells, we analyzed Caco-2 cells under several different conditions by using BAC aCGH. The CGH profile for Caco-2-a, which was used as the control, is shown in Fig. 2. These cells showed no significant difference in comparison to Caco-2 cells purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK) or from DS Pharma Biomedical Co., Ltd. (Osaka, Japan; data not shown). Caco-2-b cells, which were analyzed at passage number 63, and Caco-2-c cells, which had been cultured on the microporous membranes, showed no remarkable differences in CGH profile in comparison to Caco-2-a. Other culture conditions, such as fewer passage times than the 63 passages for Caco-2-b and use of larger diameter membrane than that used for Caco-2-c, gave similar CGH profiles (data not shown). These results indicate that the Caco-2 cell line, in comparison to the HeLa cell, is a chromosomally stable cell line, even though it was established from a cancer cell. Therefore, the Caco-2 cell line would be considered a suitable cell line for use in a validation system of intestinal drug absorption, as verified from the aspect of chromosomal stability assessed by BAC aCGH.

The ACBRI-519 cell line, which was established from normal human intestinal epithelial cells, was regarded as an alternative of the Caco-2 cell line. CGH profiles showed no significant differences between ACBRI-519 and Caco-2 cells, as also shown in Fig. 2. According to the result of BAC aCGH, ACBRI-519 and Caco-2 cells would be regarded as the same cell line. Indeed, Yamamoto et al. [21] reported that the IL-8 response to oxidative stress was almost the same between Caco-2 cells and ACBRI-519 cells. Thus, BAC aCGH can be used to recognize and to distinguish cell lines.
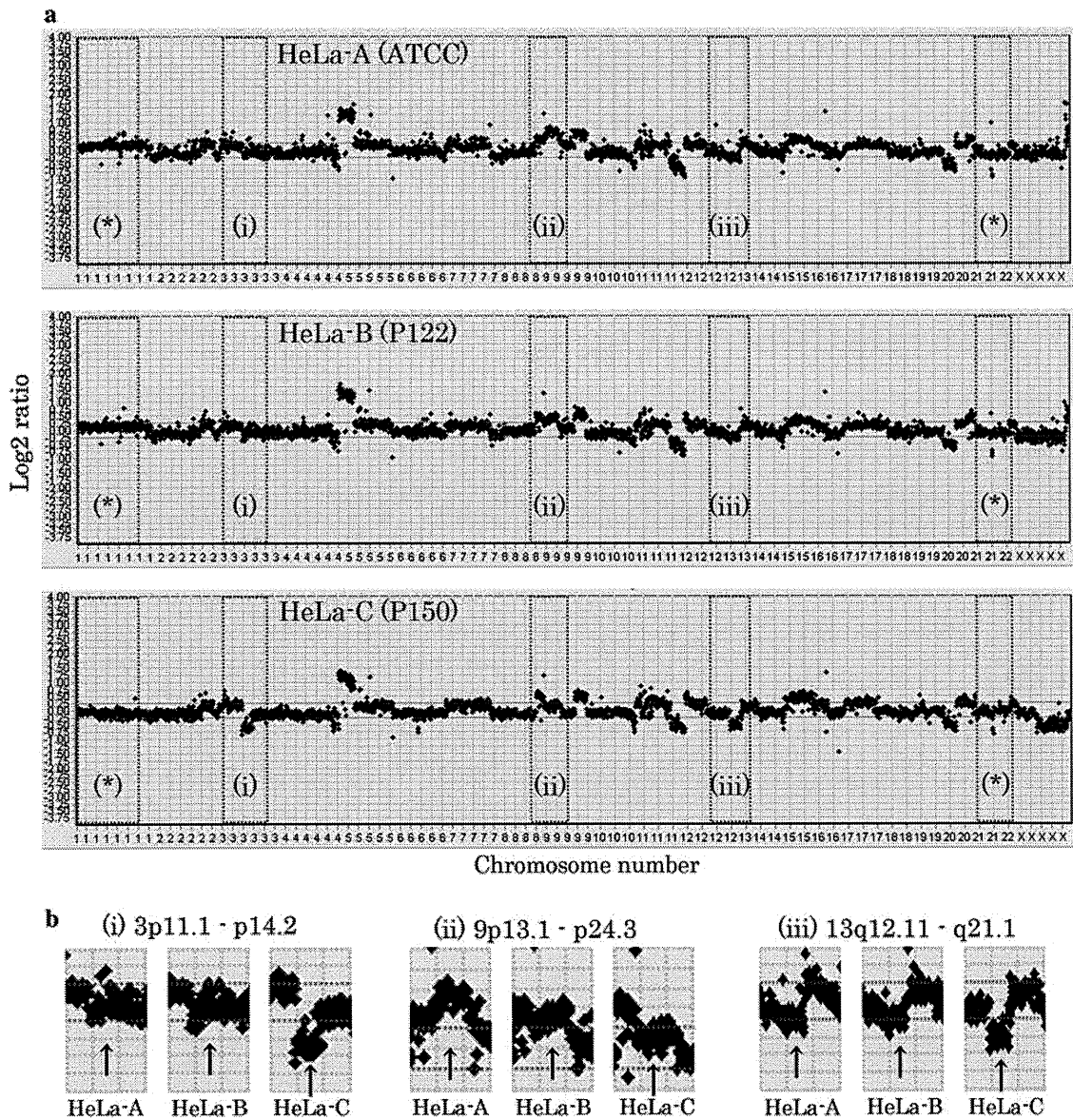
Fig. 1 BAC aCGH profiles of three HeLa cells. **a** *Upper panel* BAC aCGH profile of HeLa-A (ATCC), *middle panel* HeLa-B (JCRB, after 122 passages), *lower panel* HeLa-C (JCRB, after 150 passages). (*i*)–(*iii*) Correspond to **b**(*i*) to (*iii*), respectively. An *asterisk* indicates a tendency for DNA copy number alterations. The *ordinate* indicates the $\log_2$ ratio of Cy5/Cy3 and abscissa, the chromosome number (also applies to Figs. 1b, 2, 3). **b** Three remarkable regions of DNA copy number aberrations. *Arrows* point to regions of remarkable DNA copy number loss

Evaluation of MSCs

MSCs have been widely used clinically in the field of regenerative medicines; for instance, they are used for the treatment of osteoarthritis, bone tumor, acute myocardial infarction, and graft-versus-host disease [16, 17, 22, 23]. Because the tumorigenesis of MSCs is still a controversial issue, the safety evaluation of MSCs is very important [9–11]. BAC aCGH is a powerful method for detecting DNA copy number aberrations, which are strongly associated with tumorigenesis. In this study, we analyzed MSCs

that already had been used clinically without tumor formation for osteoarthritis patients [16]. As shown in Fig. 3, the CGH profiles of MSC-1, MSC-2, and MSC-3 followed the baseline linearly; the SD values for these CGH profiles were 0.028 ± 0.060 for MSC-1, 0.043 ± 0.072 for MSC-2, and 0.029 ± 0.063 for MSC-3. In the case of MSC-4, which was passaged three more times than MSC-3, it also followed the baseline linearly (SD value was 0.018 ± 0.073). These results indicate that these MSCs did not have any chromosomal instability including DNA copy number aberrations. Therefore, BAC aCGH was able to confirm the

Table 1 Average of $\log_2$ ratios for the regions showing DNA copy number alterations in three types of HeLa cells

Region	Average of $\log_2$ ratio					
	Chr. 1	3p11.1–p14.2	9p13.1–p24.3	13q12.11–q21.1	Chr. 21	Chr. 22
HeLa-A (ATCC)	0.120	−0.114	0.581	−0.248	−0.180	−0.145
HeLa-B (P122)	0.080	−0.154	**0.337**	−0.245	−0.170	−0.105
HeLa-C (P150)	**−0.126**	**−0.635**	**0.126**	**−0.511**	*−0.146*	*−0.029*
Number of clones	299	34	63	50	68	100

Chr. indicates whole region of the chromosome; P122 and P150, analysis after 122 and 150 passages, respectively; number of clones, the number of BAC clones in the corresponding region; bold type, the value of the $\log_2$ ratio decreased in comparison to that for HeLa-A; italic type, the value of the $\log_2$ ratio increased in comparison to that for HeLa-A
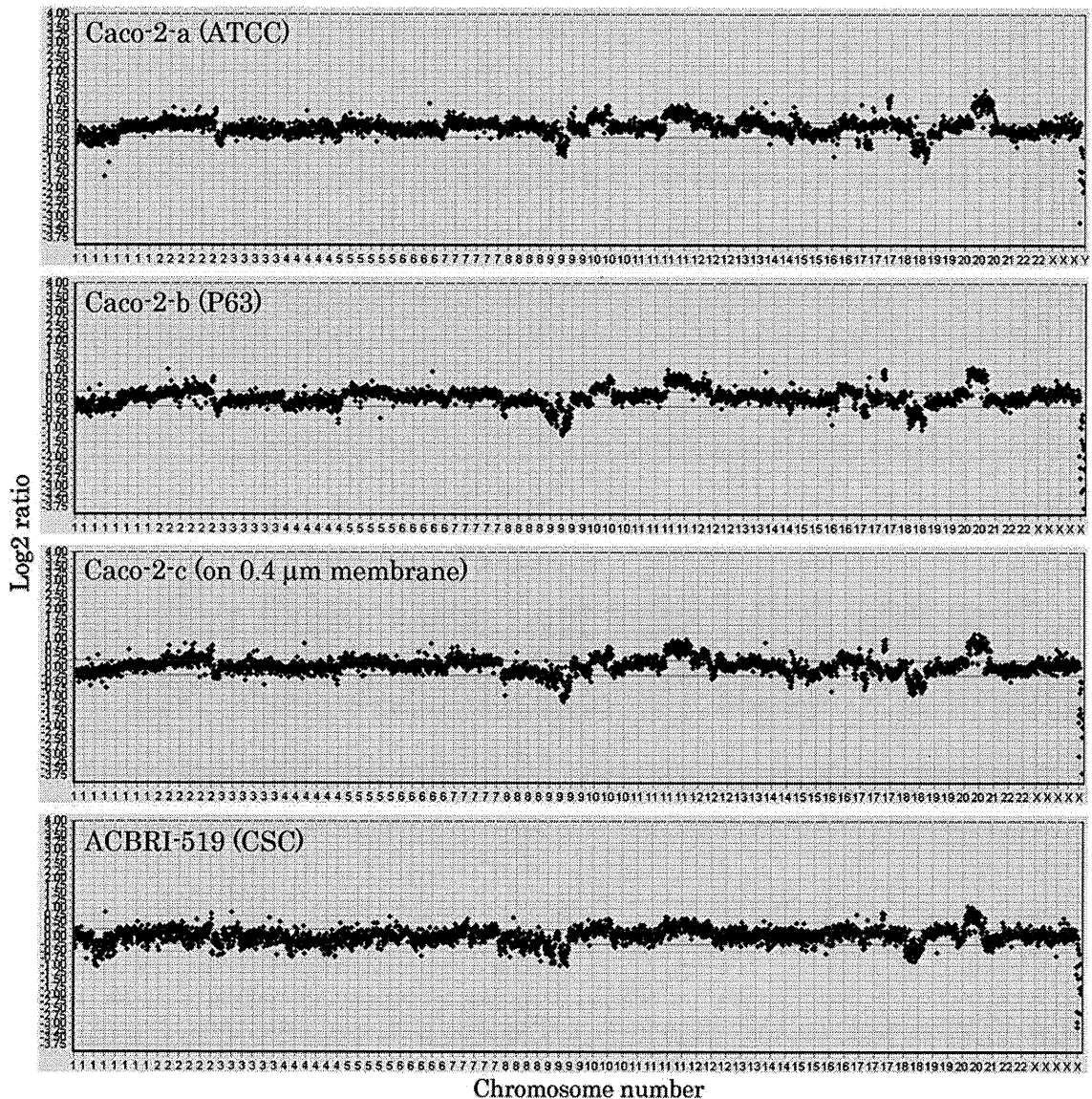

Fig. 2 BAC aCGH profiles of 3 Caco-2 cells and ACBRI-519 cells. Sequentially from the upper panel are the BAC aCGH profile of Caco-2-a (ATCC), -b (after 63 passages), -c (cultured on 0.4-μm diameter microporous membrane), and ACBRI-519 (Cell System Corp.)

safety of these MSCs at the genome level, especially DNA copy number change, which correlates well with tumorigenesis. In Fig. 3, several BAC clones that were outside the normal range (considered as normal from −0.3 to 0.3, see "Materials and methods") were possible copy number variants (CNV). These clones were confirmed as CNV loci
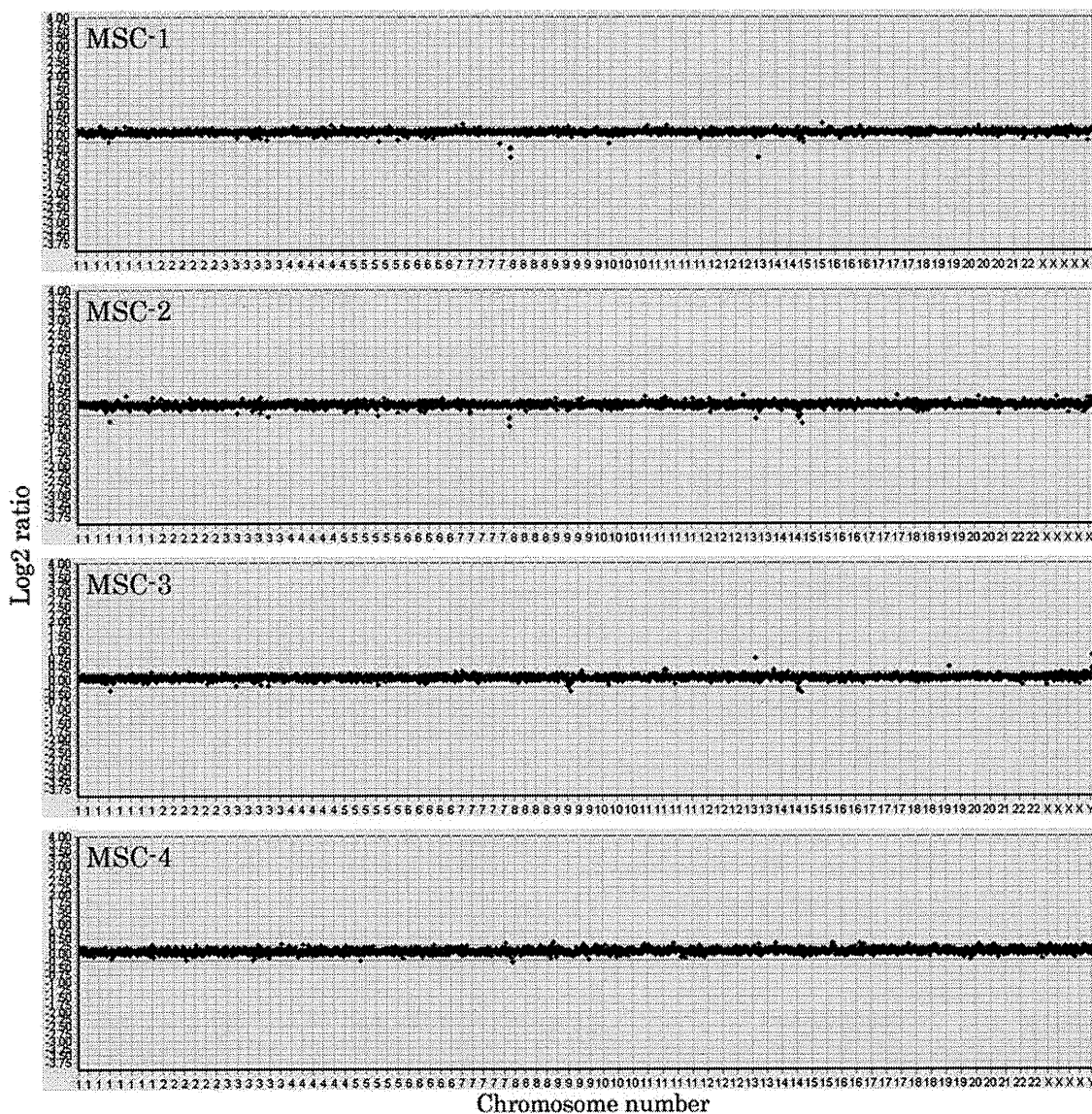
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Fig. 3 BAC aCGH profiles of four clinically used MSCs. Sequentially from the upper panel the BAC aCGH profiles are shown of MSC-1 (69-year-old female after three passages), -2 (16-year-old female after 3 passages), -3 (34-year-old male after 4 passages), and -4 (derived from the same individual as MSC-3, after 7 passages)

or in their proximity (data not shown), according to the Database of Genomic Variants website of the University of Toronto (http://projects.tcag.ca/variation/).

Although established cell lines are now an essential tool in biological and clinical studies, no one has seriously questioned the reliability of such cell lines until now. Actually, most investigators have noted morphological and/or biological alterations of cell lines during long-term culture. For coping with such alterations, these cell lines have been discarded and renewed from frozen stocks after a certain number of passages. However, the substantial alterations caused by long-term culture have not been seriously considered. Validation to detect cross contamination of cell lines has been made by using various

methods, such as HLA typing, DNA polymorphism, DNA fingerprinting, karyotyping, STR profiling, and SNPs [24–29]. In particular, STR profiling, which was developed for forensic sciences [30], was proposed as an international reference standard for human cell lines [28]. These methods can only detect at limited partial regions of the human genome. However, tumorigenesis is known to correlate with various chromosomal instabilities including DNA copy number changes throughout the entire human genome. Therefore, to avoid overlooking the possibility of tumorigenesis, it is necessary to validate cells by using BAC aCGH, as it can analyze all regions in the entire human genome. In this study, we investigated chromosomal stability and instability of established cell lines,

HeLa cell, Caco-2 cells, and MSCs derived from normal human bone marrow by performing BAC aCGH. Our results indicate that BAC aCGH is a suitable tool for validation of cell lines. Thus, we propose that BAC aCGH is a superior method for evaluation of the genomic stability of established cell lines as well as various kinds of cells and suggest that all kinds of cellular investigations should include validation of chromosomal stability by performing BAC aCGH.

References

1. Kallioniemi A, Kallioniemi OP, Sudar D, et al. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science. 1992;258:818–21.
2. Pinkel D, Segraves R, Sudar D, et al. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat Genet. 1998;20:207–11.
3. Nowak NJ, Gaile D, Conroy JM, et al. Genome-wide aberrations in pancreatic adenocarcinoma. Cancer Genet Cytogenet. 2005;161:36–50.
4. Saito S, Ghosh M, Morita K, Hirano T, Miwa M, Todoroki T. The genetic differences between gallbladder and bile duct cancer cell lines. Oncol Rep. 2006;16:949–56.
5. Hirasaki S, Noguchi T, Mimori K, et al. BAC clones related to prognosis in patients with esophageal squamous carcinoma: an array comparative genomic hybridization study. Oncologist. 2007;12:406–17.
6. Furuya T, Uchiyama T, Adachi A, et al. The development of a mini-array for estimating the disease state of gastric adenocarcinoma by array CGH. BMC Cancer. 2008;8:393–403.
7. Saito S, Morita K, Hirano T. High frequency of common DNA copy number abnormalities detected by bacterial artificial chromosome array comparative genomic hybridization in 24 breast cancer cell lines. Hum Cell. 2009;22:1–10.
8. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143–7.
9. Alison MR, Islam S. Attributes of adult stem cells. J Pathol. 2009;217:144–60.
10. Bernardo ME, Zaffaroni N, Novara F, et al. Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. Cancer Res. 2007;67:9142–9.
11. Rubio D, Garcia-Castro J, Martin MC, et al. Spontaneous human adult stem cell transformation. Cancer Res. 2005;65:3035–9.
12. Gey GO, Coffman WD, Kubicek MT. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res. 1952;12:264–5.
13. Masters JR. HeLa cells 50 years on: the good, the bad and the ugly. Nat Rev Cancer. 2002;2:315–8.
14. Fogh J, Wright WC, Loveless JD. Absence of HeLa cell contamination in 169 cell lines derived from human tumors. J Natl Cancer Inst. 1977;58:209–14.
15. Artursson P, Palm K, Luthman K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv Drug Deliv Rev. 2001;46:27–43.
16. Ohgushi H, Kotobuki N, Funaoka H, et al. Tissue engineered ceramic artificial joint-ex vivo osteogenic differentiation of patient mesenchymal cells on total ankle joints for treatment of osteoarthritis. Biomaterials. 2005;26:4654–61.
17. Morishita T, Honoki K, Ohgushi H, Kotobuki N, Matsushima A, Takakura Y. Tissue engineering approach to the treatment of bone tumors: three cases of cultured bone grafts derived from patient's mesenchymal stem cells. Artif Organs. 2006;30:115–8.
18. Pinto M, Robine-Leon S, Appay MD, et al. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line. Caco-2 in culture. Biol Cell. 1983;47:323–30.
19. Hidalgo IJ, Raub TJ, Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology. 1989;96:736–49.
20. Hilgers AR, Conradi RA, Burton PS. Caco-2 monolayers as a model for drug transport across the intestinal mucosa. Pharm Res. 1990;7:902–10.
21. Yamamoto K, Kushima R, Kisaki O, Fujiyama Y, Okabe H. Combined effect of hydrogen peroxide induced oxidative stress and IL-1α on IL-8 production in Caco-2 cells (a human colon carcinoma cell line) and normal intestinal epithelial cells. Inflammation. 2003;27:123–8.
22. Chen SL, Fang WW, Qian J, et al. Improvement of cardiac function after transplantation of autologous bone marrow mesenchymal stem cells in patients with acute myocardial infarction. Chin Med J. 2004;117:1443–8.
23. Ringdén O, Uzunel M, Rasmusson I, et al. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. Transplantation. 2006;81:1390–7.
24. O'Toole CM, Povey S, Hepburn P, Frankc LM. Identity of some human bladder cancer cell lines. Nature. 1983;301:429–30.
25. Jeffreys AJ, Wilson V, Thein SL. Individual-specific 'fingerprints' of human DNA. Nature. 1985;316:76–9.
26. Gilbert DA, Reid YA, Gail MH, et al. Application of DNA fingerprints for cell-line individualization. Am J Hum Genet. 1990;47:499–514.
27. MacLeod RA, Dirks WG, Matsuo Y, Kaufmann M, Milch H, Drexler HG. Widespread intraspecies cross-contamination of human tumor cell lines arising at source. Int J Cancer. 1999;83:555–63.
28. Masters JR, Thomson JA, Daly-Burns B, et al. Short tandem repeat profiling provides an international reference standard for human cell lines. Proc Natl Acad Sci USA. 2001;98:8012–7.
29. Demichelis F, Greulich H, Macoska JA, et al. SNP panel identification assay (SPIA): a genetic-based assay for the identification of cell lines. Nucleic Acids Res. 2008;37:2446–56.
30. Oldroyd NJ, Urquhart AJ, Kimpton CP, et al. A highly discriminating octoplex short tandem repeat polymerase chain reaction system suitable for human individual identification. Electrophoresis. 1995;16:334–7.

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Growth factor-defined culture medium for human mesenchymal stem cells

SUMIYO MIMURA[1,2,#], NAOHIRO KIMURA[3,#], MITSUHI HIRATA[1], DAIKI TATEYAMA[1],
MIDORI HAYASHIDA[1], AKIHIRO UMEZAWA[4], ARIHIRO KOHARA[1], HIROKI NIKAWA[2],
TETSUJI OKAMOTO[3] and MIHO K. FURUE*[,1]

[1]JCRB Cell Bank, Laboratory of Cell Cultures, Department of Disease Bioresources, National Institute of
Biomedical Innovation, Osaka, [2]Department of Oral Biology and Engineering, Division of Oral Health
Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, [3]Department of
Molecular Oral Medicine and Maxillofacial Surgery, Division of Frontier Medical Science, Graduate School
of Biomedical Sciences, Hiroshima University, Hiroshima and [4]Department of Reproductive Biology and
Pathology, National Research Institute for Child Health and Development, Tokyo, Japan

ABSTRACT Human bone marrow-derived mesenchymal stem cells (hMSCs) are potential cellular
sources of therapeutic stem cells as they have the ability to proliferate and differentiate into a wide
array of mesenchymal cell types such as osteoblasts, chondroblasts and adipocytes. hMSCs have
been used clinically to treat patients with graft vs. host disease, osteogenesis imperfect, or
alveolar cleft, suggesting that transplantation of hMSCs is comparatively safe as a stem cell-based
therapy. However, conventional culture medium for hMSCs contains fetal bovine serum (FBS). In
the present study, we developed a growth factor-defined, serum-free medium for culturing
hMSCs. Under these conditions, TGF-β1 promoted proliferation of hMSCs. The expanded hMSC
population expressed the human pluripotency markers SSEA-3, -4, NANOG, OCT3/4 and SOX2.
Furthermore, double positive cells for SSEA-3 and a mesenchymal cell marker, CD105, were
detected in the population. The potential to differentiate into osteoblasts and adipocytes was
confirmed. This work provides a useful tool to understand the basic biological properties of
hMSCs in culture.

KEY WORDS: mesenchymal stem cell, serum-free culture, TGF-β1

Introduction

Bone marrow-derived cells can differentiate into osteoblasts *in vitro* and *in vivo* (Friedenstein *et al.*, 1966) and thus are considered a useful source of stem cells for bone regeneration. Recently, many studies have reported that human bone marrow contains a distinct cell fraction referred to as multipotent mesenchymal stem cells (hMSCs) which can give rise to a wide array of mesenchymal cell types, including bone, fat, and cartilage"(Pittenger *et al.*, 1999). However, hMSCs can differentiate along some ectodermal and endodermal cell lineages such as neuronal cells and liver cells (Pittenger *et al.*, 1999; Dezawa *et al.*, 2004; Dezawa *et al.*, 2005). Further, a recent study reported that hMSCs have the ability to generate the multiple cell types derived from the three embryonic germ layers (Kuroda *et al.*,

2010). It has been estimated that hMSCs comprise about 0.001 to 0.01% of total bone marrow mononuclear cells (Pittenger *et al.*, 1999). For use in cell-based therapies, hMSC populations require extensive *in vitro* expansion to obtain sufficient numbers. The conventional culture medium for hMSCs is composed of a basal nutrient medium supplemented with fetal bovine serum (FBS) (Haynesworth *et al.*, 1992; Lennon DP, 1996). Although these traditional culture conditions provide robust undifferentiated hMSC expansion, the ill-defined components of FBS is undesirable for clinical applications and also hampers analysis of the cell biological mechanisms that control cell behavior.

Abbreviations used in this paper: hES cells, human embryonic stem cells; hMSCs, human mesenchymal stem cells.

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*Address correspondence to: Miho Kusuda Furue. Laboratory of Cell Cultures, Department of Disease Bioresources, National Institute of Biomedical Innovation, 7-6-8 Saito Asagi, Ibaraki, Osaka, 567-0085, Japan. Fax: +81-72-641-9851. e-mail: mkfurue@nibio.go.jp #Note: These authors contributed equally to this work.

We and others previously described serum-free media consisting of minimum essential components suitable to propagate and accurately analyze the characteristics of differentiated cells (Hayashi and Sato, 1976; Furue and Saito, 1998; Sato *et al.*, 2002; Furue *et al.*, 2005; Furue *et al.*, 2008; Hayashi *et al.*, 2010). One of these media, hESF9, supports the serial cultivation of undifferentiated human embryonic stem (hES) cells in the absence of feeder cells and thus provides an experimental system for elucidating cellular responses to specific environmental stimuli (Furue *et al.*, 2008; Na *et al.*, 2010). For example, either FGF-2 or heparin promotes proliferation of hES cells in a concentration-dependent manner although these effects were not detected under conventional culture conditions. Thus, a defined serum-free medium consisting of minimum essential components should be useful in elucidating hES/iPS cell responses to specific cues that control self-renewal, differentiation, and lineage selection (Furue *et al.*, 2010).

Because hMSCs have multipotent properties similar to hES cells, we speculated that hMSCs should be able to grow in similar culture conditions as hES cells. In the present study, we demonstrated that addition of TGF-β1 to the defined serum-free medium for hES cells supports the robust proliferation of hMSCs. The hMSC population expanded in the absence of serum expressed the mesenchymal cell markers CD44, CD73, CD90, and CD105. Further, they expressed human pluripotency surface markers, SSEA-3, -4, TRA-2-54, and also the transcription factors of *NANOG*, *OCT3/4*, and *SOX2*. We show that the serum-free expanded hMSCs can differentiate into osteoblasts and adipocytes. This work sets the stage for serum-free hMSC cell culture and thereby provides a useful tool to understand the basic biological characteristics of hMSCs.

Results

In this study we used a human bone marrow-derived hMSC line designated UE7T-13 (JCRB 1154). The life span of these

cells was prolonged by infecting them with a retrovirus containing human papillomavirus E7 and telomerase reverse transcriptase (hTERT) cDNAs (Mori *et al.*, 2005; Shimomura *et al.*, 2007; Ishii *et al.*, 2008; Takeuchi *et al.*, 2007). We first tested the ability of hESF9 medium, which we had developed for use with hES cells, to support the growth of UE7T-13 cells. The cells were harvested using trypsin/EDTA, from cultures in conventional medium containing 10% FBS (POWERDBY10) and transferred to 0.1% gelatin-coated dishes in hESF9 medium. However, UE7T-13 cell growth was quite slow. We then investigated the effects of various growth factors on proliferation of the cells. UE7T-13 cells were seeded on 0.1% gelatin in hESF9 in the absence of FGF-2 and heparin (hESF9(-/-)), containing increasing concentrations of FGF-1, FGF-2, TGF-β1, activin A, or leukemia inhibitory factor (LIF) (Fig. 1). Both FGF-1 and FGF-2 promoted UE7T-13 proliferation in a dose-dependent manner, and the greatest effect was seen at 10 ng/ml FGF-2. Neither LIF nor activin A affected on UE7T-13 cell proliferation, but TGF-β1 slightly stimulated UE7T-13 proliferation. Next all five factors (FGF-1, FGF-2, TGF-β1, activin A, and LIF) or four factors with increasing concentrations of heparin were added to UE7T-13 cultures (Fig. 2). When either FGF-2 or TGF-β1 was withdrawn from the cultures, the cell numbers decreased significantly. Heparin promoted cell proliferation in a dose-dependent manner. This result suggested that addition of FGF-2 and TGF-β1 to hESF9(-/-) medium, is critical for UE7T-13 proliferation, and heparin also enhanced cell growth. hESF9 medium supplemented with TGF-β1 was designated hESF10.

L-ascorbic acid-2-phosphate (Asc 2-P) in hESF9 medium supported hES cells. However, it is known to promote hMSC cell differentiation into osteoblasts. Therefore, we examined whether the presence of Asc 2-P in hESF10 medium promoted osteoblastic differentiation of UE7T-13 cells. We analyzed the expression of *bone sialoprotein* (*IBSP*), *osteocalcin* (*BGP*), *osteonectin* (*SPOCK2*), and *osteopontin* (*SPP1*) in UE7T-13 cell cultured in hESF10 with or without Asc 2-P and in conventional medium (Fig. 2). These osteoblast genes were expressed at significantly lower levels in cells cultured in the serum-free media than in those cultured in the conventional medium. These results suggest that the serum-free medium is suitable for hMSC maintenance. *IBSP* gene expression was higher in the cells cultured in the
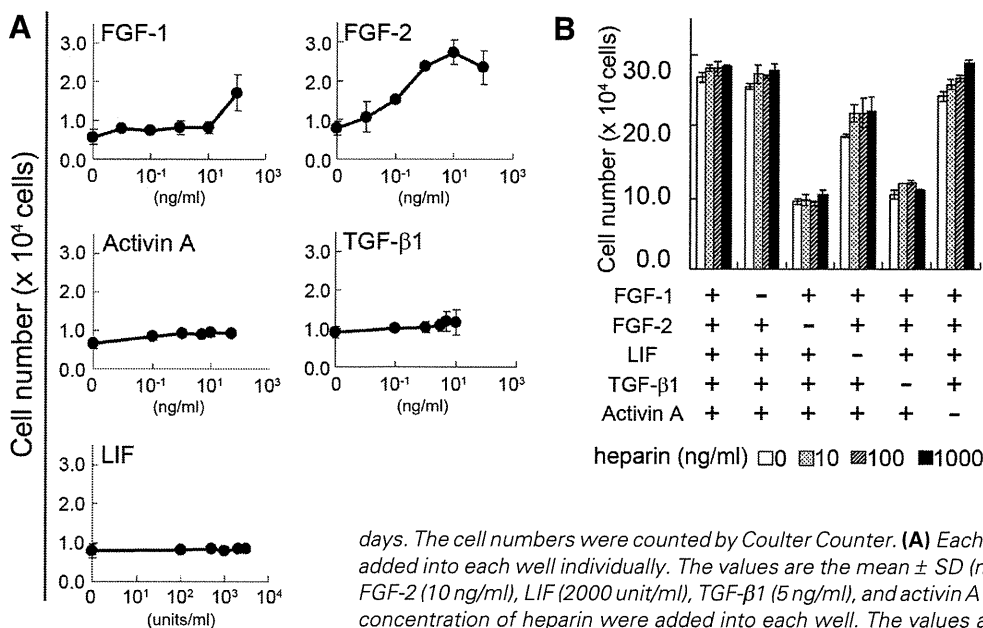

Fig. 1. Effect of growth factors on UE7T-13 cell proliferation in defined serum-free culture conditions. *After the UE7T-13 cell grown in the conventional culture conditions (POWERDBY10) were cultured in hESF9(-/-) overnight, the cells were seeded in a 24-well plate coated 0.1% gelatin in hESF9(-/-) at 1 x10⁴ cells per well and cultured for 6 days. The cell numbers were counted by Coulter Counter. (A) Each growth factor at indicated concentration was added into each well individually. The values are the mean ± SD (n=3). (B) All five factors of FGF-1 (100 ng/ml), FGF-2 (10 ng/ml), LIF (2000 unit/ml), TGF-β1 (5 ng/ml), and activin A (10 ng/ml) or four factors of them with varying concentration of heparin were added into each well. The values are the mean ± SD (n=3).*