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宇宙環境の人体影響評価と防護に関する研究;放射線晩発影響の防護

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Evaluation of Human Risk in Space Environment and Its Protection; Protection of Radiation Late Effects

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Abstract: To study the human risk of cosmic environment (including radiations) in the flying body and space base, (1) Morphological and functional effects (including changes in gene expression) of raiations on human tissues maintained in super-SCID mice, (2) Microsatellite mutations and leukemia in the offspring of mice in the space environment, and (3) Protection of radiation-induced disorders by food and supplement, and effects of space environment (including micro-gravity) on human diseases are carried out by using specific mouse models.　The first two projects are ready to be carried out in the space environment.　As for Project 3, we demonstrated protection of leukemia and congenital malformations by AHCC (Active Hexose Correlated Compound) treatment, as it was done by macrophages activated by Pyran and BCG.　In humans, AHCC definitely helps cancer patients in reducing side effects of chemotherapeutic drugs, getting a sense of wellbeing and improved intake maintains general condition. Furthermore, AHCC protected radiation hazards by radiotherapy on the skin and mucous membrane of head and neck cancer patients

Key words; Space environment, Cosmic radiations, Human risk, Super-SCID-human mice, Transgenerational effects, AHCC (Active Hexose Corlated Compound), Prevention of cancer and malformation, Protection of radiation hazards, Radiotherapy and Chemotherapy

1. はじめに

人類は、将来、宇宙生活の必要性に迫られることが考えられ、最先端かつ安全な宇宙飛行技術の開発とともに、人類が宇宙生活を行うにあたり不可欠なのが、宇宙環境および宇宙放射線（宇宙基地、飛翔体内のヒト被曝の主たる放射線である中性子線）による人体影響、即ち、重力変化等の生体影響や忘れた頃に頭をもたげてくるがんや生活習慣病の防御である。宇宙環境（含、宇宙放射線）による人体影響の評価と防護研究のため、「宇宙環境の人体影響評価と防護に関する研究」研究チームでは、20 年以上にわたり、

1）SCID プロジェクト：ヒト臓器・組織機能を数年にわたり継代維持できる超重度複合免疫不全マウス（super-SCID マウス）を用い、ヒトがマウスを宇宙に運ぶのでなく、人体実験を避けるためマウスがヒト臓器・組織をおんぶして宇宙に運び、宇宙環境のヒト組織の形態、機能、遺伝子変異、遺伝子発現への影響を調べるための地上研究、
2）継世代プロジェクト：人類の宇宙での生活を余儀なくされた場合を考慮し、少数のＮ５♂マウスを宇宙に運び、帰還後正常♀マウスと交配し、多数の子孫を作成し、宇宙放射線等宇宙環境の次世代におよぼす影響、特に、がん、突然変異、発生異常の発生を調べる地上研究、
3）宇宙創薬プロジェクト：　がん等各種生活習慣病、情動行動異常等自然発症モデルマウスや安全性高感度検出モデルマウスを用いた宇宙環境（含、宇宙放射線）に対する生体反応と防護に関する地上研究を行ってきた。

これら研究の基盤は、我が国独自の発見、開発によるものであり、人類が宇宙環境利用、あるいは、

宇宙環境で生活するためには避けて通れない研究課題であり、宇宙生活や宇宙よりの帰還後を想定した地上研究を実施し成果を報告してきた。現在、我が国の哺乳動物個体の打ち上げ実験は中断しているため、Bersimbay 博士との共同研究を含め、いつでも宇宙実験が出来るよう常備体制を維持している。

上記 3 本柱の内、3 番目の疾患モデルマウスを用いた宇宙医学（創薬）研究のうち、重力変化に対する生体反応に関する研究は、パラボリックフライトでも一部目的が達せられる。医薬基盤研究所の野村プロジェクト特有の情動行動異常モデルマウスを用いμG による行動異常と遺伝子発現の変化に関する共同研究を三菱重工と行い、重力変化がパニック状態を増強すること、大脳、小脳に急激な遺伝子発現の変化をもたらすことを発見し第 27 回シンポジュウムでも報告した。本年度は、宇宙環境において、放射線による晩発障害（発生異常、がん等）に対する食品やサプリメントによる防護に関する地上研究の成果を発表する。福島原発事故の対策にもなりうる。

２． 放射線誘障害に対する防護研究

１）放射線防護剤

放射線防護剤の研究は Table 1 に示すごとく、古くからなされている。放射線の急性毒性を対象にしている。しかし、生体への毒性が強く、核戦争時での兵士の防護が主目的であった。また、放射線療法、化学療法の際に、使用することも考えられるが、正常組織への毒性があまりにも強すぎる。最近、CBLB502 という新たな防護剤が開発され、話題となっている。動物実験レベルでは放射線防護剤として急性毒性も少なく、核戦争時、放射線治療時、化学療法時の正常組織の放射線急性障害の防護に役立つのでないかと期待されている。しかし、CBLB502 そのものの晩発影響（がん等）も確認しておかねばならない。いずれにしても、宇宙放射線や、原発事故時の放射能汚染から正常人を防護するものは存在しない。

Table 1. Radioprotectors

Inhibition of Radiation Induced Disorders

"Radioprotector "

An agent that provides protection against the toxic biological effects of ionizing radiation, mostly to Acute Toxicity but not to Late Effects

Use for Nuclear War, Nuclear Accidents, and also Radiotherapy and Chemotherapy

Sulfhydryl compounds; Very toxic, can not use in human.

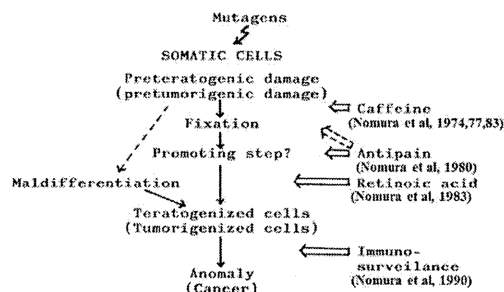
Amifostine (WR-2721); Toxic, can not use in normal human as radioprotector, but permitted to use for suppression of side effects of cancer sedatives (FDA, USA).

CBLB502; A novel radioprotector (Science, 2008). An agonist of TLR-5. May be usefull as radioprotector in normal animal tissue and nuclear war.

２）放射線の晩発効果（がん、発生異常等）の予防

原発事故当初に放出される放射性ヨウ素に対する非放射性ヨウ素剤の直前あるいは同時投与による放射性ヨウ素の甲状腺への取り込みを事前に抑えることにより、甲状腺の内部被ばくを抑え、甲状腺がんの発生を予防することは必須の処置であり有効である。

我々は、化学物質、放射線によるがん、発生異常発生に対応する予防法を研究してきた（Fig. 1, Table 2）。初期の損傷（DNA 損傷等）の修復課程、促進の抑制過程、（分化過程）、がん化、先天異常化細胞の監視過程である。最初に、紫外線や４NQO 誘発の突然変異が誤りがち修復を抑制する Caffeine により抑制されることから、損傷細胞が除去された結果として、がん、発生異常も抑えられることを証明した。しかし、アルキル化剤や放射線障害には全く無効かつかえって障害を増加させることがわかった。すなわち作用物質特異的で放射線障害には無効である。次に、プロテアーゼインヒビターの Antipain により、がんも発生異常も抑制することを証明した。また、Vitamin A, D, Retinoic Acid も抑制効果が確認されたが、放射線誘発のがん、発生異常には抑制効果はみられない。



Immune surveillance may kill or eliminate altered cells causing cancer and malformations and the lesion is replaced with reserved normal cells.

Fig.1. Bio-defense system to protect cancer and malformation (Nomura, 1985)

Table 2. Suppression of radiation-induced late effects (mutation, malformation, and cancer)

1. Error-prone repair inhibitors, *etc;*
Caffeine, Theophylin, Theobromin, Methyl-xanthines
(Nomura, Nature,1974,76, Cancer Res., 1977, 83)
Agent dependent!
Antipain (Protease Inhibitor, Nomura, Cancer Res. Etc, 1980, 1983)

2. Vitamins, *etc.*;
Vitamine A, C, D, Retinoic Acid (Nomura, Cancer Res, 1983), **Nicotinamide** (Gotoh, Nomura, Muta. Res., 1988, 93)

3. Immune-system;
Pyran, P. acnes, BCG, Activated-Macrophages, *etc.*
(Nomura *et al*, 1990), AHCC (Nomura *et al*, this study)

そこで、化学物質や放射線で変化した細胞（がん化した細胞、発生異常前駆細胞を含む）を除去する目的で、マクロファージの活性化物質あるい

は活性化マクロファージを投与、注射したところ見事に抑制・予防できた。この過程での効果はエラーフリーであり、実験的に放射線誘発発生異常の予防に成功した最初の例である。以下に、Pyran、BCG 前投与およびこれら物質で活性化したマクロファージの注入によりウレタンおよび放射線誘発発生異常を予防した例を示す(Figs. 2, 3, and 4)。

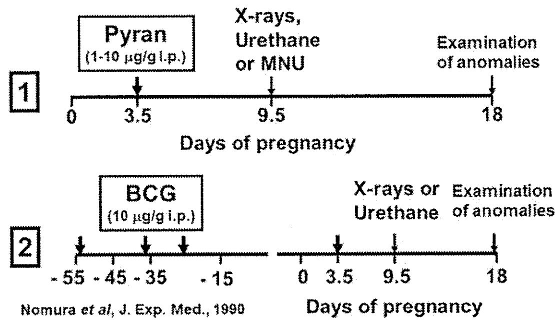


Fig. 2. Pyran or BCG pretreatment to prevent congenital malformations; experimental procedures.

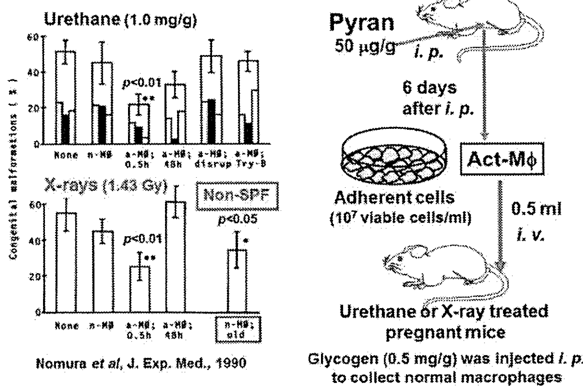


Fig. 3. Suppression of X-ray and urethane-induced malformation by Pyran-activated macrophages

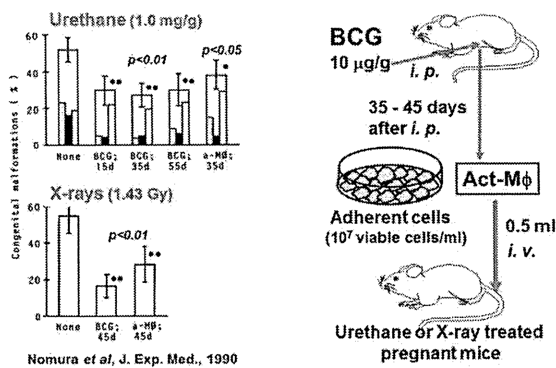


Fig. 4. Suppression of X-ray and urethane-induced malformation by BCG pretreatment and BCG-activated macrophages

3．ＡＨＣＣによる放射線誘発発生異常の予防

AHCC (Active Hexose Correlated Compound：担子菌菌糸体培養抽出物) は、Basidiomycetes mycelia polysaccharide immune enhancer であり、その主成分はαグルカンである。 AHCC (2%水溶液) を Pyran の場合と同様に、妊娠 3 日目と 5 日目 N5 マウスに前投与 (腹腔内注射) し、9 日目にガンマ線 1.4 Gy を全身照射した。妊娠 18 日目に帝王切開し、胎児の死亡、発生異常を調べた。放射線誘発発生異常は有意に抑制された (Figs. 5 and 6) 。

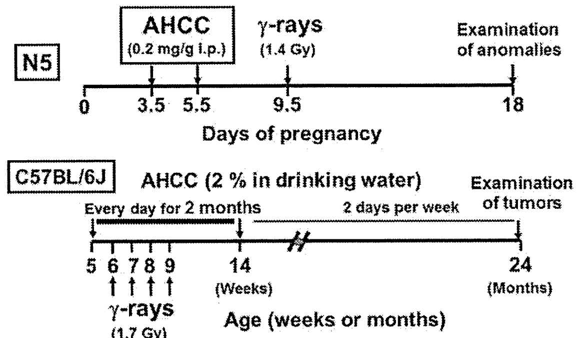


Fig. 5. AHCC pretreatment to inhibit radiation-induced malformation and tumors in mice; experimental procedure

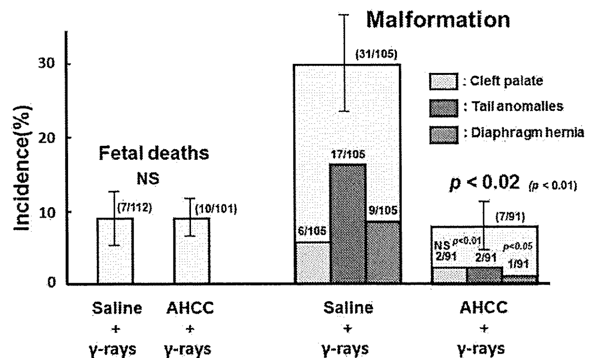


Fig. 6. AHCC pretreatment suppresses radiation-induced congenital malformation in N5 mice

4．放射線誘発腫瘍に対するＡＨＣＣの効果

放射線誘発白血病を高発する C57BL/6J/Nos マウスに ^{137}Cs ガンマ線 1.7 Gy を生後 6、7、8、9 週齢で 4 回照射し、2%AHCC 水溶液を連日 2 か月間経口投与、以降週 2 日間 24 週齢まで経口投与した。その結果、担癌マウス、白血病マウスは、AHCC 投与群では、AHCC 非投与群に較べ、有意に減少したことを第 27 回シンポジュウムで報告した。放射線誘発がんを抑制した最初の例である。自然発生腫瘍に対しても抑制効果が少し見られ、詳細な実験は不可能に近いが、低線量放射線によるがんに対しても有効であると思われる。

5．ヒト放射線障害に対するＡＨＣＣの効果

NEIGRIHMS (ノースイースタンインドラ・ガンジーヒューマンメディカルサイエンス地域研究所、インド)において、頭頸部癌患者の化学療法時に、

AHCC投与群(25例)と、非投与群(25例)を比較して、AHCC投与群の方が有意にQOL等の改善が見られた。ほとんどの患者で食欲の改善がみられ、輸血を必要とした12例がAHCC投与群では3例に減少、22例には、悪心・嘔吐、白血球減少など化学療法の副作用の明らかな減少が見られた（Int. J. Clinical Medicine）。

放射線療法患者各25例においても、AHCC投与群では、食欲不振の有意な減少とともに、放射線照射部位の皮膚の剥離、粘膜の炎症、すなわち正常組織への損傷が有意に低下した（Fig. 7）。

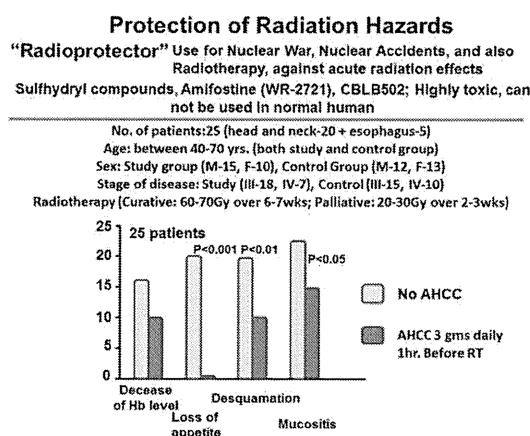


Fig. 7.　Protection of radiation-induced hazards by AHCC in human

謝辞：文科省科研費基盤研究 A、厚労省科研費、武田科学振興財団助成金、アミノアップ化学助成金、住友財団環境科学助成金、大阪癌研究会助成金および医薬基盤研究所創薬等モデル動物研究プロジェクト（野村プロジェクト）の支援を受けた。

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まだ生まれ来ぬ子孫を守れるのは 今、生きている我々自身

大阪大学名誉教授　野村　大成

7月末、林勝彦氏から福島原発事故に関し、京都フォーラムでの講演を依頼された。原発事故に関する講演依頼とメディアの要請は、安全を強要するものが多くすべて拒否していたが、林氏が「言いたい放題に言ってくれた方がよい」とのことだったので引き受けた。8月に入り、案内を頂いた時、「公共」という文字を見つけ一瞬身構えた。公共、国、公益という名目のもとに、いかに多くの犠牲者を出したか計り知れない。開催の前日になりフォーラムの事務局に問い合わせたところ、哲学、地学、NGO等いろんな分野から40数名が集まり、フリーディスカッションするとのことでした。初日は欠席し、夜半よりスライドと資料作りに専念し、やっと2日目の朝7時半になんとか用意できたので、会場にタクシーで駆けつけた。

2日目は、「福島原発事故を公共哲学する」ことであった。科学ジャーナリスト、元知事、JCO事故を救った住田先生の講演があり、討論が活発に行われ、途切れる時間もなく座長は打ち切るのに難儀していた。驚くべきことは、私が言いたい放題言ってやると身構えていたことを演者、討論者に先に言われてしまい、手も挙げられない状態であった。まさに善意、良心の塊のような発言がつづき、これでは討論にならないとも思った。そのうち、私の順番となり、「微量放射線の人体に与える影響」という事務局より与えられた題目で講演を始めた。〝問われる安全の哲学—良心〟というサブタイトルをつけた。原子力行政に対しては公共の哲学ではなく、〝安全の哲学〟という不可侵の哲学が必要であることを身で感じていたからである。

放射線の人体に与える影響は、歴史的、科学的事実に基づき、国際機関の勧告等を受け入れ規制してきた。放射線等のヒトへのリスクの約8割を占めるのが癌（2007年勧告では9割5分）であり、〝直ちの影響〟ではなく、数十年後に発生し、被ばく線量に対し直線的に増加する致死がんのリスクで決められている。ヒトでは100mSv以下で有意の増加の証拠がない。即ち〝微量放射線〟は科学的には未知の世界でもある。従って、国民の健康のために、より安全を保つようLNT（直線しきい値なし）モデルで、被ばく線量限度を決めた。御存じのように、公衆は1mSv、職業被ばくは20mSv／年（5年平均、1年最大50mSv）である。

このたびの東北大震災、特に福島原発事故に際しては、政府、メディア、それを取り巻く識者の発表には、Chernobyl、Sellafield、JCO事故等の経験は全く反映されておらず、長らく〝放射線の人体影響〟を研究してきた私には耐えがたい屈辱感があった。排出量、汚染濃度を示さず、〝直ちに健康に影響はない〟（少なくとも1000mSv以上でなければ直ちの影響は起こらない。放射線審議会のいう下血などは10000mSv以上）の政府、放射線審議会等の発表に終始し、大手メディア、学者までもがこれに追従、チェルノブイリ、JCOの経験者、原子炉工学、人体影響のエキスパートの名もみなかった。私は知らないが、戦前の情報統制とはこのようなものであったのか。原子力の安全神話のもとに、如何にしてこのような社会制度を築き上げたのか、公共哲学は検証しなければならない。

テレビ、新聞等の取材は、すべて拒否していたが、事故後1週間がたち、某通信社に放射線を知る旧友が残っていたので、思い切ってメモを送った。また、5月下旬には、衆院の震災対策プロジェクトチームの代表より講演依頼があり、〝言いたい放題言う〟を条件に引き受けたぐらいである。今回、分野を超えて〝安全の哲学〟を理解して頂く機会がもてたのは幸せだった。この言葉は、原子力の安全神話？（特に省庁再編後）のもとに原子力の安全性を審査する委員会（放射線審議会、原子力委員会、原子力安全委員会、原子力安全・保安院等）の〝独立委員会〟としての機能が失われたことにより、もんじゅ事故（1995年）、JCO事故（1999年）が続いた時に、未来の不安について言った言葉である。これは原子力に限ったことではないことは、翌22日の講演、討議を聞くとよくわかる。公害（鉱毒、有機水銀）でも同じことであった。箕浦教授は紀元前以来の津波被害の歴史を地質学的に解析し、今回の津波災害を予測した見事なデータを拝見した。まさに〝目からうろこ〟という状態で、住田先生いわく、〝10年前にこの事実を原子力委員会が知らされていたら、大震災、少なくとも原発事故は予防できた。〟自然災害、公害、原子力事故、全てにおいて多くの歴史的事故の証拠、記載が残っている。予知していながら、人類は災害を防止できなかったのか。

その本質を公共哲学は探る必要がある。原子力に関しては、原子力推進機関の中に安全委員会（原子力安全保安院）をおき、安全審査の独立機能をなくし、原子炉は安全とばかりに、国は東大を除き原子力工学を廃止した。また、原子力の人体影響を研究する放射線基礎医学講座も研究費のためなのか大阪大学を除いて自然消滅した。工、医ともに安全性の教育、研究の場を日本はなくしたわけである。これでは、原子力関係の事故が頻発したのも当然の結果である。なのに学者を含め、市民は誰も異議を唱えなかった。

ここではたと気付いたのは、いくら歴史的事実が存在しても、これを受け入れるのは人であり、その人を決めるのは誰であるかにより安全性は大きく揺らぐ。安全性に関しては、各省庁、企業の利害関係で決めるものでない。

私が環境、次世代研究の序文に必ず使う言葉がある。〝科学の進歩により、人類は多くの恩恵を受けた。それと引き換えに、地球環境は破壊され、この地に生を受けたか弱き生きものに多くの障害をもたらした。それを予知し、防御するのも人類文明の進歩でなくてはならない。〟また、〝まだ生まれ来ぬ子孫を守れるのは、今生きている我々自身である。〟

講演、討議に参加して、私のエッセイのタイトルを思い出し、講演の時に急遽配布した。英国セラフィールドにある核物質再処理工場（青森の六ヶ所村と同じ）の従業員の子供に白血病が多発するとGardner教授が発表（1990年）した。私の昔の研究（Nature, 1982）が主として引用されたので、Nature誌より依頼を受け論文を書いた。その論文に思いもしなかったタイトルがつけられていた。〝Of Mice and Men〟である（1990）。これはスコットランドの農民詩人Robert Burnsが、1785年11月の朝、畑を耕していて誤って二十日ネズミ（マウス）の巣を壊してしまった時にしたためた詩である。概略を以下に記した。

マウスに寄せて
—心地よい巣穴から
鋤で掘り起こしてしまった
1785年11月

俺は悲しくてならぬ—
人間どもがのさばって
自然界の互いの結び付きを
壊してしまったのが。
評判がひどく悪いのも
無理はない。
だからこそ
おまえは俺を見て、
びっくりしたのだ。
この地球上に生を享けた
命限りある
哀れな仲間同士なのに。

マウスにせよ、人間にせよ、
周到に立てた計画が
しばしば思った通りには
ならず
あてにした悦びにかわって
悲しみと苦しみだけが
残るのだ。

そして未来には—
はっきりとは見えぬが—
恐ろしいことを予感するのだ。

ロバート・バーンズ

マウスに寄せて（英語原文）

“To a Mouse”, on turning her up
in her Nest, with the Plough,
November, 1785

I'm truly sorry Man's dominion
Has broken Nature's social union,
An' justifies th' ill opinion,
Which makes thee startle,
At me, thy poor, earth-born companion,
An' fellow-mortal!

The best laid schemes o' Mice an' Men
Gang aft a-gley,
An' lea'e us nought but grief an' pain,
For promis'd joy!

An' forward, tho' I canna see,
I guess an' fear!

Robert Burns

当時、〝この世で最も小さなか弱い〟哺乳動物はマウスといわれていた。産業革命がもたらす障害をうたった時である。この〝Mice and Men〟は Wordsworth、Steinbeck、そして私の論文のタイトルにつけられた。環境保護の標語にも使われた。何故、人類は過ちを繰り返そうとするのか。歴史的事実、科学的事実が役立たなかったとすれば、今この地に生を受けた我々が、次世代のために対策をなしとげなければならないことを意味する。

第104回公共哲学京都フォーラム
「東日本大震災を公共哲学する」

日時：2011年8月20日(土)・21日(日)・22日(月)
場所：神戸ポートピアホテル　本館地下1F　生田の間

（敬称略）

○8月20日(土)

1. 日本の今昔（ソクサル）（内肌）
李秉冑　LEE Byung Yong（写真作家）
2. 公共性を模索する宗教—東日本大震災後の動向
木村敏明（東北大学大学院文学研究科准教授）
3. 災害と外国人—母国に「逃げる」ことを中心に—
郭基煥（東北学院大学経済学部准教授）
4. 公共哲学としての災害モニュメント
—東北関東大震災被災地を世界遺産に
芳賀満（東北大学高等教育開発推進センター教授）
5. ボランティアを通して見えてきた
『復幸』のキーワード
児島永作（復幸連絡会研究員）

○8月21日(日)

6. 科学報道を検証する
柴田鉄治（科学ジャーナリスト）
7. 原発と地方自治
佐藤栄佐久（元福島県知事）
8. 今後の原子力規制機構のあり方
住田健二（元原子力安全委員会委員長代理／大阪大学名誉教授）
9. 微量放射線の人体に与える影響　問われる安全の哲学；良心
野村大成（大阪大学名誉教授）
10. 脱原発は可能か
林勝彦（元NHKエンタープライズ21 EXプロデューサー）

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RESEARCH ARTICLE

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

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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

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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

[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_2PO_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.

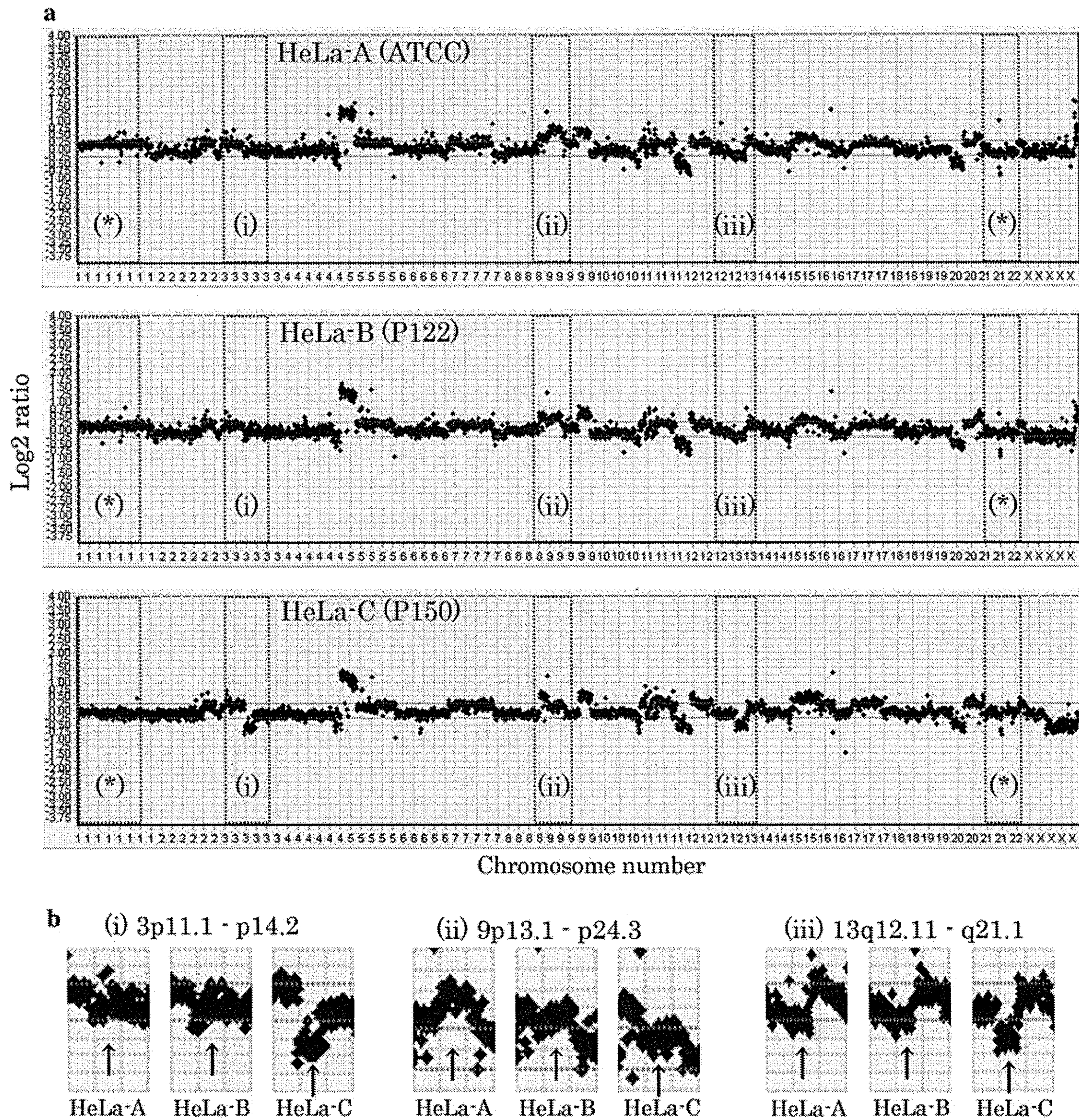

Fig. 1 BAC aCGH profiles of three HeLa cells. **a** *Upper panel* BAC aCGH profile of HeLa-A (ATCC), *middle panel* HeLa-B (JCRB, after 122 passages), *lower panel* HeLa-C (JCRB, after 150 passages). (*i*)–(*iii*) Correspond to **b**(*i*) to (*iii*), respectively. An *asterisk* indicates a tendency for 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

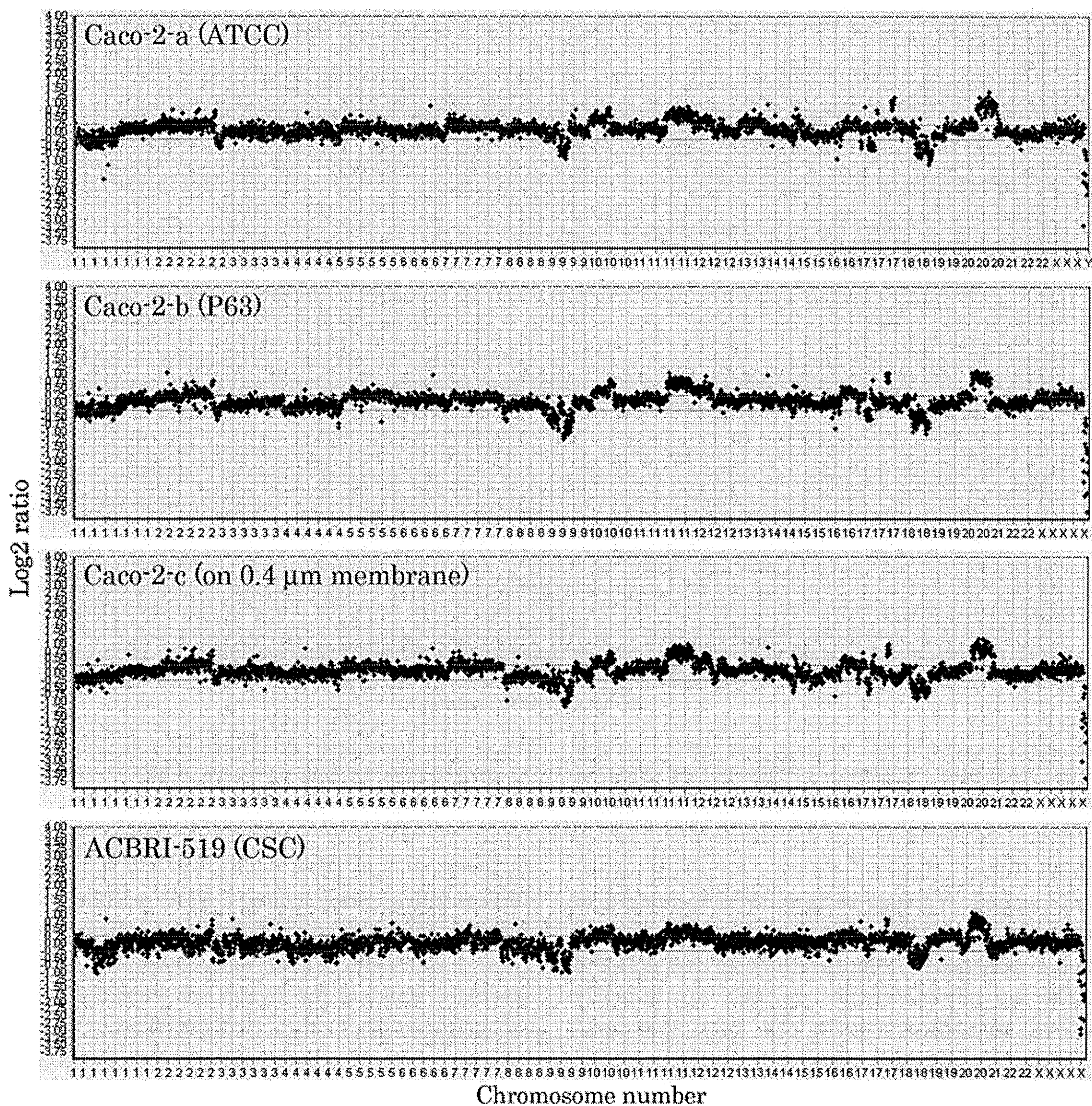

Fig. 2 BAC aCGH profiles of 3 Caco-2 cells and ACBRI-519 cells. Sequentially from the upper panel are the BAC aCGH profile of Caco-2-a (ATCC), -b (after 63 passages), -c (cultured on 0.4-μm diameter microporous membrane), and ACBRI-519 (Cell System Corp.)

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

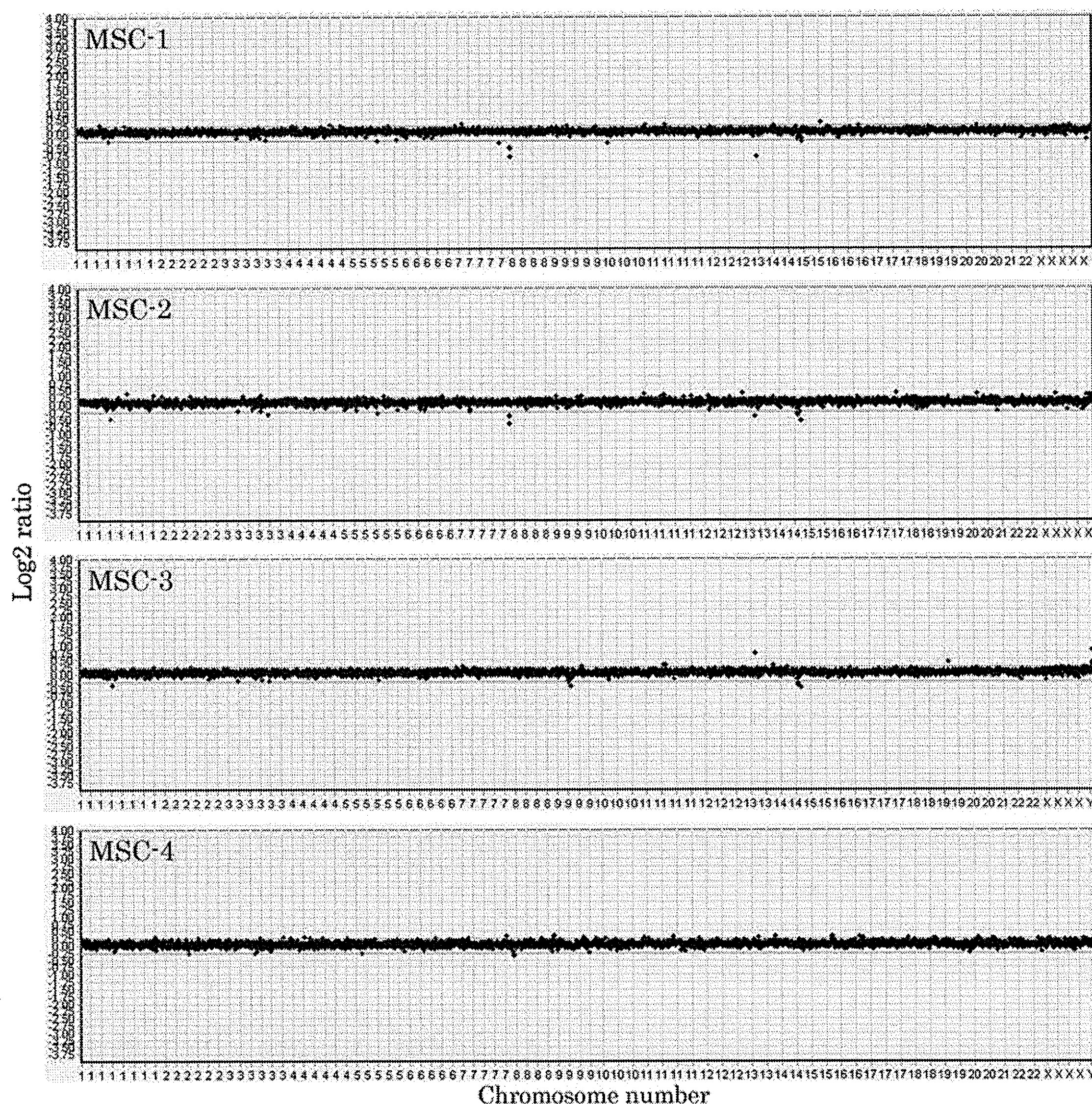

Fig. 3 BAC aCGH profiles of four clinically used MSCs. Sequentially from the upper panel the BAC aCGH profiles are shown of MSC-1 (69-year-old female after three passages), -2 (16-year-old female after 3 passages), -3 (34-year-old male after 4 passages), and -4 (derived from the same individual as MSC-3, 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.

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Growth factor-defined culture medium for human mesenchymal stem cells

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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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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

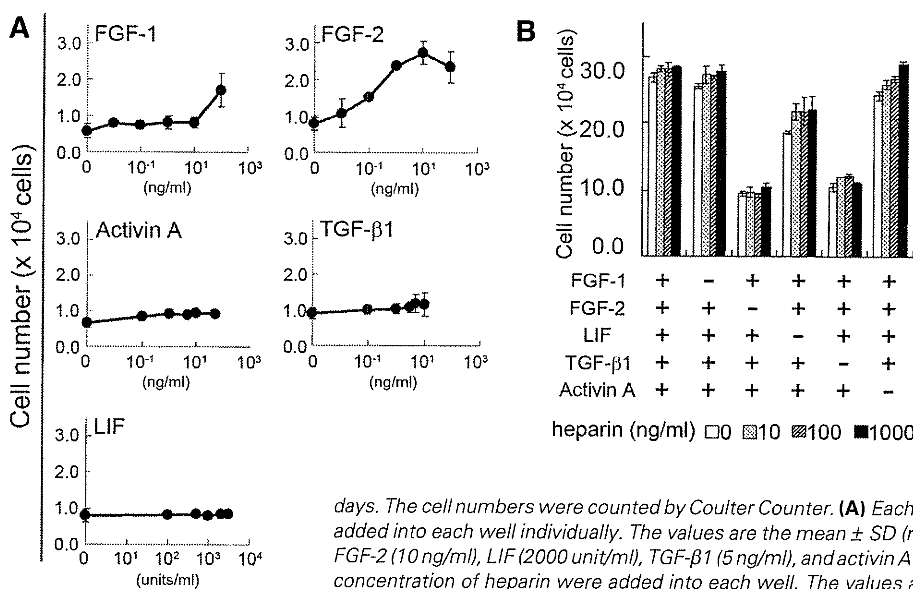

Fig. 1. Effect of growth factors on UE7T-13 cell proliferation in defined serum-free culture conditions. *After the UE7T-13 cell grown in the conventional culture conditions (POWERDBY10) were cultured in hESF9(-/-) overnight, the cells were seeded in a 24-well plate coated 0.1% gelatin in hESF9(-/-) 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).*

presence of Asc 2-P. These results suggested that Asc 2-P promoted differentiation of UE7T-13 cells into osteoblasts. We removed Asc 2-P from hESF10 medium for hMSCs, and designated the new formulation D-hESF10.

To confirm the characteristics of UE7T-13 cells expanded in the absence of serum, we performed flow cytometry with antibodies to markers for hMSCs and pluripotent cells (Fig. 3A). Cells grown in D-hESF10 medium were positive for CD44, CD73, CD90, CD105, and TRA-2-54 (tissue non-specific alkaline phosphatase antibody), but negative for CD45 (a marker of all hematopoietic cells) and CD56 (a neural cell adhesion molecule). We further stained the cells with antibodies to CD105 and SSEA-3 (Fig. 3B). The immunocytochemical analysis showed that SSEA-3$^+$/CD105$^+$ double positive cells were present in the UE7T-13 population grown in D-hESF10 although cells positive for either CD105 or SSEA-3 were also detected in the population. The cell growth rate in D-hESF10 was comparable to that in conventional culture conditions (Fig. 4).

We subsequently examined the properties of UE7T-13 cells serially passaged in D-hESF10 medium. The morphology of serum-free expanded UE7T-13 cell populations was comparably small, spindle-shaped cells compared with that in conventional medium (Fig. 5A). The expression of hMSC and hES cell pluripotency markers were determined by real-time PCR analysis (Fig. 5B) in UE7T-13 cells cultured for 4 passages in D-hESF10 medium. The expression of hMSC markers, *CD105*, *THY1*, and *integrin β1* (*ITGB1*), and the hES cell pluripotency markers, *OCT3/4* (*POU5F1*) and *NANOG* were similar in the cells cultured in D-hESF10 compared with those in the cells cultured in conventional culture conditions. *SOX2* expression was significantly higher in cells cultured in D-hESF10 compared with cells cultured in conventional culture conditions. On the other hand, the expression levels of *IBSP*, *BGP*, *SPOCK2*, and *SPP1* were significantly lower in cells cultured in D-hESF10 compared with those in the cells cultured in conventional culture conditions. These results suggest that serum-free expanded UE7T-13 cells retain an undifferentiated phenotype.

We determined the differentiation capacity of the serum-free expanded UE7T-13 cells. After the UE7T-13 cells were cultured in D-hESF10 for 7 passages, the cells were cultured in medium designed to induce differentiation into osteoblasts or adipocytes (Fig. 6). Culturing in osteoblastic differentiation medium induced the formation of nodules that stained positive with Alizarin red, suggesting that the cells had the potential to differentiate into osteoblasts. When the cells were cultured in

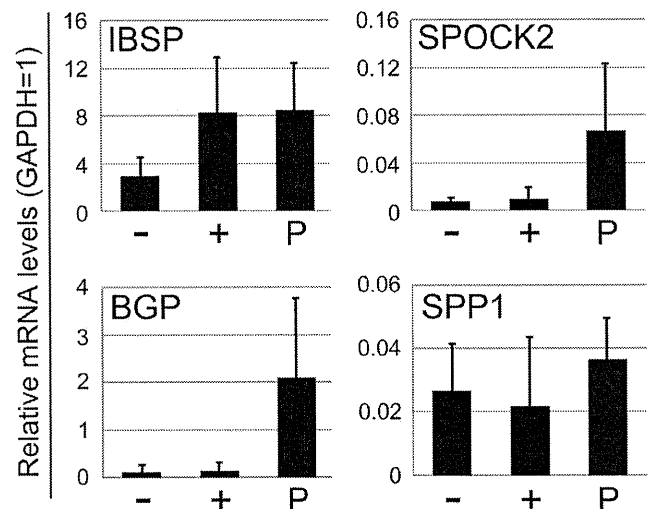

Fig. 2 (above). The effect of culture conditions on osteoblastic marker expression. *The gene expression in the cells cultured on gelatin in hESF10 without (-) or with (+) Asc 2-P for 6 days, in comparison with the cells grown in POWERDBY10 (P) was analyzed by the quantitative RT-PCR. The gene expression was normalized by the amount of* GAPDH. *The values are the mean ± SD (n=3).*

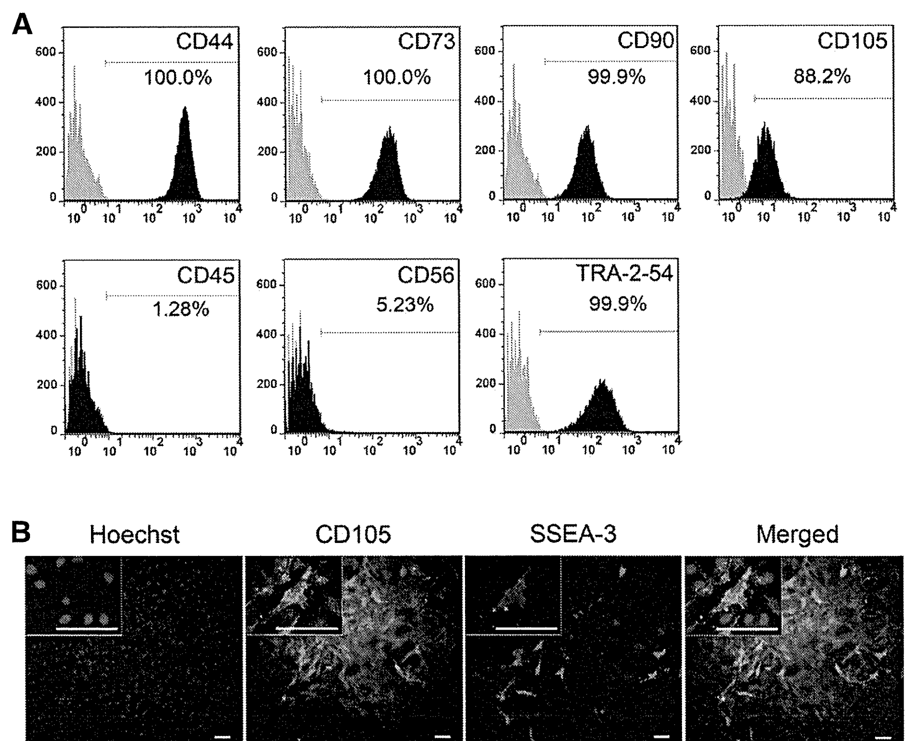

Fig. 3. Expression of hMSC markers in UE7T-13 cells. (A) *Flow cytometric profiles for CDs in UE7T-13 cells. hMSC marker expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days was analyzed by flow cytometric analysis. Antigen histogram (black); control histogram (gray); the horizontal bar indicates the gating used to score the percentage of antigen-positive cells.* **(B)** *Immunocytochemical analysis of SSEA-3 and CD105 expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days. Scale bars, 100 μm.*

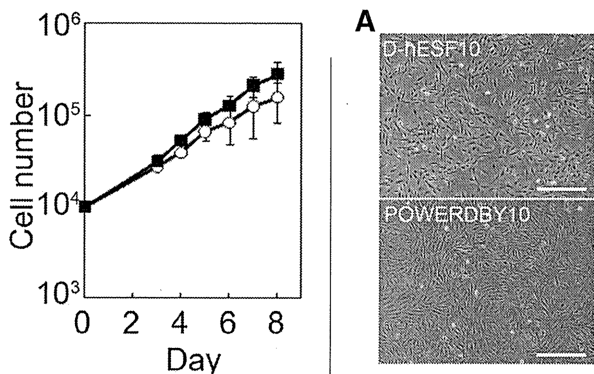

Fig. 4 (above left). A comparison of the growth of different UE7T-13 cells in the defined serum-free medium and conventional culture conditions. *The cells were seeded in a 24-well plate coated with gelatin in D-hESF10 (open circle), or in a 24-well plate in POWERDBY10 (closed square) at a cell density of 1x10⁴ cells per well. Cell numbers were counted every day. The values are the mean ± SD (n=3).*

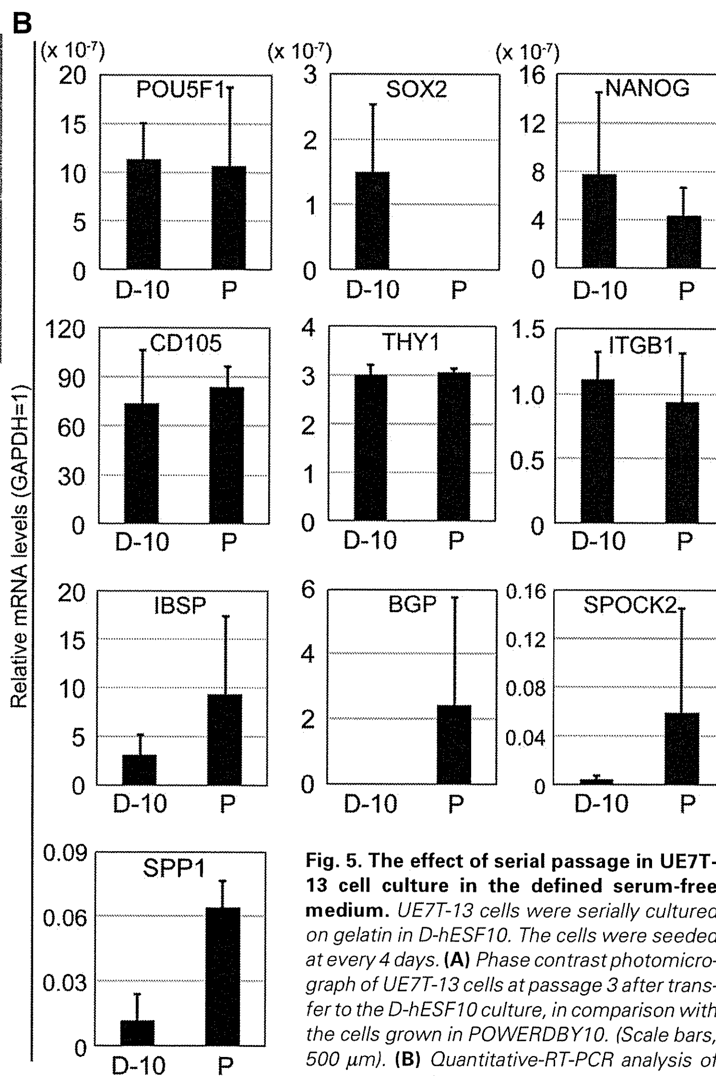

Fig. 5. The effect of serial passage in UE7T-13 cell culture in the defined serum-free medium. *UE7T-13 cells were serially cultured on gelatin in D-hESF10. The cells were seeded at every 4 days.* **(A)** *Phase contrast photomicrograph of UE7T-13 cells at passage 3 after transfer to the D-hESF10 culture, in comparison with the cells grown in POWERDBY10. (Scale bars, 500 μm).* **(B)** *Quantitative-RT-PCR analysis of gene expression in UE7T-13 cell cultured on gelatin in D-hESF10 at passage 4 (D-10), in comparison with the cells grown in POWERDBY10 (P). The name of each gene is noted in each bar graph. Gene expression was normalized with respect to* GAPDH. *The values are the mean ± SD (n=3).*

adipocytic differentiation medium, Oil red O-positive cells appeared. Taken together these results suggest that the serum-free expanded UE7T-13 cells have maintained the capacity to differentiate into osteoblasts or adipocytes.

Discussion

Developing clinical serum-free media for maintaining and expanding human stem cells is a major research topic in regenerative medicine. Our current results indicate that it is possible to culture hMSCs on gelatin in a defined medium, designated D-hESF10, in which human recombinant insulin, human transferrin, a low concentration of fatty acid-free bovine albumin conjugated with oleic acid, FGF-2, and TGF-β1 are the protein components. The basal medium ESF was developed for mouse ES cells (Furue *et al.*, 2005). For hES cell culturing, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) was removed from ESF but Asc 2-P was added (Furue *et al.*, 2008). For propagating hMSCs, Asc 2-P was removed from the hES cell culture medium because we found that Asc 2-P increased osteoblastic marker expression in hMSCs. These findings indicated that signaling by Asc 2-P in hMSCs is different from that in hES cells.

FGF-2 is a heparin-binding growth factor which stimulates the proliferation of a wide variety of cells. The biological activity of FGF-2 is efficient in the concentration range of 0.1 to 10.0 ng/ml. Addition of FGF-2 has been shown to increase the growth rate and life span of hMSCs from different species (Tsutsumi *et al.*, 2001; Benavente *et al.*, 2003), suggesting that FGF-2 play an important role in self-renewal of hMSCs. In hES cells, FGF-2 is a crucial to maintain the undifferentiated state (Amit *et al.*, 2004; Hoffman and Carpenter, 2005). We previously reported that FGF-2 at 10 ng/ml together with heparin supported the cell proliferation of hES cells in serum-free without feeders (Furue *et al.*, 2008). In this study, we found that FGF-2 at 10 ng/ml together with heparin supported the cell proliferation of hMSCs in a serum-free medium. These findings suggest that they share the same signal pathway to support self-renewal. Heparin at 1 mg/ml promoted hMSC cell proliferation, and we previously reported that heparin at 1 mg/ml inhibited hES cell proliferation. Thus the sensitivity to heparin is different between hMSCs and hES cells.

The TGF-β1 pathway has been reported to be important in hMSC differentiation into the osteogenic and chondrogenic lineages (Li and Xu, 2005; Kulterer *et al.*, 2007). While we have shown that TGF-β1 alone did not promote cell proliferation of hMSCs, the combination with FGF-2 and heparin enhanced cell proliferation of hMSCs. Chase *et al.* reported the combination of TGF-β1, FGF-2, and PDGF-BB in a commercial serum-free medium for the expansion of hMSCs although the optimal concentrations of these factors were not disclosed. The cell growth rate in D-hESF10 medium was similar with that in the conven-

tional culture conditions suggesting that addition of TGF-β1 and FGF-2 is sufficient to replace serum in supporting hMSC cell growth. A culture medium consisting of the minimum components necessary to support survival and proliferation would be beneficial to understand the characteristics of naïve hMSCs. Therefore, we think that addition of PDGF-BB is not crucial for an hMSC culture medium.

Several studies reported that two distinct cell morphologies are seen in early-passage hMSC cultures: small, spindle-shaped cells that are rapidly self-renewing and large, flat cells that replicate slowly and appear more mature (Mets and Verdonk, 1981; Colter *et al.*, 2001; Sekiya *et al.*, 2002). The morphology of serum-free expanded UE7T-13 cell population contained comparably small, spindle-shaped cells. However, specific undifferentiated markers of hMSCs have not been identified yet (Pochampally *et al.*, 2004). Further, although the cells are cloned, cells within an individual colony are heterogeneous in morphology, growth rates, and efficiency with which they differentiate (Mets and Verdonk, 1981; Bruder *et al.*, 1997; Colter *et al.*, 2001). The International Society for Cellular Therapy (ISCT) has proposed three criteria to define hMSCs (Dominici *et al.*, 2006). hMSC population must be positive at least for several antigens such as CD105, CD73, and CD90, and negative for CD45. CD105 is usually used to identify an hMSC population. Many studies reported that hMSCs also expressed hES cell pluripotency markers, SSEA-3, -4, NANOG, OCT3/4, and alkaline phosphatase (Pochampally *et al.*, 2004; Roubelakis *et al.*, 2007; Battula *et al.*, 2008; Conrad *et al.*, 2008; Pang *et al.*, 2010). We also detected the expression of NANOG, OCT3/4, and SOX2. These findings suggested that hES cell pluripotency markers may be universal stem cell markers in humans. Dezawa's group recently reported that double positive CD105 and SSEA-3 cells have the ability to generate multiple cell types derived from the three embryonic germ layers (Kuroda *et al.*, 2010). We also confirmed the existence of CD105 and SSEA-3 double positive cells in the hMSC population expanded in D-hESF10. In this study, we confirmed the differentiation potential of hMSCs to generate osteoblasts or adipocytes, but in the future we will examine the ability of hMSCs to generate cells from all three germ layers.

To facilitate the transition of human stem cell biology from basic research to clinical application all the components of maintenance and differentiation media should be publicly disclosed so they can be evaluated by many researchers. A commercial xeno-free serum-free medium for hMSCs was reported recently (Chase *et al.*, 2010). However, the non-disclosure of components is problematic as the medium formulation cannot be usefully modified or improved. Because all the components of D-hESF10 medium are disclosed here, the medium can be modified to study signaling pathways involved in maintaining multipotency and to develop differentiation protocols.

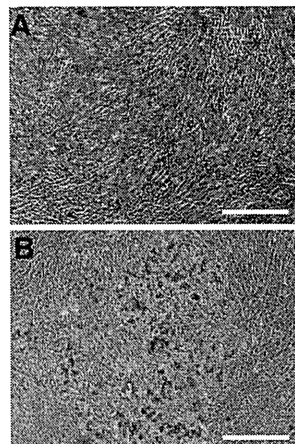

Fig. 6. The differentiation ability of UE7T-13 cell grown in the defined medium. *The UE7T-13 cells were serially cultured in D-hESF10 at passage 7, and then cultured in the differentiation medium.* **(A)** *Osteoblastic differentiation was induced in osteoblastic medium for 20 days. The nodules were stained with Alizarin Red S (red).* **(B)** *Adipocytic differentiation was induced in adipocytic medium for 24 days. The cells were stained by Oil red O staining (red). Scale bars: 500 μm.*

Materials and Methods

Cell Cultures

An immortalized hMSC line UE7T-13 (Mori *et al.*, 2005) (JCRB 1154, JCRB Cell Bank, Osaka, Japan) was used in this study. Cells were maintained on 100 mm dish (BD Falcon, Oxnard, CA) in POWERDBY10 (MED-SHIROTORI, Tokyo, Japan) that was also used in the experiments as a control medium. The cells were harvested with 0.25% trypsin in 1 mM EDTA-4Na.

Serum-free Cell Culture Media

hESF9 comprises ESF basal medium (Furue *et al.*, 2005) without HEPES supplemented with nine defined factors: Asc 2-P, 6-factors (human recombinant insulin, human transferrin, 2-mercaptoethanol, 2-ethanolamine, sodium selenite, oleic acid conjugated with fatty acid-free bovine serum albumin (FAF-BSA)), bovine heparan sulfate sodium salt, and human recombinant FGF-2 (Sigma, St. Louis, MO), as described previously (Furue *et al.*, 2008) (Supplementary Table 1). ESF basal medium without HEPES supplemented with Asc 2-P (hESF-GRO), and ESF basal medium without HEPES and Asc 2-P (hESF-DIF) were purchased by the Cell Science & Technology Institute (CSTI, Sendai, Japan). All other reagents were from Invitrogen (Carlsbad, CA) and Sigma. D-hESF10 medium consists of hESF-DIF medium supplemented with 6-factors, FGF-2, heparin, and TGF-β1 (R&D Systems, Minneapolis, MN). To harvest cells, 0.25% trypsin in 1 mM EDTA-4Na was used and the trypsin was inactivated with 0.1% soybean trypsin inhibitor (Sigma). For differentiation into osteoblasts or adipocytes, the cells were cultured according to the instruction by the suppliers (Lonza, Basel, Switzerland). The differentiated cells were stained by Alizarin Red S (Wako Pure Chemical Industries, Osaka, Japan) or Oil Red O (Wako).

Cell proliferation

Before the serum-free experiments, cells grown in POWERDBY10 were incubated by in hESF9 medium without heparin and FGF-2 (hESF9(-/-)) overnight to starve the effect of serum. Cells were replaced at the cell density of 1×10^4 cells /well on 24-well plate (BD Falcon) coated with 0.1% porcine gelatin solution (Millipore, Billerica, MA) and cultured in hESF9(-/-) medium in the presence of varying growth factors. The cell numbers were counted by Coulter Counter (Beckman Coulter, Hialeah, FL).

Gene expression

A detailed reverse transcription-polymerase chain reaction (RT-PCR) protocol was described previously (Furue, *et al.*, 2005). Total RNA was extracted from hMSCs using RNeasy Mini Kit (Qiagen, Hilden, Germany) and SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the provider's instructions. Q-RT-PCR was carried out using the TaqMan gene expression Master Mix on in ABI PRISM 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) according to the supplier's instructions (ABI). Specific primers-probe set were listed in Supplementary Table 2. Expression levels were all normalized by the expression level of *GAPDH*. The relative level of each gene in cDNA of undifferentiated hES cells was defined as "1." The KhES-3 cell line was used as a control; the cells were obtained from the Institute for Frontier Medical Science, Kyoto University, and the Review Board of the National Institute of Biomedical Innovation approved this research.

Antigen expression

For *in situ* immunocytochemisty, the cells were immunostained with antibodies, as described previously (Draper *et al.*, 2002; Furue *et al.*, 2008). In this study, fluorescence images were acquired using by IN Cell Analyzer 2000 (GE Healthcare, Buckinghamshire, England). Flow cytometry was performed with BD FACS Canto flow cytometer (Becton Dickinson, San Jose, CA) as described previously (Draper *et al.*, 2002; Furue *et al.*, 2008). In this study, the labeled primary antibodies were used, but the biding of anti-SSEA-3, anti-CD56, and Tra-2-54 antibodies was visualized with RPE-conjugated goat anti-mouse Ig (Dako, Carpinteria, CA) or Alexa Fluor 647 goat anti-rat IgM (Invitrogen). The primary antibodies used are listed in Supplementary Table 3.

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