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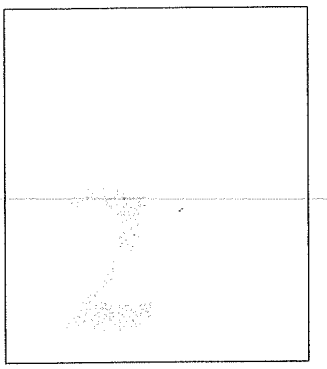

間葉系幹細胞の基礎

(2) 間葉系幹細胞の性質

加藤 幸夫* 久保 裕嗣** 清水 正和**
五十嵐 晃** 辻 紘一郎** 河本 健*
中島 歩*3

要旨 ヒト骨髄由来の間葉系幹細胞(MSC)は,多分化能をもち低酸素に耐性である.このようなMSCの特徴"stemness"は,一群の遺伝子によって支持されているかもしれない.またMSC特徴的遺伝子の発現は,MSCが存在する場所により部分的に変化すると思われる.顎骨由来のMSCは腸骨由来のMSCとはある程度異なる遺伝子発現パターンを示した.また脂肪,軟骨分化能の程度も異なっていた.そしてMSCは腎臓の再生にも役立つかもしれない.

<Key point>

stemness

組織特異的分化マーカー

多分化能

self-renewal

非対称分裂

I. "stemness"共通遺伝子は存在するか?

幹細胞のもっともはっきりした特徴は,組織特異的分化マーカーが発現していないことである.しかしこれでは幹細胞を規定できない.

幹細胞は,多分化能を潜在的に維持して,self-renewalの能力をもつ.またある状況では非対称分裂をして,一方の娘細胞のみが分化細胞となる.幹細胞がすべて分化してしまっては,幹細胞が体内から枯渇するからである.これらのプロセス(stemnessという言葉で表現されている)は,幹細胞では共通のメカニズムで制御されているかもしれない.またもしそうなら,すべての幹細胞で共通なstemness遺伝子が存在するかもしれない. 実際,2002年に二つのグループが,胚性幹細胞(ES細胞),造血幹細胞(HSC),神経幹細胞(NSC)で共通して発現している遺伝子をstemness遺伝子として報告した.しかし二つの報告で共通しているstemness遺伝子は数個しかなかった[1),2)].一方,別の

Key words: ステムニス,マイクロアレー解析,顎骨,腸骨,腎疾患

* 広島大学医歯薬学総合研究科口腔生化学 *3同 分子内科学(〒734-8553 広島市南区霞1-2-3)
** 株式会社ツーセル

グループによって皮膚上皮の幹細胞のstemness遺伝子も探求されたが，上記の幹細胞と共通して亢進している遺伝子の数は少数であった[3]．これまでの研究で，“stemness”遺伝子が同定できなかった理由は，純化した幹細胞集団を用いていなかったためかもしれない．あるいはmicroarray解析に技術的な問題があったのかもしれない[4]．

microarray解析

一方，われわれもmicroarray解析によって，ヒト骨髄由来の間葉系幹細胞（MSC）に選択的に高レベルに発現する遺伝子を同定したが（後述），これらのMSC特徴的遺伝子群と上記の幹細胞で発現が亢進している遺伝子との間で共通性は少なかった．したがって，すべての幹細胞に共通なstemness遺伝子があるのではなく，各幹細胞はそれぞれ特有のstemness遺伝子を有していると考えられる．たとえばES細胞では*Oct4*, *Nanog*, *Sox2*が未分化維持に必須であるが，他の幹細胞では，これらに代わる遺伝子があるのではないだろうか．

II. MSCの多分化能および各種の骨由来の骨髄MSCの性質の違い

骨髄でMSCは，多くが未分化なまま静的状態で維持される．しかし骨髄は出生後に発生，成熟するので，MSCはもともとは骨原基の周囲組織に存在したはずである．あるいは造血幹細胞のように遠い組織（肝臓）から骨髄へ移動したかもしれない．また出生して骨髄が完成した後，骨髄MSCの一部が，骨芽細胞，軟骨細胞あるいは脂肪細胞へと分化する．骨髄MSCがどのようなシグナルに応答して，どのような機構で分化するのか，また増殖と分化のバランスについても未だ不明な点が多い．さらにMSCは造血支持能をもち[5]，この能力と多分化能がどのように共存するのかも不明である．

造血支持能

一方で，すべての骨髄でMSCは共通であるかも不明である．顎骨由来のMSCは強力な骨分化能をもつものの，軟骨，脂肪分化能が低かった[6]．さらに腸骨，大腿骨，脛骨，顎骨由来の骨髄MSCの遺伝子プロファイルを比較したところ，大多数のMSCマーカーはこれらで共通していたものの，数個の遺伝子が顎骨MSCとそれ以外のMSC（腸骨，大腿骨，脛骨由来）で有意に異なる発現パターンを示した[7]（五十嵐ら，投稿中）．顎骨MSCは外胚葉の神経冠由来であるのに対して，腸骨，大腿骨，脛骨MSCは中胚葉（体節）由来と考えられるので，起源の違いが性質の違いを規定しているのかもしれない．

遺伝子プロファイル

外胚葉

中胚葉

また骨膜，軟骨膜，滑膜，脂肪に存在する骨，軟骨分化能をもつ細胞と骨髄MSCとの関係も不明である．これらのMSC様細胞は，由来組織の違いにより分化能の程度が異なる．たとえば滑膜由来MSCは軟骨分化が顕著であり，脂肪由来MSCは脂肪分化能が高いとされている．

滑膜由来MSC

脂肪由来MSC

一方，骨髄MSCは，少なくとも試験管内では，筋芽細胞，神経様細胞，肝

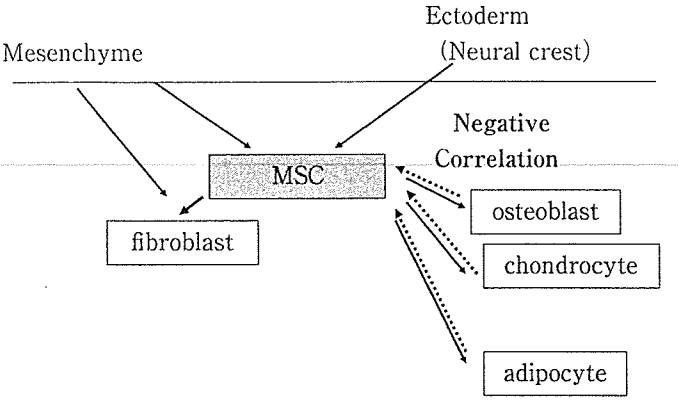

図　MSC system

MSC と線維芽細胞の発現プロファイルは類似してるが，MSC のみが分化スイッチをもっている．また MSC の遺伝子発現プロファイルは可逆的に変換しうる．つまり可塑性が大きい．

セメント細胞

臓様細胞へも分化した[8]．また歯周組織欠損部へ移植すると，セメント細胞，歯根膜細胞へも分化した[9]．このような予想外の細胞への MSC の分化は，分化転換（transdifferentiation）かもしれないもののメカニズムとして興味深い．これらの疑問を追究するために，われわれは，ヒト骨髄 MSC の遺伝子発現プロファイルを検討した．

III. MSC の発現プロファイル

DNA microarray

MSC，線維芽細胞，MSC 由来の骨芽細胞，軟骨細胞，脂肪細胞の全遺伝子（約 54,000 プローブ）の発現プロファイルを DNA microarray を用いて解析すると，意外なことに MSC は線維芽細胞ともっともよく似ていた．つまり両者で弱いものの有意な正の相関があった．一方，MSC は骨芽細胞，軟骨細胞，脂肪細胞とは有意な負の相関があった（図）．つまり全体としていうと，MSC で発現が亢進している遺伝子は，分化すると低下して，MSC で抑制されていた遺伝子は，分化すると発現が亢進するものが多いことが示唆された．なお骨芽細胞と軟骨細胞はもっとも高い相関係数を示した．これはともに石灰化基質を大量に産生するからかもしれない．

相関係数

IV. MSC の発現プロファイルは系列決定の前の中立的な位置を維持している

全遺伝子の発現レベル

MSC の遺伝子発現パターンの特徴を捉えるために，MSC で上方，あるいは下方制御されている遺伝子を同定する方法がよくとられている．しかしわれわれはそれに加えて，MSC での全遺伝子の発現レベル（中間値を基準にした相対

値）の平均値，SD，範囲，および遺伝子発現レベルの分布曲線（各発現レベルに対応する遺伝子数をプロットしたもの）の尖度（kurtosis：ピークの裾野の広がりの程度を示す）および歪度（skewness：正規曲線からのゆがみ度を示す）を計算することで，分化前／後を比較した．MSCと各種分化細胞とは，平均値はほとんど同じであるものの，範囲とSDは最小であった．さらにkurtosis，skewnessも小さい数値を示した．分化すると，各遺伝子は中間値よりも上方あるいは下方制御され，その分化系列に独特の遺伝子発現レベルの分布曲線を示すようになる．MSCで範囲，SD，kurtosis，skewnessが最小であったことは，MSCでは各遺伝子の上方，下方発現が低レベルであり，転写調節システムが分散と歪みの小さい中立位置にあることを示唆している．

転写調節システム

なお分化後にSDが増加する原因遺伝子を追究するために，機能別に遺伝子を分類した後でその遺伝子群の発現レベルの平均値とSDを計算した．軟骨，骨および線維芽細胞では細胞外基質カテゴリーの遺伝子の平均値とSDが増加し，脂肪ではエネルギー代謝カテゴリーの遺伝子の平均値とSDが著明に増加していた．一方，MSCではすべてのカテゴリーでSDが低かったが，転写因子のカテゴリーでも遺伝子の発現レベルのSDが分化細胞と比較して最小であった．このことからも，MSCでは転写調節が最小限にしか行われていないことが判明した．

細胞外基質
エネルギー代謝
転写因子

V. 腎疾患のMSCによる治療は可能か？

1998年にヒトES細胞のクローンが樹立され[10]，ES細胞を用いた臓器再生への期待が高まった．しかしES細胞は自己の細胞でないため，たとえ臓器が作られたとしても，免疫抑制剤が必要である．これに対して，自家MSCであれば拒絶反応がない．また腎臓は中胚葉由来の臓器であるため，骨髄MSCを用いた腎臓再生が可能かもしれない．しかし腎臓は複雑な組織構造をもつので尿細管上皮やメサンギウム細胞を再生しても，腎臓全体を再生することにはならない．

拒絶反応
尿細管上皮
メサンギウム細胞

それにもかかわらず，MSCを用いた腎疾患の治療についての報告が最近になって出始めた．Kaleらは急性尿細管壊死モデルにおいて，骨髄中の$Lin^{-}Sca\text{-}1^{+}$の細胞が尿細管細胞の再生に寄与することを報告した[11]．このほかにも虚血再還流モデルを用いて骨髄由来幹細胞の寄与を示唆した報告もあり，MSCを経静脈的に投与した群で病態の改善が報告されている．しかしMSCが尿細管細胞へ分化したというよりもMSCがもつ抗炎症効果によって病態が改善したようである[12]．

急性尿細管壊死モデル

一方，Yokooらは，尿管芽が発芽する時期のラット胎児を取り出し，試験管

内で全胎培養を行い，さらにこの胎児より後腎原器を取り出して6日間器官培養した．つまり子宮外で尿管芽が発芽する前のステージから成熟腎臓を形成させる実験系を確立した．さらに尿管芽が発芽する直前の中間中胚葉はglial cell line-derived neurotrophic factor（GDNF）を発現しているため，上記の系にGDNFをアデノウイルスによって強制発現させたMSCを移植すると，移植されたMSCが腎臓の各種の細胞へ分化してネフロンや周囲の間質が形成されることを示した[13]．この観察は，腎臓疾患にもMSCが役立つ可能性を示している．

中間中胚葉

GDNF

わが国の透析患者は24万人を超え，毎年3万人が新規の透析導入となっている．正常腎の10%の機能が回復されれば，これらの患者は透析から離脱できる．さらに透析患者での，二次性副甲状腺機能亢進症に伴う骨病変，閉塞性動脈硬化症などの合併症の治療にもMSC移植が有効かもしれない．

透析患者

VI. 骨髄MSCのその他の性質

骨髄MSCは，増殖能，低酸素耐性能，多分化能，造血支持能をもつとともに，分化シグナルに高感度で応答するスイッチ機能をもつ．低酸素に関連して，HIF1の標的分子であるDEC1，DEC2，LOXL2，LOX[14),15)]はMSCで5～10倍もの高レベルに発現している．増殖能以外についてはMSCと線維芽細胞とは明らかに異なる．骨髄MSCの増殖能はむしろ線維芽細胞よりも低かった．そして増殖を抑制する方向に作用するp16などの癌抑制遺伝子の発現が高レベルであった[16]．また線維芽細胞は，MSCへと変換できないのに対して，骨芽細胞，軟骨細胞および脂肪細胞は，少なくとも特殊な培養条件下では，MSCへ回帰することができる．このようなMSCの幹細胞としての特徴を支持する遺伝子の解明が今後の課題である．

低酸素耐性能

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Summary

Characterization of bone marrow mesenchymal stem cells

Yukio Kato*, Hiroshi Kubo**, Masakazu Shimizu**, Akira Igarashi**, Koichiro Tsuji**, Takeshi Kawamoto*, Ayumu Nakashima*3

Bone marrow mesenchymal stem cells (MSC) have multi–lineage differentiation potential and are resistant to hypoxia. MSC activity may be supported by "stemness" genes, whose expression is modulated by the *in vivo* location of MSC. Jaw–derived and ilium–derived MSC showed different gene expression profiles and different chondrogenic and adipogenic potentials. MSC may be useful for regenerative medicine to treat some renal diseases.

Key words：stemness, microarray analysis, jaw, ilium, renal diseases

**Department of Dental and Medical Biochemistry,*
*3*Department of Molecular and internal medicine Graduate School of Biomedical Sciences, Hiroshima University*
***TWO CELLS, Co., Ltd.*

Complete removal of HIV-1 RNA and proviral DNA from semen by the swim-up method: assisted reproduction technique using spermatozoa free from HIV-1

Shingo Kato[a], Hideji Hanabusa[c], Satoru Kaneko[d], Koichi Takakuwa[e], Mina Suzuki[e], Naoaki Kuji[b], Masao Jinno[f], Rie Tanaka[a], Kenichi Kojima[c], Mitsutoshi Iwashita[f], Yasunori Yoshimura[b] and Kenichi Tanaka[e]

Background: Use of antiretroviral drugs has reduced the mortality rate for HIV infection and many HIV-discordant couples wish to have children. It is possible for an HIV-infected man to father children without risk of HIV transmission if HIV-free spermatozoa can be obtained from his semen.

Methods: An improved swim-up method was used to collect HIV-free spermatozoa from the semen of HIV-positive males. Diluted semen was layered over a Percoll solution with a continuous density gradient of 30–98%, and then centrifuged. The bottom layer was collected by cutting the end from the tube and the sperm suspension was collected using the swim-up method. Spermatozoa were tested by nested polymerase chain reaction (PCR) for HIV-1 RNA and DNA, with a detection limit of one copy. Spermatozoa were used for assisted reproduction in 43 couples.

Results: HIV-1 RNA and proviral DNA were not detected by nested-PCR assay in all 73 of the collected spermatozoa samples from 52 patients. The HIV-1-negative sperm was used for *in vitro* fertilization in 12 couples and for intracytoplasmic sperm injection in 31 couples. No detection of HIV-1 RNA or proviral DNA in the culture medium of the fertilized eggs was confirmed again before embryo transfer. Of the 43 female partners, 20 conceived and 27 babies were born. HIV antibodies, HIV RNA and proviral DNA were negative in all of the females and babies.

Conclusions: HIV-negative spermatozoa could be obtained from semen of HIV-positive men. The method involves no risk of HIV transmission to female partners and their children.

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Keywords: assisted reproductive techniques, HIV-1, sperm, HIV-free spermatozoa

From the [a]Department of Microbiology and [b]Department of Obstetrics and Gynecology, Keio University School of Medicine, Tokyo, the [c]Department of Hematology, Ogikubo Hospital, Tokyo, the [d]Department of Obstetrics and Gynecology, Tokyo Dental College, Ichikawa City, Chiba Prefecture, the [e]Department of Obstetrics and Gynecology, Niigata University, Ichibancyo Asahimachidoori Niigata City, Niigata Prefecture and [f]Department of Obstetrics and Gynecology, Kyorin University, Mitaka City, Japan.

Correspondence to Dr Hideji Hanabusa, Tokyo Ogikubo Hemophilia Center and Department of Hematology, Ogikubo Hospital, 3–1–24 Imagawa Suginami-ku, Tokyo 167–0035, Japan.

E-mail: hanabusa@muh.biglobe.ne.jp

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Introduction

Since the mid-1990s, the use of HAART has spread, and the AIDS mortality rate has decreased by more than 80% in the industrialized world [1]. HIV infection/AIDS is becoming a controllable chronic infection and HIV-infected individuals are now living longer. Many HIV-positive people are getting married and wishing to have children.

Semprini *et al.* [2] reported that they had conducted artificial inseminations in more than 2000 HIV-discordant couples (HIV-positive male and HIV-negative female) using their swim-up method, and that no HIV transmission was observed. However, their method may be suboptimal because it has not been proven to remove HIV RNA completely, and they did not measure proviral DNA in infected cells in the semen. Zhang *et al.* [3] reported that HIV may be present as proviral DNA in seminal cells in HIV-infected men who have achieved undetectable levels of viral RNA in plasma with HAART, and this HIV could be capable of sexual transmission. It has not been determined whether HIV is attached to spermatozoa or whether spermatozoa can be infected with HIV [4,5]. Therefore, contraception is recommended for HIV-discordant couples, even if HIV RNA is undetectable in plasma [3].

Authorities in different countries have different opinions concerning the use of assisted reproductive technology using spermatozoa collected by the swim-up method [6–8]. However, it would be possible for an HIV-infected man to father children without risk of HIV transmission if HIV-free spermatozoa can be obtained from his semen. This study examines an improved swim-up method for isolating HIV-free sperm and its use in assisted reproductive methods.

Methods

This clinical study was approved by the ethics committees of Niigata University, Ogikubo Hospital, Keio University and Kyorin University. All of the couples visited the Hematology Department of Ogikubo Hospital and received counselling and explanations of the clinical study. Informed consent was obtained from all participating couples. Semen was obtained by masturbation, and then tested for sperm concentration, motility and deformity.

Percoll preparation

An isotonic solution of Percoll (Amersham Life Science, Tokyo, Japan) was made by dissolving 980 g Percoll in 10.0 ml 2.0 mol/l Hepes-NaOH, pH 7.4, 10.0 ml human serum albumin (25%w/v), 0.05 g fosfomycin and 0.05 g cefarotin. The resulting 98% Percoll solution was sterilized with a Millipore filter (0.45 μm pore size).

Semen pretreatments

The procedure is shown in Fig. 1. Ejaculates were diluted twice with Hanks solution, followed by standing in a test tube for 10 min to precipitate filterable micro-calculus, then filtered through an ART filter (20 μm clearance; ART filter, Nipro, Osaka, Japan) to remove fibers, micro-calculus and mucinous debris. The upper phase of sperm suspension was loaded onto 6 ml Percoll linear gradient from 98% to 30% in a separable fine-neck tube (Nipro) and centrifuged at 400 × *g* for 30 min. The separable fine-neck tube was made of glass, and its bottom was squeezed to minimize the volume of sediment. To recover the sperm precipitated in the bottom tip, the top of the tube was plugged with a rubber cap, and the middle of the squeezed bottom was snapped off with an ampoule cutter.

Motile sperm were separated by the modified swim-up method. A fine glass capillary was inserted in 2 ml of the medium in a vial, then a needle tip was introduced to the bottom through the inner capillary. The motile sperm were allowed to swim up at 37°C in an incubator with 5% CO_2–air. After 60 min, 1 ml of upper layer was collected, containing the sperm that had swum up.

The sperm suspension was divided into two portions. One was used for HIV assessment, and the other was cryopreserved with KS-II medium [9] in a liquid nitrogen container.

Standard HIV-1 materials

MOLT-4 cells infected with HIV_{LAI} and its culture supernatant were used as standards for HIV-1-infected cells and virus stock, respectively. RNA purified from virus stock and the pNL4-3 plasmid [10] were the standards for HIV-1 RNA and DNA, respectively. The concentrations of the standard HIV-1 DNA and RNA were determined by spectrophotometry and the null-class equation of the Poisson distribution of the reverse transcriptase (RT)-nested polymerase chain reaction (PCR). Cells were counted using a Burker–Turk hemocytometer (Emergo, Landsmeer, the Netherlands). The virion concentration was considered to be half the virus RNA concentration.

Detection of HIV-1 RNA and DNA

The samples of sperm suspension, culture medium or plasma were centrifuged at 35 500 × *g* for 1 h at 4°C. RNA and DNA were extracted from the precipitate using QIAamp UltraSens Virus Kit (Qiagen, Tokyo, Japan). One fourth of the eluate was tested in quadruplicate by RT-nested PCR as follows. The RT reaction was performed by incubation at 42°C for 10 min in a

) µl solution consisting of 1× PCR buffer II (10 mmol/l ris-HCl pH 8.3, 50 mmol/l KCl; Perkin Elmer Life ciences, Yokohama, Japan), 3 mmol/l $MgCl_2$, .2 mmol/l each dNTP, 0.1 µmol/l primer GA1R 5′-CCCAGGATTATCCATCTTTTATAG-3′, 1595–572 [10]), 4 U RNasin (Promega, Tokyo) and 20 U uperScript II (Invitrogen, Tokyo, Japan). The whole RT roduct was subjected to a first-round PCR in a 50 µl olution consisting of 1× PCR buffer II, 4 mmol/l $MgCl_2$, 0.2 mmol/l each dNTP, 0.2 µmol/l primers GA1F (5′-TGTTAAAAGAGACCATCAATGAGG-3′, 388–1411) and GA1R and 0.5 U AmpliTaq (Perkin-Elmer). Then, 1 µl of the first-round PCR product was used in the second-round PCR in a 50 µl solution containing primers GA2F (5′-GGCCAGATGAGA-GAACCAAGG-3′, 1465–1485) and GA2R (5′-CATCCTATTTGTTCCTGAAGGGTAC-3′, 1535–1511) and the other components in first-round PCR. The primers were located in *gag* p24. The thermal profile of PCR in GeneAmp PCR System 9700 (Applied Biosystems, Tokyo, Japan) was 94°C for 2 min; three cycles of 94°C for 5 s,48°C for 10 s and 72°C for 15 s; 22 cycles of 94°C for 5 s, 60°C for 10 s and 72°C for 15 s; with a final cycle of 72°C for 1 min and then the mixture kept at 4°C. The PCR products were electrophoresed through a 2.0% agarose gel in the presence of 0.5 µg/ml ethidium bromide and photographed under ultraviolet illumination. Throughout the procedure, the medium used for washed sperm or fertilized eggs was the negative control and this medium with 10 virions added was the positive control. The whole process took approximately 5 h. For samples of peripheral blood mononuclear cells (PBMC), DNA was extracted using QIAamp DNA Kit (Qiagen) and 0.5 µg of the DNA was tested in triplicate by the PCR procedures omitting reverse transcription. Competitive RT-nested PCR was performed as previously described [11].

Infectivity of HIV-1 during incubation

After incubation at 37°C under 5% CO_2 for various periods, the virus stock was added to 5×10^6 stimulated donor PBMC in 1 ml RPMI 1640 medium supplemented with 30% immobilized fetal calf serum and 70 U/ml human recombinant interleukin 2 (Shionogi, Osaka, Japan), and further incubated for 5 days. The culture supernatants were tested for p24 concentration with VIDAS HIV P24 II (BioMérieux, Tokyo, Japan).

Clinical study

If the HIV-1 testing for virion RNA and proviral DNA was negative, the other portion of frozen sperm was thawed for use in assisted reproduction. Mature eggs were obtained by means of ovulation-inducing drugs, and then placed in a dish containing 3 ml RPMI culture medium (20% albumin). The HIV-1–negative sperm solution was introduced to eggs by means of *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), and the dish containing treated eggs was incubated at 37°C under 5% CO_2 for 48 h. Before embryo transfer, the culture medium for the fertilized egg was collected and tested for the presence of HIV-1 again. Only when HIV-1 RNA and DNA assays by nested PCR were negative was embryo transfer conducted. All the female partners who underwent assisted reproductive therapy, even those who did not conceive successfully, were tested for HIV antibodies, HIV-1 RNA and proviral DNA in the blood at 1 and 3 months after the assisted reproductive technique and after delivery. The babies were tested for HIV RNA and proviral DNA in umbilical cord blood at birth and in blood until 6 months after birth.

Results

Sensitivity of the HIV-1 RNA/DNA test

The procedure to detect a single copy of either HIV-1 virion RNA or proviral DNA in sperm suspensions (the HIV-1 RNA/DNA test) was developed by selecting and improving techniques in three main steps (collection of HIV-1 virions and infected cells by centrifugation, extraction of viral RNA and DNA with silica-gel-membrane technology, and the detection of the viral RNA and DNA by nested PCR) to achieve zero apparent loss in recovery at each step. First, the exact virion concentration of the standard HIV_{LAI} virus stock was determined by direct RT-nested PCR at endpoint dilution by using the null-class equation of the Poisson distribution. Then, one virion of HIV_{LAI}, on average, was added to 1 ml Sydney IVF medium (Cook, Tokyo, Japan) and the whole procedure was initiated. When one fourth of the eluate from an extraction column was examined (replicated four times) with RT-nested PCR, 12 of

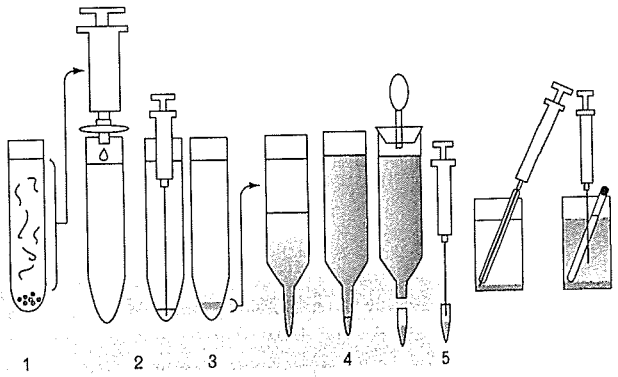

Fig. 1. Revised swim-up method to remove HIV completely. (1) The semen is diluted and debris allowed to precipitate. (2) The suspension is filtered, 0.1 ml Percoll added to the bottom, and the tube is centrifuged. (3)The sperm sediment is layered onto a linear gradient of Percoll (30–98%). (4) After centrifugation, the sediment is recovered by cutting the tube. (5) The sperm suspension is introduced into the bottom of the culture medium using a microtube. (6) The sperm that swim up are recovered.

20 samples exhibited at least one band in four reactions (Fig. 2). Next, a single MOLT-4 cell chronically infected with HIV_{LAI} was added to 1 ml Sydney IVF medium and subjected to the HIV-1 RNA/DNA test without reverse transcription. RT-nested PCR showed that 6 of 10 samples exhibited positive reactions. The ratios of positive reactions for virions (60%) and infected cells (60%) were in close agreement with that predicted from the Poisson distribution (63%), providing evidence that the protocol has the ability to detect RNA/DNA in a single virion as well as in a single infected cell when present in as much as 1 ml of IVF medium. To study the influence of the presence of sperm in the medium on the sensitivity of the test, two sets of five samples containing 0.5, 1, 2, 4 and 8×10^6/ml spermatozoa in Sydney IVF medium were tested; one set was mixed with 50 virions and the other set with 100 infected cells. The numbers of virion RNA and proviral DNA from sperm-containing samples that were determined by competitive PCR varied in the range 75–112 copies (note two RNA copies/virion) and 96–122 copies, respectively, in a manner that was not dependent on the sperm quantity. These results strongly suggest that the protocol can detect a single virion or infected cell even in the presence of up to 8×10^6 spermatozoa per sample.

Removal of HIV-1 virions and infected cells from mixed semen by sperm-washing

To assess the efficiency of sperm-washing procedures with Percoll density gradient centrifugation and swim-up for removal of HIV-1 from semen, HIV-1 virions or HIV-1–infected cells were added to healthy donor semen. When 2×10^7 virions HIV-1 were mixed with 1.6 ml healthy donor semen containing 6.3×10^7 spermatozoa/ml, 63 copies of HIV-1 RNA were detected after centrifugation but no HIV-1 RNA was detected after swim-up. When 5×10^5 HIV-1–infected cells were mixed with 1.6 ml of healthy donor semen containing 6.3×10^7 spermatozoa/ml, no HIV-1 DNA was detected after either centrifugation or swim-up. The sperm suspension collected after swim-up was 1.0 ml in volume and contained 50 000 spermatozoa of 100% motility.

Decay of infectivity of HIV-1 during incubation

A virus solution of HIV_{LAI} was incubated in culture medium for various periods and the p24 production ability was quantified in stimulated PBMC to evaluate the stability of HIV-1 *in vitro* with regard to infectivity. Infectivity decreased semiexponentially with a half-life of approximately 13 h.

Results of the clinical study

A total of 52 HIV-1-positive individuals participated in the clinical study (Table 1); 29 were haemophiliacs and 23 had become infected through sexual contact. The median age was 33 years (range, 27–44) in the 16 untreated individuals, 34 years (range, 28–41) in patients receiving antiretroviral drugs and with viral load $\geq$ 50 copies/ml, and 32 years (range, 20–51) in patients receiving HAART and with viral load $<$ 50 copies/ml. Median plasma viral load was 17 500 copies/ml (range, 70–100 000) in the untreated group and 1500 copies/ml (range, 54–31 000) in patients receiving treatment and with a viral load $\geq$ 50 copies/ml.

Among 48 patients whose partner had assisted reproductive therapy, the median plasma viral load was 17 500 copies/ml (range, 70–100 000) in 15 patients in the untreated group, 4800 copies/ml (range, 54–31 000) in 10 patients receiving antiretroviral treatment and with viral load $\geq$ 50 copies/ml, and $<$ 50 copies/ml in 23 patients taking HAART. Median CD4 cell count was 365 cells/μl (range, 66–1071) in the untreated group, 457 cells/μl (range, 60–652) in patients receiving antiretroviral drugs and with viral load $\geq$ 50 copies/ml, and 399 cells/μl (range, 41–792) in patients receiving HAART and with viral load $<$ 50 copies/ml. The median sperm count of the HIV-positive males was 47×10^6/ml (range, 0–82) in the untreated group, 41×10^6/ml (range, 0–65) in patients receiving antiretroviral drugs and with viral load $\geq$ 50 copies/ml, and 35×10^6/ml (range, 0–120) in patients receiving HAART and with viral load $<$ 50 copies/ml.

Azoospermia occurred in four patients, who were excluded from this study.

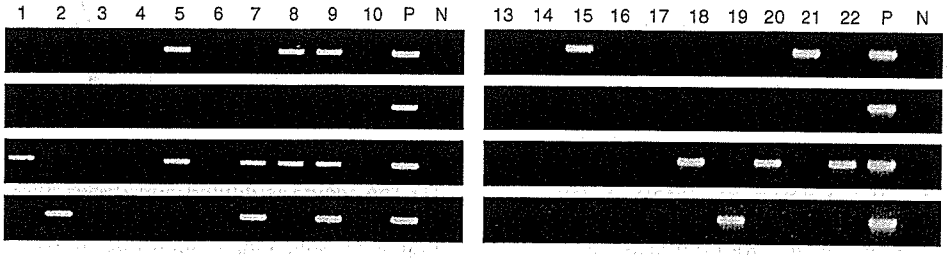

Fig. 2. Reverse transcriptase nested polymerase chain reaction (RT-nested PCR) capable of detecting a single copy of HIV-1 RNA. One virion of $HIV\text{-}1_{LAI}$, on average, was added to 20 sets of 1 ml Sydney IVF medium and then tested with the RT-nested PCR. When one fourth of the eluate from an extraction column was examined (in quadruplicate) with RT-nested PCR, 12 of 20 sets (lanes 1–10 and 13–22) exhibited at least one band in four reactions. Lanes P, positive control using 10 copies of $HIV\text{-}1_{LAI}$ RNA; lanes N, negative control with no HIV-1 RNA.

ble 1. Characteristics of male patients with HIV infection.

		Men treated with HAART	
	Untreated men ($n = 16$)	Viral load ≥ 50 copies/ml ($n = 13$)	Viral load < 50 copies/ml ($n = 23$)
edian age [years (range)]	33 (27–44)	34 (28–41)	32 (20–51)
oute of infection			
Haemophilia	10	8	11
Sexually transmitted	6	5	12
ledian HIV viral load in serum [copies/ml (range)]	17 500 (70–100 000)	1500 (54–31 000)	< 50
D4 cell count [cells/μl (range)]	365 (66–1071)	457 (60–652)	399 (41–792)
perm concentration [$\times 10^6$/ml (range)]	47 (0–82)	41 (0–65)	35 (0–120)
zoospermia (No.)	1	2	1

n all patients, the median motility rate was 28%, and the nedian incidence of morphologically normal spermato-:oa was 12%. The median concentration of spermatozoa n patients (excluding the four patients with azoospermia) was 42×10^6/ml (range, 3–120) and 52×10^6/ml (range, 0–170) spermatozoa were collected after the Percoll centrifugation. The median motility rate was 28% and 45% before and after the Percoll procedure. Following the swim-up method, there were 1.5×10^6/ml (range, 0–11) collected spermatozoa, and the motility rate was 100%. Spermatozoa could be collected by the swim-up method in 73 semen samples from the 48 patients. No HIV-1 RNA or proviral DNA was detected in any sperm suspensions collected after the swim-up procedure. The HIV-1-negative sperm was used for IVF in 12 couples and for ICSI in 31 couples. HIV-1 RNA or proviral DNA could not be deptected in the culture medium of the fertilized eggs before embryo transfer. Of the 43 female partners, 20 conceived and 27 babies were born. HIV antibodies, HIV RNA and proviral DNA were negative in all of the females and babies.

Discussion

This study demonstrated that it is possible to detect a single copy of HIV-1 RNA or proviral DNA, and that HIV-negative spermatozoa can be obtained from the semen of HIV-positive males with the careful use of density gradient centrifugation and the swim-up technique. There has been no HIV-1 transmission in any of the female partners who underwent IVF or ICSI, nor in any of the babies.

Some studies have indicated that HIV can bind and enter into spermatozoa [4,5,12,13]. However, CD4 is not expressed on the surface of spermatocytes or spermatozoa [14,15]. Brogi *et al.* [4] have reported that HIV can attach to the surface glycoprotein of spermatozoa. In children at birth, the infection route is considered to be mother to child [16], and there is no case report of a child or embryo who has been infected with HIV via spermatozoa. It has also not been proven that a spermatid could be infected with HIV during spermatogenesis. This study showed that spermatozoa collected by the swim-up method were neither infected with HIV-1 nor had HIV-1 attached to them.

Semen contains spermatozoa, seminal plasma, white blood cells, microbes, metallic crystals and fibres of underwear. If components with higher density than spermatozoa are in a sample at centrifugation, those components may bring viruses and infected cells down to the bottom sperm fraction. Therefore, in our technique, we left diluted semen undisturbed to settle heavy components, and then took the sperm-containing upper fraction. If the sperm fraction (the bottom layer) following Percoll centrifugation is pipetted through the other denser layers, as is commonly done, HIV may contaminate the sperm fraction via the tube wall. In this study, we sealed the top of the tube after centrifugation and collected the sperm fraction by cutting off the bottom layer, which prevented contamination from the higher layers.

Gomibuchi *et al.* [17] reported that their method could not reduce HIV-1 RNA in semen to < 100 copies/ml in 55.6% of patients. Kuji *et al.* [18] have reported that the use of endotoxin-free Pureseption for semen processing had a lower elimination rate for HIV than the Percoll method. Some groups have used a swim-up technique in which the spermatozoa collected after centrifugation with a separating solution were washed with a culture medium and layered below the medium, followed by swim-up. Because the difference in the specific gravity of the sperm suspension and that of the culture medium is small, HIV and mononuclear cells may easily diffuse to the top layer during the swim-up method [17]. The actual procedures of the swim-up method, such as semen-washing techniques, the materials used in centrifugation, the concentrations of separating solutions, and the methods used to collect the bottom layer (sperm fraction) vary among researchers [17,19,20]. Therefore, it is

considered that the HIV elimination rate will also vary. Our improved swim-up method provides a safer procedure for use in assisted reproductive techniques.

Semprini *et al.* [2] have reported that HIV transmission has not occurred in over 2000 patients who underwent artificial insemination using their method. Their successful results may be explained by the fact that infectious HIV is less than 1/10 000 of all HIV virions [11,21,22] and that removal of the HIV-producing mononuclear cells by the swim-up method is a major factor in reducing infection risk. We have reported that a female was infected with HIV-1 after six artificial insemination procedures using sperm prepared only by centrifugation in another hospital [23]. Artificial insemination should not be performed when inadequate HIV elimination methods are used or when the absence of HIV is not confirmed by highly sensitive tests.

Most HIV-infected patients in this study had low sperm counts and sperm motility rates, and provided a small number of spermatozoa after the swim-up method. As we try to achieve higher virus elimination rates, the number of collected spermatozoa becomes small. Ohl *et al.* [24] reported no pregnancies after artificial insemination using sperm obtained by the swim-up method. If it takes too long for PCR procedures, or if spermatozoa are frozen, the fertilization ability of the spermatozoa may be decreased and the probability of pregnancy may be low. It is difficult to confirm rapidly the removal of HIV-1 RNA and DNA in spermatozoa actually used for artificial insemination. CD4 and chemokine receptors are not expressed on eggs [25] and, therefore, eggs cannot become infected with HIV in the sperm suspensions collected using the swim-up method even if HIV is present in the suspension. If the suspensions are contaminated with a small amount of HIV, the infectivity of the HIV would still decrease to below 1/10 after a 2 day incubation. In addition, in IVF or ICSI, it is possible to confirm the absence of HIV-1 in the culture medium of fertilized eggs before embryo transfer. Therefore, we conducted IVF or ICSI using frozen spermatozoa that had been confirmed negative for HIV-1.

In conclusion, we have demonstrated that it is possible to collect spermatozoa with evidence of the absence of HIV-1 RNA and proviral DNA from semen of HIV-infected males. Whatever method is used for assisted reproductive technique and for removal of HIV from semen to reduce the risk of secondary transmission, it is essential to confirm the absence of HIV-1 RNA and proviral DNA in the sperm preparation used for the assisted reproductive technique with the most sensitive tests possible.

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Mesenchymal progenitor cells derived from chorionic villi of +human placenta for cartilage tissue engineering

Xiaohong Zhang [a], Ayako Mitsuru [a], Koichi Igura [a], Kenji Takahashi [a], Shizuko Ichinose [b], Satoru Yamaguchi [c], Tsuneo A. Takahashi [a,*]

[a] *Division of Cell Processing, The Institute of Medical Science, The University of Tokyo (IMSUT), Tokyo, Japan*
[b] *Instrumental Analysis Research Center for Life Science, Tokyo Medical and Dental University, Tokyo, Japan*
[c] *Yamaguchi Hospital, Nishifunabashi, Funabashi, Chiba, Japan*

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Abstract

Human mesenchymal stem cells are currently being studied extensively because of their capability for self-renewal and differentiation to various connective tissues, which makes them attractive as cell sources for regenerative medicine. Herein we report the isolation of human placenta-derived mesenchymal cells (hPDMCs) that have the potential to differentiate into various lineages to explore the possibility of using these cells for regeneration of cartilage. We first evaluated the chondrogenesis of hPDMCs in vitro and then embedded the hPDMCs into an atelocollagen gel to make a cartilage-like tissue with chondrogenic induction media. For in vivo assay, preinduced hPDMCs embedded in collagen sponges were subcutaneously implanted into nude mice and also into nude rats with osteochondral defect. The results of these in vivo and in vitro studies suggested that hPDMCs can be one of the possible allogeneic cell sources for tissue engineering of cartilage.

Keywords: Placenta; Mesenchymal cells; Chondrogenesis; Cartilage; Tissue engineering

Mesenchymal stem cells from various sources are capable of differentiating into different cell lineages under proper culture conditions [1–3] and have generated a great deal of interest because of their potential use in regenerative medicine. Recently, the human placenta, umbilical cord, and amnion appeared on the stage in the search for MSCs, because of their easy availability with fewer ethical problems compared to other types of cells [4–6]. Umbilical cord blood contains high numbers of hematopoietic stem/progenitor cells and, like bone marrow, has been used to treat various hematological diseases such as leukemia and aplastic anemia, as well as inherited diseases [7,8]. Cord blood also contains mesenchymal cells and there is a report of the existence of multipotential stem cells (unrestricted somatic stem cells), though the number of MSCs is reported to be much smaller than in bone marrow, and it is also difficult to isolate them consistently [9–11]. The human placenta, umbilical cord, and amnion are discarded after the delivery of infants as medical waste. Since the information necessary for cord blood transplantation, i.e., genetic diseases in donors and their families, viral screening, contamination of microorganisms, etc, needs to be obtained by cord blood banks routinely, cells from the placenta and cord blood should be among the safest of allogeneic cell sources. HLA data such as HLA-A, -B, and -DR of the newborn are also obtained by the banks. Therefore, we chose chorionic villi from the fetal part of the human placenta as a target mesenchymal cell source.

Once cartilage is damaged, little restoration occurs because the tissue has little self-healing capacity. Many attempts have been made to repair defects of cartilage due to trauma, osteochondritis, and other conditions by

* Corresponding author. Fax: +81 3 5449 5452.
E-mail address: takahasi@ims.u-tokyo.ac.jp (T.A. Takahashi).

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transplanting chondrocytes or periosteum, and by osteochondral grafts or meniscal allografts [12–15]. However, the results were not satisfactory. The tissue engineering of cartilage has progressed significantly in recent years and bone marrow represents the main source of MSCs for both experimental and clinical studies. However, the use of these cells entails problems such as the necessity of harvesting BM from donors, individual variation [16], limitation to autologous use, and difficulty for hereditary disease patients, all of which underscore the need for alternative sources of autologous and allogeneic MSCs for medical use.

We have reported that mesenchymal progenitor cells from chorionic villi in the human placenta can differentiate into osteoblasts, chondrocytes, adipocytes, and neural cells under different induction conditions in vitro [17], and hPDMCs also have the ability to support the proliferation of hematopoietic stem cells as feeder cells [18]. Therefore, hPDMCs appear to be a possible source of MSCs for use in regenerative medicine. The aim of this study was to analyze the potential for chondrogenic differentiation of hPDMCs and examine whether the hPDMCs could be used as a source of allogeneic mesenchymal cells for tissue engineering of cartilage.

Materials and methods

Isolation and culture of human hPDMCs. This study was approved by the Institutional Review Board of IMSUT. Term placentas were collected after obtaining written informed consent from donors. The processing of the placenta started within 8 h of delivery. To isolate hPDMCs from chorionic villi, the explant culture method was used as described previously [17]. In brief, the amnion and chorionic plate were removed from the placenta, after which the fetal villi were cut into small pieces, washed thoroughly in phosphate-buffered saline (PBS), and then attached to dishes with no coating. Finally, DMEM (low glucose) with 10% FBS and 1% antibiotics/antimycotics was added to the plates. After incubation at 37 °C in a 5% CO_2 atmosphere for 2 or 3 weeks, the cells that migrated were harvested with 0.25% trypsin/1 mM EDTA solution and counted using a hemocytometer. For expansion, the harvested cells were reseeded at a density of 2×10^3 cells/cm^2 in DMEM (low glucose) with 10% FBS and 1% antibiotics/antimycotics, and the culture medium was replaced 2 times every week. The cells used in this study were within 5–15 population doublings (approximately three to six passages).

The human bone marrow-derived mesenchymal cells (hBDMCs) used in this study were purchased from BioWhittaker (Walkersville, MD) and cultured in DMEM (low glucose) with 10% FBS and 1% antibiotic/antimycotics.

Clones of hPDMCs. To analyze the chondrogeneic differentiation of subclones of hPDMCs, the cells were seeded at the density of 1×10^3 cells per 100-mm diameter dish culture in conditioned medium with 10 ng/ml recombinant human basic fibroblast growth factor (bFGF) added as a cloning medium. The culture was maintained in the cloning medium until the formation of well-defined colonies. The subclones were harvested using sterile cloning rings and expanded in the cloning medium.

Flow cytometry. The hPDMCs were harvested using a 1-mM EDTA (pH 7.4) solution. For analysis, cells were stained by combination of antibodies and propidium iodide (PI): FITC-conjugated CD44, CD31, and HLA-class I; PE-conjugated CD73, CD29, CD105, and Tie-2; APC-conjugated CD45, CD34; FITC-mouse IgG1; PE-IgG1, APC-IgG1, and PI. After exposure to labeled antibodies, cells were washed with ice-cold PBS (−) and resuspended in ice-cold PBS (−). The expression of the corresponding cell surface antigen was assayed by FACS Calibur using CELL Quest software (BD). The data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Pellet culture. For chondrocyte differentiation, a pellet culture system was used [19]. Briefly, 2×10^5 cells were placed in a 15-ml polypropylene tube and centrifuged into a pellet. The pellet was cultured at 37 °C with 5% CO_2 in 500 μl of chondrogenic medium containing 10 ng/ml transforming growth factor-β_3 (TGF-β_3) and 500 ng/ml bone morphogenetic protein-2 (BMP-2) in addition to high-glucose DMEM supplemented with 10^{-7} M dexamethasone, 50 μg/ml ascorbate-2-phosphate, 40 μg/ml proline, 100 μg/ml pyruvate, and 50 mg/ml ITS + Premix (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml BSA, and 5.35 mg/ml linoleic acid). The medium was replaced every 3–4 days for 21 days. The chondrogenic medium without BMP-2 was also employed. In the control, the cells were maintained in the medium without BMP-2, TGF-β_3, and dexamethasone. After 3-week induction, the pellets were embedded in paraffin and cut into 5-μm sections, which were then stained with toluidine blue. For immunohistochemistry, an anti type-II collagen monoclonal antibody was used. The reactivity was detected using a diaminobenzidine tetrahydrochloride (DAB) substrate after incubation with an HRP-linked secondary antibody.

Culture in atelocollagen gel. To examine the chondrogenic differentiation of hPDMCs in a three-dimensional culture system, the cells were cultured in atelocollagen gel (Koken, Tokyo, Japan). The volume ratio of the induction medium to 30% atelocollagen gel was 1:4, and the final cell density was adjusted to 1×10^7/ml. The cell–collagen gel composites were cultured in the chondrogenic media as described above and incubated at 37 °C with 5% CO_2. The culture medium was replaced 2 times every week. After a 3-week culture, the cell–atelocollagen gel composite was embedded in paraffin and cut into 5-μm sections for histological analysis with toluidine blue staining.

RT-PCR. To examine the cartilage-specific gene expression, total RNA was prepared from the pellet and cell–atelocollagen gel composite after induction for 2 weeks. The pellet and cell–atelocollagen gel composite were digested with 3 mg/ml collagenase for 3 and 1 h at 37 °C, respectively. hPDMCs without induction were used as a negative control. Total RNA was extracted by using Trizol-LS following the manufacturer's instructions. RT reaction was performed with a Superscript Kit for 50 min at 42 °C, followed by incubation for 15 min at 72 °C using an oligo-dT primer. For examination of the chondrogenic-related gene expression, PCR amplification was performed by 30 cycles of 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 1 min. PCR products were analyzed by electrophoresis in 2% agarose gel containing ethidium bromide for visualization under UV and photographic recording. PCR primers were made as follows: Sox9 (forward) 5′-GAACGCACATCAAGACGGAG-3′, (reverse) 5′-TCTCGTTGATTT CGCTGCTC-3′ (631 bp product; Z46629); COL2A1 (forward) 5′-TTCAG ATATGGAGATGACAATC-3′, (reverse) 5′-AGAGTCCTAGAGTGAC TGAG-3′ (472 bp product; L10347); Aggrecan (forward) 5′-AAACCACC TCTGCATTCCAC-3′, (reverse) 5′-CCTCTCTCTCCTTGCAGGTC-3′ (560 bp product; NM013227); COL10A1 (forward) 5′-CACCAGGCA TTCCAGGATTCC-3′, (reverse) 5′-AGGTTGTTGGTCTGATAGCT C-3′ (926 bp product; NM009925); BMP-2 (forward) 5′-CAGAGACCC A CCCCCAGCA-3′, (reverse) 5′-CTGTTTGTGTTTGGCTTGAC-3′(688 bp product; NM007553); BMP-6 (forward) 5′-CTCGGGGTTCATAAG GTGAA-3′, (reverse) 5′- ACAGCATAAACATGGGGCTTC-3′ (412 bp product; NM001718); β-actin (forward) 5′-TGACGGGGTCACCCACA CTGTGCC-3′, (reverse) 5′-TAGAAGCATTTGCGGTGGACGATG-3′ (660 bp product; NM001101).

Transmission electron microscopic examination. The ultrastructural changes were examined by transmission electron microscopy in the pellets cultured for a week. The cultures were terminated by fixing the pellets with 2.5% glutaraldehyde in 0.1 M PBS for 2 h. The cells were washed overnight at 4 °C in the same buffer and postfixed with 1% OsO_4 buffered with 0.1 M PBS for 2 h. The pellets were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections were double-stained with uranyl acetate and lead citrate, and then examined with an H-7100 transmission electron microscope (Hitachi, Hitachinaka, Japan).

Subcutaneous transplantation of hPDMC in collagen sponge into nude mice. Chondrogenesis of hPDMCs in vivo was examined by transplantation of hPDMCs into subcutaneously into nude mice. We chose the collagen sponge (5 mm in diameter by 3 mm in thickness, BD Biosciences 354513) as a scaffold to perform the in vivo experiment because it could be easily inserted did not need to be anchored by covering it with periosteum. One $\times 10^6$ cells in 100 μl medium were seeded into the collagen sponge and the composite was cultured for 2 weeks in chondrogenic medium and then transplanted into subcutaneous pockets of nude mice (5 weeks old). After 3 weeks, the composite was taken out, embedded in paraffin, and cut into 5-μm sections for histological analysis by toluidine blue staining.

Transplantation of hPDMC in collagen sponge into articular osteochondral defect in nude rats. For another experiment, a defect in articular cartilage was created as described previously [20]. Briefly, after nude rats (weight around 300 g) were anesthetized, a 3-cm-long scalp skin cut was made at the midline of the parapaellar skin. The soft tissue was dissected to expose the capsule, the capsule was incised, and the patella was dislocated laterally to expose the patella groove of the femur. A defect, 2 mm in diameter and penetrating the subchondral bone plate, was prepared on the patellar groove of the femur with a microdrill. The lesion was flushed with saline and dried with gauze. The hPDMC-loaded collagen sponge was inserted into the defect. The patella was repositioned, and the medial aspect of the capsule was closed with a nylon suture. The joint was not splinted and the rat was allowed to move freely in a cage. An empty defect served as a control. Six weeks after the surgery, the animals were sacrificed and the femoral condyles were dissected and fixed in 10% neutral-buffered formalin for 24 h. Then 0.5 M EDTA (pH 7.4) was used to decalcify the samples (for ≈7 days). The samples were paraffin-embedded, cut into 5-μm sections, and histology was examined by toluidine blue staining. The presence of human cells in the repaired tissue was confirmed by using an antibody specific for human β-2 microglobulin, a component of the class I antibody complex, as described previously [21]. In brief, the primary antibody of β-2 microglobulin conjugated with FITC was diluted 1:10 and used for staining cells. The reactivity was detected using a new fuchsin substrate system after incubation with an APL-linked anti-FITC secondary antibody.

Reagents. Culture medium and chemicals were purchased from the following companies.

Dulbecco's modified Eagle's medium (DMEM), dexamethasone, ascorbate-phosphate, proline, pyruvate, propidium iodide, and the alkaline phosphatase-conjugated anti-FITC antibody were purchased from Sigma Chemical (St. Louis, MO), fetal bovine serum (FBS) from Moregate BioTech (Bulimba, Australia), antibiotics, antimycotics, and 0.25% trypsin/1 mM EDTA solution from Gibco, Life Technologies (Grand Island, NY), Trizol-LS, oligo-dT primers, and Superscript II from Invitrogen Life Technologies (Carlsbad, CA), recombinant human BMP-2 from Yamanouchi Pharmaceutical (Tokyo, Japan), recombinant human TGF-β_3 from R&D System (Minneapolis, MN), recombinant human basic fibroblast growth factor (bFGF) from PeproTech EC (London, UK), type II collagen antibody from Lab Vision (Fremont, CA), antibodies of CD44, CD73, CD31, HLA-class I, HLA-DR, ITS+ Primix and human β_2-microglobin antibody from BD Biosciences (PharMingen, CA), antibodies of CD29, CD105, and CD34 from Beckman Coulter (Tokyo, Japan), antibody of Tie-2 from Nichirei (Tokyo, Japan), and the horseradish peroxidase (HRP)-linked antibody, new fuchsine substrate system, diaminobenzidine tetrahydrochloride (DAB) substrate, and toluidine blue solution from Dako (Kyoto, Japan).

Results

Isolation, expansion, and characterization of hPDMCs

The average number of hPDMCs that migrated per piece of chorionic villi by the explant culture method was $\approx 1 \times 10^4$ cells after 20 days and these cells had fibroblast-like shapes with a heterogeneous cell population (Fig. 1A). The phenotypes of hPDMCs were negative for hematopoietic- and endothelial-related cell antigens, such as CD31, CD34, CD45, CD133, and Tie2. They had high expression of mesenchymal progenitor-cell-related antigens, such as CD29, CD44, CD73, CD105, CD90, and HLA-class I, but not HLA-class DR (Fig. 1B). That hPDMCs were isolated without contamination by maternal cells was confirmed by XY chromosome analysis using FISH as described previously [17]. In subclonal culture, clones were isolated from culture dishes and expanded for the analysis.

Chondrocyte differentiation in pellet culture

When the hPDMCs were pelleted into a micromass and differentiated in serum-free medium in the presence of BMP-2, TGF-β_3, and dexamethasone, condensation of the pellet into a single aggregate was observed on the next day. The condensed pellet grew continuingly during culture for 3 weeks and it became white and opaque, with glistening and transparency (Fig. 2A). The pellet from the culture in the induction medium with TGF-β_3 and dexamethasone was smaller than the pellet from the culture in the medium with BMP-2 (Fig. 2B). Paraffin sections of these pellets showed that cartilage matrix was synthesized. The appearance of metachromatic matrix was demonstrated by toluidine blue staining (Fig. 2C), and type II collagen, the specific protein of chondrocyte, was demonstrated by immunohistochemical staining, indicating the occurrence of chondrogenesis.

RT-PCR was used to examine the expression of genes related to chondrogenic differentiation (Fig. 3A), such as Sox9, a major regulator of cartilage-specific genes. Expression of COL2A1, aggrecan, COL10A1, BMP-2, and BMP-6 was detected in the pellets cultured for 2 weeks. TEM examination showed that the cells in the 1-week cultured pellet were oblong with large, euchromatic, ovoid nuclei, and filled with endoplasmic reticula. These cells produced large quantities of extracellular fibers (Fig. 3B).

The pellets of cultured hBDMCs and hPDMCs were similar in size and weight after 3 weeks of induction (Figs. 4A and B). Moreover, chondrogenic differentiation analysis of subclones showed that they grew glistening and transparent (Fig. 4C), indicating that they had potential for chondrogenic differentiation.

Chondrogenic differentiation of hPDMCs in atelocollagen gel

The cell–atelocollagen gel became white, glistening, and harder than the original gel after 3 weeks of culture (Fig. 5A). In histological examination, metachromatic territorial matrixes were observed in atelocollagen gel stained by toluidine blue (Fig. 5B, top). Cells with lacuna formation were examined in the newly formed matrix (Fig. 5B, bottom). RT-PCR confirmed the expression of genes related to the chondrogenic differentiation of hPDMCs in atelocollagen gel after 2 weeks of culture (Fig. 5C).

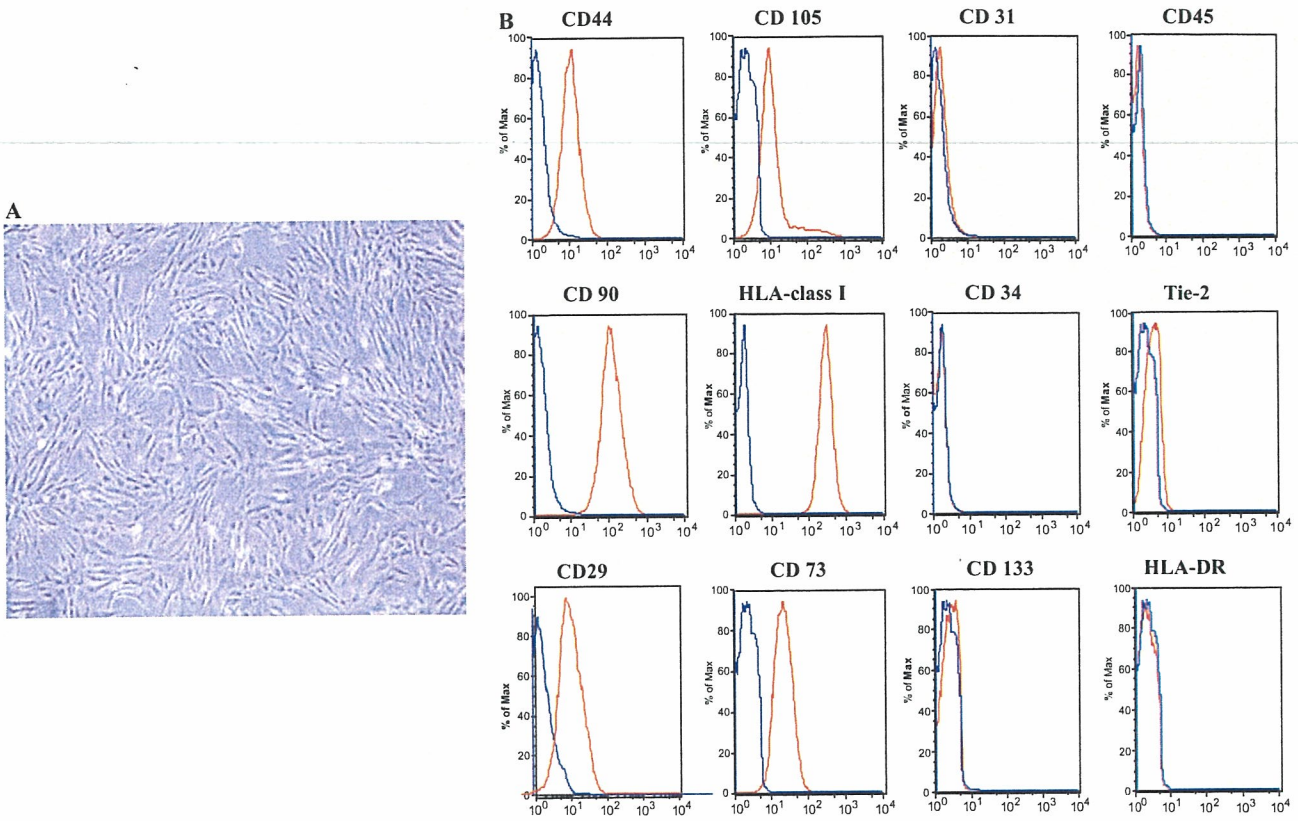

Fig. 1. Morphology of hPDMCs. (A) Proliferated cells show fibroblast-like morphology. Magnification: 40×. (B) Phenotype of hPDMCs. FACS demonstrated that the hPDMCs were positive for CD29, CD44, CD105, CD90, CD73, and HLA-class I but negative for CD31, CD34, CD45, CD133, and HLA DR.

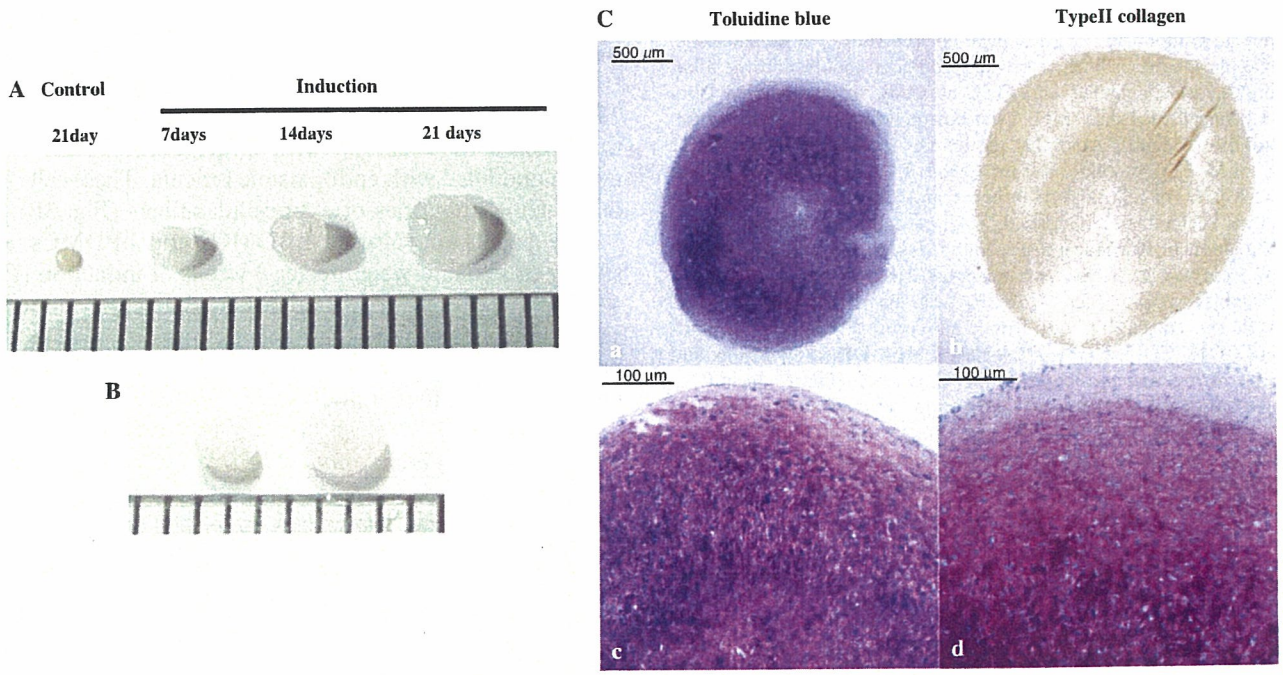

Fig. 2. Pellet culture of hPDMCs. (A) Differentiation of pellets on day 7, 14, and 21. The pellets increased in size up to 3 weeks and became white and opaque with glistening and transparency. A 1-mm scaled ruler is shown. (B) Three-week cultured pellets in the induction medium with BMP2 (right) and without BMP-2 (left). (C) Histological analysis of 3-week cultured pellets. The pellets were embedded in paraffin, sectioned, and stained with toluidine blue (a, c, and d) and the antibody for type II collagen (b). (a, b, and d) With BMP-2, (c) without BMP-2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

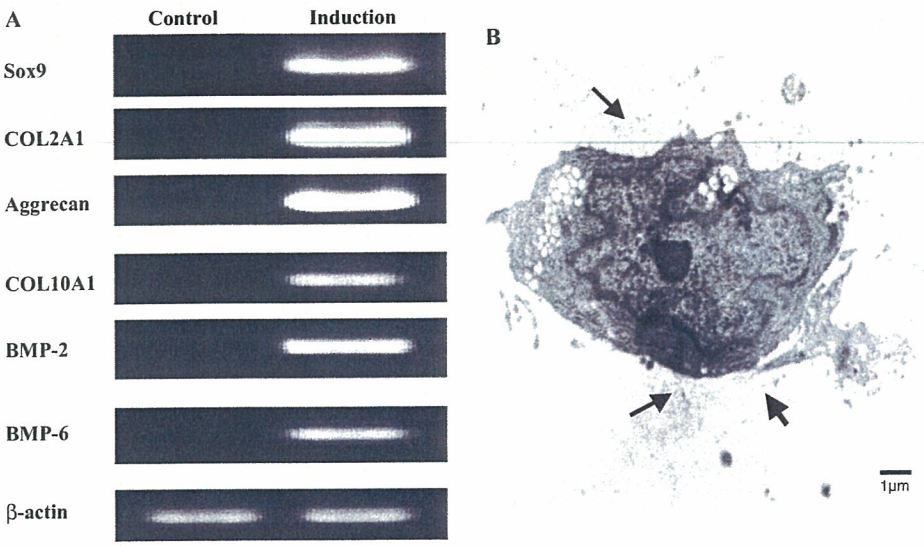

Fig. 3. RT-PCR analyses and TEM examination. (A) RT-PCR analyses for gene expression related to the chondrogenic lineage. Total RNA was extracted from pellets on day 14. Negative control was obtained from the non-induced hPDMCs. (B) TEM observation of 1-week cultured pellet. The cell is fibroblastic with an elongated, oblong phenotype having a large, euchromatic, and ovoid nucleus. The cell contains much endoplasmic reticulum. The cell produced a large number of extracellular fibers (indicated by arrows).

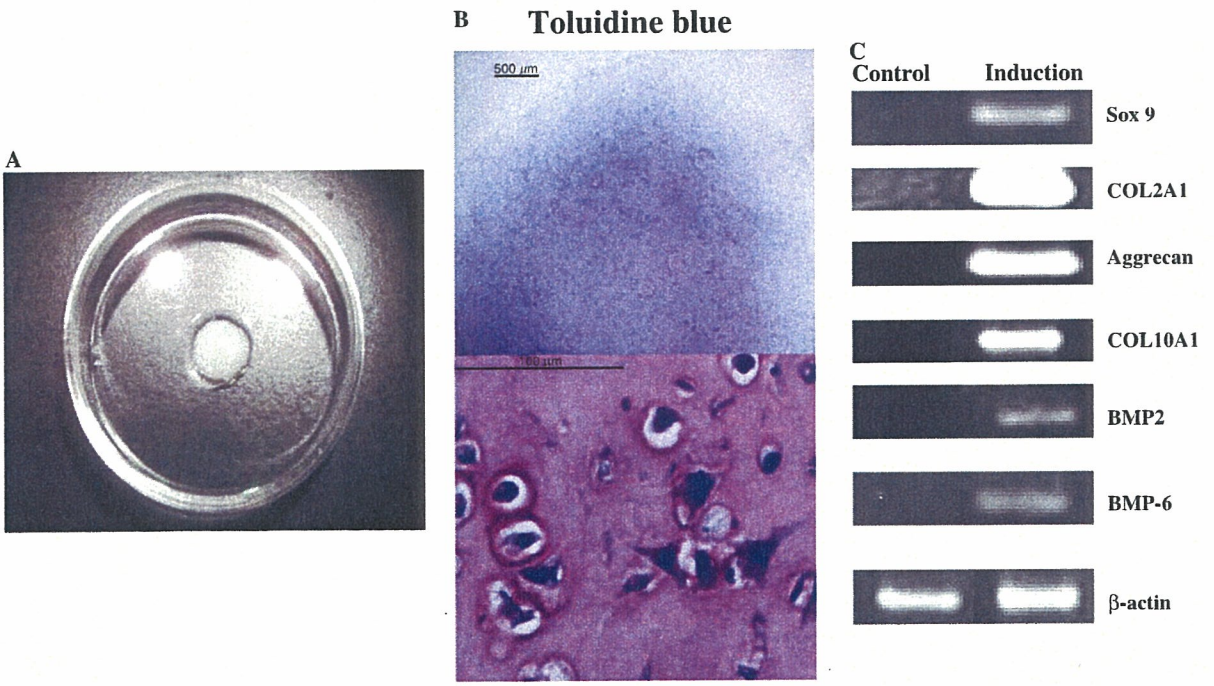

Fig. 4. Culture in atelocollagen gel. After 3-week induction, the cell–collagen gel composites (center of the picture, in a 30-mm dish) became harder than the original gel. (A) Paraffin gel section stained with toluidine blue. Metachromatic matrix was stained by toluidine blue in atelocollagen gel. In the bottom picture, the magnification of the square part, chondrocyte-like cells with formation of lacunae are shown. Top: 10×, Bottom: 250×. (B) RT-PCR confirmed the expression of genes related to chondrogenic differentiation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Chondrogenesis of hPDMCs in subcutaneous transplants

At harvest, 3 weeks after subcutaneous transplantation of the pre-induction hPDMC-loaded collagen sponges into nude mice, the composite was larger than at implantation and had changed to white and glistening, transparent stiff tissue (Fig. 6A). Chondrocyte-like cells within lacunae and a large amount of extracellular matrix were observed in the composite (Fig. 6C).

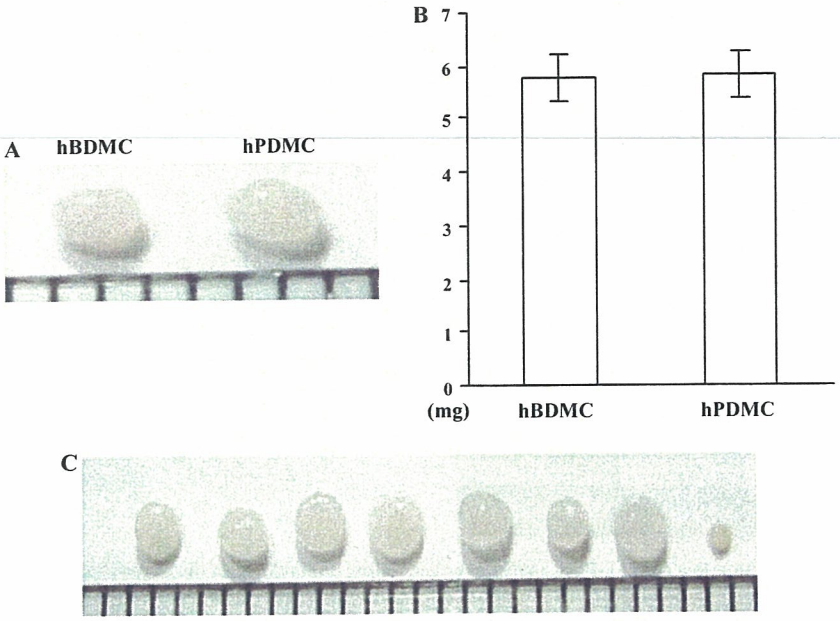

Fig. 5. Chondrogenic differentiation. (A) Pellets of hPDMCs and hBMDMCs were cultured for 21 days. A 1-mm scaled ruler is shown. (B) Weight of hPDMCs and hBMDMCs pellets on day 21. Data are showed as means ± SD ($n = 3$). (C) The clones were cultured in the cloning medium with 10 ng/ml bFGF. Chondrogenic differentiation ability of clones was determined by the pellet culture at day 21. A 1-mm scaled ruler is shown. Pellets made from all 7 clones selected increased in size and showed a glistening transparent appearance. An hPDMC pellet cultured without induction medium (right) was used as a negative control. A 1-mm scaled ruler is shown.

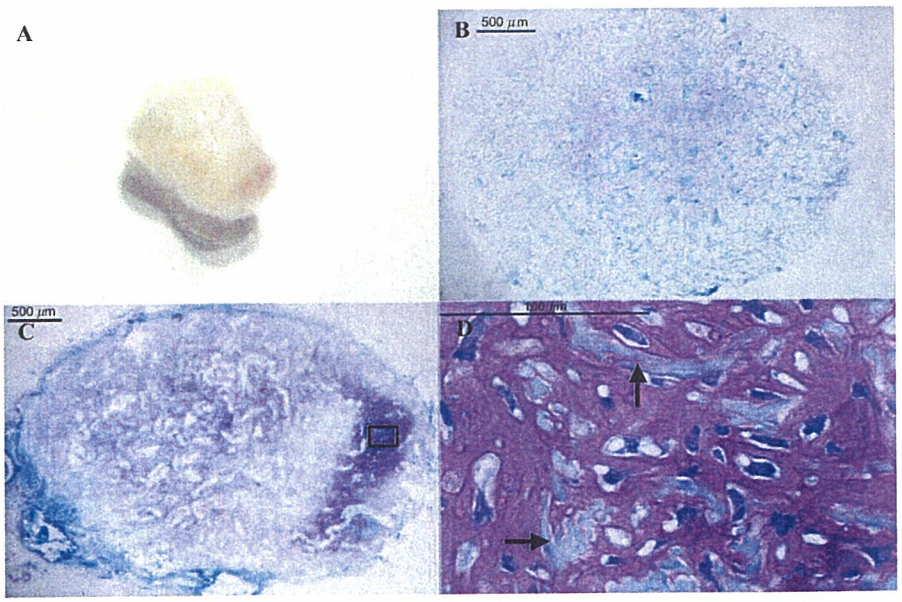

Fig. 6. In vivo chondrogenesis of hPDMCs loaded in collagen sponges in nude mice. The hPDMCs were loaded into a collagen sponge and cultured in vitro for weeks in chondrogenic medium. These sponges were implanted into subcutaneous pockets in nude mice for another 3 weeks. (A) After 3-week implantation, the PDMCs loaded in the sponge were removed from the mice. The composite was white and stiff-like cartilage tissue. (B) At 2 weeks of in vitro culture, cells filled the pores of the collagen sponge. Some local spots showed the formation of extracellular matrix detected by toluidine blue staining. (C) Histological analysis showed a large quantity of extracellular matrix formed between collagen sponge fibers in the implanted cell/collagen composite. (D) High magnification of (C). The cells are round and polygonal within the lacunae and extracellular matrix is strongly stained by toluidine blue. Magnification: (A) 2×, (B,C) 10×, and (D) 250×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Chondrogenesis of hPDMCs in osteochondral defect transplantation

In the osteochondral defect transplantation, the original defect was covered with stiff reparative tissue, which was white, and had a smooth surface at 6 weeks after surgery (Fig. 7A). Histological analysis showed that the reparative tissue filled the defect and closely adhered to the residual part of bone (Fig. 7B). All reparative regions were positive for toluidine blue. Less intense metachromatic staining was observed at the edge of the reparative tissue indicating the formation of hypertrophic cartilage. In the bottom part, the strongest metachromatic staining and round cells showed hyaline cartilage appearance. Differentiating hPDMCs in the defect were confirmed by positive staining for an antibody specific for human β_2-microglobulin (Fig. 7D).

Discussion

We isolated hPDMCs from chorionic villi of the fetal part of the human placenta at 38–40 weeks of gestation using the explant method. The surface epitopes of hPDMCs expressed mesenchymal progenitor-related antigens such as CD29, CD44, CD105, CD90, and CD73 [1,2,22,23], and were negative for hematopoietic and endothelial-related antigens such as CD31, CD45, CD34, Tie-2, and CD133. The cells expressed HLA-class I, but not HLA-DR. The hPDMCs could be differentiated into chondrocytes in induction medium with TGF-β_3 and dexamethasone, and combination with BMP-2 dramatically enhanced the production of cartilage extracellular matrix (Fig. 2B). These results were similar to those in a previous report using bone marrow and synovium-derived MSC [24,25]. RT-PCR showed expression of cartilage-specific extracellular matrix molecules (Fig. 3A), including COL2A1 and aggrecan, and the chondrogenic transcription factor Sox9 in the in vitro chondrogenesis of hPDMCs. BMP-2 and BMP-6 were expressed in culture after induction for 2 weeks, in spite of the fact that exogenous BMP-2 was provided continuously. The expression of BMP-2 and -6 has been reported in the chondrogenesis of BDMCs in vitro [24] but not in MSCs derived from synovium [25]. Endogenous BMP signals may be important for chondrogenesis of hPDMCs. Gene expression of COL10A1, a marker of hypertrophic chondrocytes, was found at 2 weeks. This was similar to the results observed during in vitro chondrogenesis of cells from bone marrow [24], adipose tissue [2], and synovium-derived MSCs in culture [25]. A similar result was also obtained from synovium-derived MSCs without BMPs [26,27]. The expression of COL10A1 seems to be characteristic of in vitro chondrogenesis of MSCs, regardless of the original tissue.

The hPDMCs could be differentiated into chondrocytes in atelocollagen gel under chondrogenic media. It has been reported that transplanting chondrocytes into a newly formed matrix of atelocollagen gel can promote restoration of the articular cartilage of the knee [28]. We attempted to

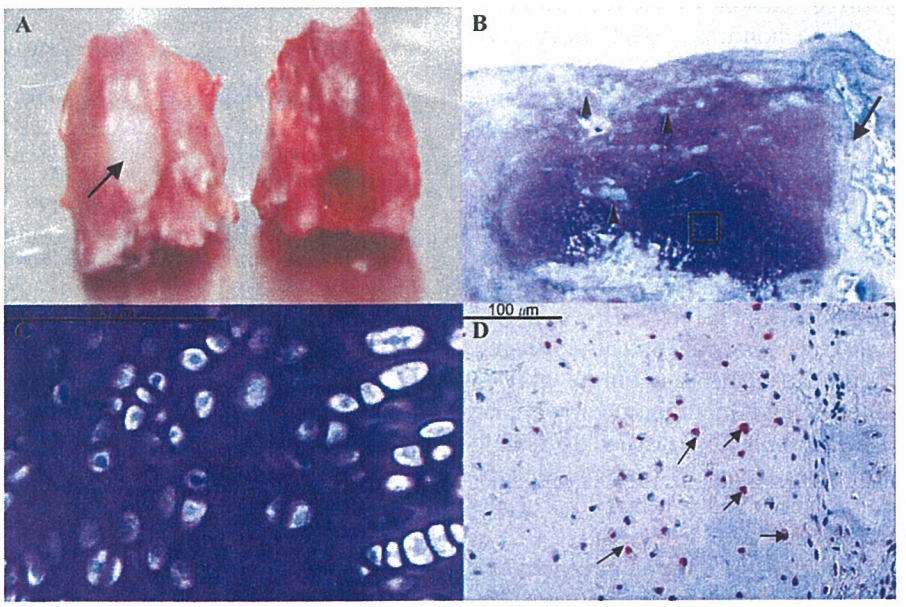

Fig. 7. Chondrogenesis of hPDMCs loaded on collagen sponge in osteochondral wound healing in a nude rat. (A) Left: The appearance of junctions of the nude rat. The hPDMCs loaded in the collagen sponge and transplanted to the defect the junction have a glass-like appearance with a smooth surface and stiffness (Arrows indicate the reparative tissue) Right: The junction where no composite was transplanted remains an empty hole. (B) Histological analysis showed that the reparative tissue filled the defect and closely adhered to the residual portion, covering all of the defect. Less intense metachromatic staining was observed at the edge of the reparative tissue. In the bottom part, round cells and high metachromasia can be observed. Arrow: junctional area between repair (left) and residual (right) part; arrowheads: collagen sponge fibers. (C) High magnification of the square in section B. Hyaline cartilage-like cells with strong metachromasia can be observed with a hyaline cartilage-like appearance. (D) Cartilage-like cells were stained with an antibody specific for human β-2 microglobulin. Arrows indicate positive cells. Magnification: (B) 10×, (C) 250×, and (D) 100×.

make cartilage-like tissue by culturing hPDMCs in atelocollagen gel and our results suggested the possibility that hPDMCs might be usable as an alternative cell source for this cellular therapy because the number of chondrocyte isolates from the knee is limited.

Transplantation of the preinduction hPDMC-loaded collagen sponge in vivo resulted in the production of cartilage with cells within lacunae surrounded by a large amount of metachromatic matrix compared to in vitro culture (Fig. 6). This indicated that differentiated hPDMCs could produce a substantial cartilage matrix in the in vivo environment. In transplantation into the osteochondral defect, reparative tissue had a cartilage-like surface and filled up the entire defect (Fig. 7A). The formation of hypertrophic repair cartilage was observed at the top part, but the bottom one had the strongest metachromasial staining and round cells, which indicated the formation of hyaline cartilage by hPDMCs (Fig. 7C). The formation of a hypertrophic cartilage surface in osteochondral defect has been reported in several clinical studies using BDMC (hypertrophic cartilage cannot function as normal hyaline cartilage and even ossification) [29,30]. Induction of mesenchymal progenitor cell differentiation into hyaline chondrocytes may be related to the potential for differentiation of the cells and induction conditions. In our investigation, the formation of hyaline cartilage in the bottom part of the reparative tissue indicated the possibility that hPDMCs could be used for repairing damaged articular cartilage. Therefore, appropriate induction conditions and a good experimental model are needed to examine the redevelopment of the articular surface as well as the formation of subchondral bone in osteochondral defect repair by hPDMCs.

According to a previous report, the pellet's size and weight are convincing indicators for in vitro chondrogenesis of MSCs [25], Our data showed that the chondrogenesis potential of hPDMCs was comparable to that of hBDMCs (Figs. 4A and B). Clonal analysis showed that the population of hPDMCs was homogeneous after several passages, because the potential for chondrogenic differentiation was evident in the examined clones (Fig. 4C). However, we found that chondrogenic differentiation potential differed in individual hPDMCs, perhaps depending on the individual cell or primary cells that randomly migrated from placental tissue pieces. Thus, an effective method to isolate mesenchymal progenitor cells from the placenta and to identify-specific surface markers of stem cells and progenitors of mesenchymal cells in hPDMC is necessary.

Recently, it has been reported that human mesenchymal stem cells modulate allogeneic immune cell responses [31–33]. If so, and if hPDMCs can differentiate into chondrocytes in vitro and in vivo, they will be one of the possible cell sources for cartilage tissue engineering. However, further studies remain necessary to precisely analyze the chondrogenesis of hPDMCs and to explore potential methods for clinical application.

Acknowledgments

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