行政院及所屬各機關出國報告

（出國類別：研究）

組織工程

——脂肪衍生之母幹細胞——

服務機關：成大醫院　整形外科

出 國 人 職　　稱：主治醫師

姓　　名：林聖哲

出國地區：美國

出國期間：89 年 8 月 14 日至 90 年 8 月 13 日

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目次

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摘要

組織工程是一門新興的領域，極具發展性及前瞻性，它結合了醫學、細胞學、生理學、生化學、免疫學及醫學工程等相關領域，綜合而獨立發展為組織工程。以前認為只有胚胎細胞才有能力發展成為體細胞，但是研究人員最近在成年人體內發現其他已經發育的組織內含有母幹細胞，能夠培養並分化為有限的幾種組織，如骨骼、軟骨、神經及脂肪細胞，而人類抽脂手術後的抽取液即有此母幹細胞存在，整形外科醫師可以廢物利用，利用自體組織來培養出特殊的細胞，既可做學術研究，亦可能於未來使用於臨床工作上，來克服一些組織缺乏或血液循環太差的重建整形外科手術上的難題。

林聖哲醫師於 2000 年赴美 UCLA 進修組織工程領域，已學成抽脂液母幹細胞的分離、純化、培養的技術，想再進一步從事特殊免疫生化染色及定量研究，期於數年內運用此技術使用於人體，此一組織工程計劃特點為不使用骨髓組織，以避免細胞取得不易及捐贈區疼痛的問題，而改用美容門診常見抽脂手術的抽脂液做為母幹細胞的來源，利用特殊的肌肉刺激劑來分化出骨骼肌細胞，利用光學顯微鏡來觀察其形態的變化，利用免疫組織生化法來檢查 MyoD1 Myosin Heavy Chain 的存在，並用 Axioskop2 儀器加以照相存檔及定量化，最後利用 cDNA 及 RT-PCR 的方法來進一步證實肌肉細胞分化的比率及正確性。

在實驗的 negative control 對照組方面，一組為把處理過的抽脂液培養在一般的培養液中做為對照，另一組把人體纖維母細胞放在實驗細胞分化促進液中，看能不能長出骨骼肌細胞。在實驗的 Positive Control 方面，我們把以前研究學者研發的骨髓母細胞和商品化的人類肌肉細胞放在肌肉分化促進液中培養，並用同一套分析方法去比

較及證實與本實驗脂肪抽取液分離的母幹細胞所分化的肌肉細胞的相同性與相異處。

目的

林聖哲博士於2000年赴UCAL組織工程實驗室研究一年，已熟悉此源自抽脂液的母幹細胞培育方式，將把它使用於培養成骨髓肌細胞，以期有一天正式使用於先天或後天的肌肉系統疾病或損傷患者身上，利用培養的骨髓肌細胞替代損傷或功能不佳的肌肉來達到以細胞基礎的治療方式，提供除了外科科外另一修復及重建肌肉功能的方法。

過程

人體脂肪抽取液是在整形外科門診抽脂手術中取得，在局部麻醉下，以 Tumesence 方法執行，局部麻醉劑內有 1% Xylocaine，epinephrine ($1:10^5$) 和生理食鹽水，抽脂機為傳統負壓機械式吸脂機，以 Tumesence 方法可以減少流血量，避免太多血液細胞雜混抽取液，細胞較不易破壞，並減低萃取母幹細胞的困難度。

處理過的脂肪抽取液(Processed Lipo-Aspirate, PLA)

首先請助理將脂肪抽取液由手術室儘快送到實驗室，先用等體積的 Phosphate Buffered Saline (PBS)多次沖洗及激烈搖盪混合，濾掉血液成份，再加上等體積 0.075% collagenase (37℃, 30 分鐘)來分解殘留血液細胞，再利用 Dulbecro's Modified Eagles Medium (DMEM)來中和 Collagenase，分裝成 50cc 的試管，並於 1200xg 下離時 10 分鐘，以取得高濃度的 stromal vascular fracture (SVF)萃取物，接著再度用 DMEM 溶解 SVF，此時需用 100 μm 的尼龍濾網過濾細胞殘存物，再放到 Control Medium (DMEM,10% FBS,1% Antibiotic/Antimycotic solution)中，於 37℃/5% CO_2 的 incubator 中放過隔夜天再把欲培養的細胞懸浮液用 PBS 沖洗以除去 non-adherent RBC，此時的萃取液稱為 Processed Lipo-Aspirate (PLA).

加入肌肉細胞分化促進液(Myogenic Medium, MM)

把 $1x10^4$ 個細胞培養在 35mm 的培養皿上，加入 Myogenic Medium (DMEM,10%FBS,5%Horse Serum,50μm hydrocortisone 和 1% Antibiotice/Ant-mycotic solution)，每週換分化促進液兩次，共六週，每天以光學顯微鏡觀察細胞形態的變化。

免疫組織生化法

每 $5x10^3$ 個細胞培養在一小格(共八小格的培養皿)，每一週做免疫組織生化法一次，方法為把一小格培養皿用 PBS 濕潤兩次，用 4% Paraformaldehyde 於 4℃下固定 2 分鐘，再加上 3% H_2O_2 5 分鐘來阻斷 endogenous peroxides 的活性，並加上 Blocking Buffer (BBPSB，1% Horse Serum 0.1% Triton/x-100) 30 分鐘來阻斷 non-specific epitope，接著加入 monoclonal antibody to human MyoD1 或 human fast twitch skeletal muscle myosin heavy chain 於 4℃中過夜，隔天用 BB 再度清洗，並加上 Horse Anti-mouse IgG Biotinylated Secondary Antibody (1:250 dilutim)兩小時，Secondary antibiotics 用 Vectastain ABC Kit，最後用 hematoxyline 3 分鐘來加強對比染色。

定量分析

利用 Axioskop 2 顯微鏡照相系統在 200 倍下計算染有 MyoD1 或 Myosin heavy chain 的細胞比率，以 one-way analysis of variance (ANOVA) 做統計分析。

CDNA 和 RT-PCR 的方法

用 superscript II Enzyme 把培養肌肉細胞的 RNA 反轉合成為 oligo d(T)-Primed cDNA，等量的 cDNA 用以做為 templates for PCR amplification (50μl Reaction volume)。Primer Pairs 如下：

MyoD1: 5'-AAGCGCCATCTCTTGAGGTA-3'

和 5'-GCGCCTTTATTTTGATCACC-3'

Myosin Heavy Chain:

5'-TGTGAATGCCAAATGTGCTT-3'

和 5'-GTGGAGCTGGGTATCCTTGA-3'

經 35 個 amplification 週期後取出樣品在 agarose gel 中分析，預期分子大小：MyoD1 為 500bp，而 Myosin Heavy Chain 為 750bp。

Negative Control

1. Human Fibroblast + Myogenic Media

2. Process Lipo-Aspirate + Control Medium

Positive Control

1.Human Skeletal Muscle Cell

2.Bone Marrow-derived Stem Cell + Myogenic Medium

心得

在重建整形外科領域中，組織缺損的重建或塑型常因適當組織捐贈的困難取得，捐贈區的後遺症及血液循環不良的因素影響而受到限制，組織工程技術的研發提供我們臨床工作者一個新的思維模式。如果能萃取具有再度分化能力的母幹細胞，加以引導培養出我們所需的特殊細胞，亦可達到治療的功效。理論上只有胚胎母幹細胞可以利用於多源性細胞的分化，一為胚胎母幹細胞，但基於道德的考慮及細胞的合法控制，只能用於動物實驗；另一為自體母幹細胞，它就沒有這些限制，尤其是來自骨髓的自體母幹細胞，已經在世界很多研究機構成功地培養，並得到卓越的突破性發展。人體骨髓源自於胚胎時期的中胚層，富含造血母細胞和間質，我們以前對造血細胞了解較多，但較少研究間質的重要性及用處，其實這些間質含有源自中胚層的母幹細胞，已被研究學家成功地培養分化出脂肪、軟骨、骨骼及肌肉細胞，但由骨髓間質所衍生的母幹細胞數目較少(<0.0005%)，且需要在體外長時間培養及繁殖，才能使用於臨床，這些步驟不僅耗時、花錢且有細胞汙染的可能性，且捐贈者會長期疼痛，因此其他組織來源的自體母幹細胞一直被尋找著，其中脂肪組織和骨髓一樣源自胚胎期的中胚層，一樣富含多樣性的間質細胞群，其細胞取得容易，可以利用需要治療的患者本身的細胞，不必借用他人細胞以避免外來組織的排斥問題，且手術時只需局部麻醉，可抽取的母幹細胞較多，捐贈區較不疼痛，這些經過處理過的脂肪抽取液可以在體外穩定的繁殖和低程度的老化，利用免疫螢光法及流量細胞測量法發現，大部份經處理過的脂肪抽雙液源自中胚層，另參雜有一些內皮細胞、平滑肌細胞及 Pericytes，經特殊溶解分離及純化後加上專屬的培養液亦與骨髓母幹細胞一樣可以分化

為骨髓、軟骨、脂肪及骨髓肌細胞，此技術在近年來於美國 UCLA 及匹茲堡大學正積極的研究，並在技術上有重大突破，將陸續發表在世界著名雜誌，林聖哲博士於 2000 年赴 UCAL 組織工程實驗室研究一年，已熟悉此源自抽脂液的母幹細胞培育方式，將把它使用於培養成骨髓肌細胞，以期有一天正式使用於先天或後天的肌肉系統疾病或損傷患者身上，利用培養的骨髓肌細胞替代損傷或功能不佳的肌肉來達到以細胞基礎的治療方式，提供除了外科科外另一修復及重建肌肉功能的方法。

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建議

在醫學方面

1.結合組織工程，創造另外一種以細胞為基礎的肌肉修補方式。

2.利用美容手術抽脂手術的丟棄物(抽脂液)加入廢物利用，達到研究新科技的目的。

在組織工程方面

1.把基礎醫學的技術有效的應用到臨床上。

2.比較脂肪來源的母幹細胞和骨髓來源的母幹細胞培養分化的肌肉細胞有何異同?

未來展望

本年度的目標為建立一套實驗的基本操作手冊，並提高肌肉細胞的分化成功率，如有時間會把此技術培養幹細胞於可分解的生醫材料上(Biodegradable Material)，如 Poly-glycolic Acid, Poly Lactic Acid 或 Collagen 上。

未來會再做動物實驗，並等無菌技術及安全性更提高以後，將使用於先天性肌肉病變和後天性肌肉損壞的病人身上，以提供除了手術方法外，另一以細胞為基礎的組織工程療法。

來去UCLA—夢想之旅　　林聖哲

人生有夢，逐夢踏實，從小生活在殷實的城市—嘉義，總是羨慕同學得以因打少棒而出國，但也目睹了嘉義七虎少棒鍛羽波麻波特時社會的人情冷暖，及長總以為若能學有所成，回故鄉開業，得以幫助故鄉人，即是此生最大志願了。然而在台大當完整形外科總住院醫師後，因成大醫院剛成立，再加上邱浩遠主任的精神感召，故得以"轉進"成大，繼續服務於大學醫院，沒有成為開業醫。初入成大，一切從頭來，沒有基本病人群，又沒知名度，因此先一頭鑽入醫工所唸書，師事周有禮教授，民國89年初，當還沈醉在所做的"顏面表情動態分析"論文為SCI雜誌所接受的喜悅時，又喜上加喜，接獲UCLA來函同意我赴美進修一年。當時會選擇UCLA的原因很多，但最主要的因素有二：一為我從小就是LA Lakers的球迷；二為UCLA同意我從事二十一世紀最Hito的研究—組織工程，一年後的今天回想起來，這個抉擇是絕對正確的，因為Lakers在美國職業籃球二連霸，我們全家得以躬逢盛會，而且UCLA在我進修半年後發表了全世界第一篇利用脂肪抽取術分離出的Stem Cells來分化為骨髓、軟骨、肌肉及脂肪細胞，雖然未能掛名其中，但有幸在論文修改其間，幫忙補充一些資料，也是與有榮焉。

我把這一年(89.8.14~90.8.13)在UCLA進修的情形分為四個階段：

一、蠻荒期(前二個月)

出國前因博士班畢業及出國申請作業兩頭忙，所以無法做太多行前準備作業，記得一家五口出國前的行李只有兩大箱，還被友人譏笑比出國短期旅行還精簡，後來勉強湊了一人一箱就直奔天使之城—洛杉磯，前兩個月為了UCLA的行政作業，房事相關的水電、瓦斯、電話、垃圾，社會安全卡的申請，小孩就學及買車，忙到體重

直降，大有不如歸去之感。因為我們不是美國居民又沒有 Credit 就算你有合法簽證(J-1 and J-2)，在美國生活也是大不易，再加上以前在成大醫工所並沒有組織工程的課程可修，剛入行，不論在知識上或技術上都大不如人，挫折感很重，真不好意思說自己是 M.D 加上 Ph.D.,幸好在同事 Min 的幫忙及鼓歷勵下，才得以漸入佳境，日起有功。

二、成長期：(前二個月到前四個月)

經二個月的奮戰後，總算把一些細胞學、生化學、基因學的程度提昇到聽得懂，並可以加入討論的地步，但在每週一的 4 小時研究報告及討論會，仍受限於英文表達能力及敏捷度的限制，只能偶而插話，但 UCLA 的醫師及醫工所教授也漸漸了解，來自台灣的我在臨床經驗及手術技巧也稱得上優良，因此會不時停下來詢問我的意見，那種受重視的感覺直到現在都還感激莫名。這時在實驗操作上，也漸漸由只能到比佛利山美容外科診所拿脂肪抽取物的卑伏，升格為幫忙分離 Stem Cell 的"二廚"，並擁有獨立的 incubator 空間可以自己培養自己想要的細胞，甚至偶而可以充當"大廚"，獨立分離出 Adipo-Derived Stem Cell 供同事分化成其他細胞。

三、成熟期：(中間六個月)

由於細胞培養的能力已被認證通過，所以得以有多餘的時間去開一些國議醫學會及參加專為UCLA整形外科住院安排的Instruction Courses, 如利用黃金比例的原則用陶土塑造臉形課程及繪畫課，由其中了解到 UCLA 對住院醫師訓練課程的多樣性及精緻度。此時也被委以重任，尋求利用除了 Collagenase 外的物理方法來分離 Stem Cell，並成功地利用超音波在固定的頻率和能量下，分離出 Stem Cell，現仍在 UCLA 的 REBAR 實驗室繼續研發中。

四、整理期：(後兩個月)

如同服役中由"破百之日"倒數饅頭般的心情，收拾行囊準備歸國

的時間漸漸接近，UCLA 的秘書 Jessica 也帶來了一個令人高興又難以回答的問題，UCLA 想讓我多留一年，但只有少許生活津貼，為了尋求這個問題的解答，我們全家開了數次家庭會議，雖然小孩已漸漸習慣美式教育，但他們還是很懷念台灣，而且終究他們還是要回台灣唸書，而我呢?或許多一年會讓我的研究之路更充實，但積蓄即將用盡，未來的一年成大勢必將我停薪(留不留職仍未定)，而我們又不像日本友人 Yoshi，第一年日本政府給全薪，第二年給 70%，且基於公平原則及未事先報備下，我們決定如期返國，在 LA 長榮機場櫃檯還被 Check-In 的地勤人員笑說，那天是我們一年期機票有效期間的最後一天。當決定回國時，心情輕鬆很多，不過也感覺時間不多，必須把以前作的實驗方法及流程更詳細記錄下來，並從根本做起，親自調配各種 Induction Media(此工作原為研究助理的工作)，以免回國後無法調配出與 UCLA 一樣的藥劑，而使細胞培養的工作功虧一潰。

回國不到一個月，發生 911 紐約雙子星大廈撞機的恐怖事件，連忙聯絡在 LA 的友人，直到全部聯絡上才安心，因為那些飛機大部份飛往 LA。在我們安全返台三個月的此時，我們由衷感激在這段期間不論在生活上或工作上給我們全家有形或無形的支持的美國親友，原諒我不能一一寫出您們的名字，就算寫出，您們大部份也看不懂中文，但別忘了我幫您們取的中文姓名以及我給您們的中文印章，那些內含著我們全家無比的感激與思念。

後記：

感謝UCLA 整形外科同仁對我及成大醫院的愛戴及支持，其中 Dr. William Shaw 已於十月底飛來成大擔任外科客座教授；Dr. Marc Hedrick 將於十二月中中華民國整形外科年會時來成大做專題演;Dr. Kawamoto 亦預於明年三月中受邀來訪，這些行程及內容容後再撰文敘述。

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Multilineage Cells from Human Adipose Tissue: Implications for Cell-Based Therapies

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ABSTRACT

Future cell-based therapies such as tissue engineering will benefit from a source of autologous pluripotent stem cells. For mesodermal tissue engineering, one such source of cells is the bone marrow stroma. The bone marrow compartment contains several cell populations, including mesenchymal stem cells (MSCs) that are capable of differentiating into adipogenic, osteogenic, chondrogenic, and myogenic cells. However, autologous bone marrow procurement has potential limitations. An alternate source of autologous adult stem cells that is obtainable in large quantities, under local anesthesia, with minimal discomfort would be advantageous. In this study, we determined if a population of stem cells could be isolated from human adipose tissue. Human adipose tissue, obtained by suction-assisted lipectomy (*i.e.*, liposuction), was processed to obtain a fibroblast-like population of cells or a processed lipoaspirate (PLA). These PLA cells can be maintained *in vitro* for extended periods with stable population doubling and low levels of senescence. Immunofluorescence and flow cytometry show that the majority of PLA cells are of mesodermal or mesenchymal origin with low levels of contaminating pericytes, endothelial cells, and smooth muscle cells. Finally, PLA cells differentiate *in vitro* into adipogenic, chondrogenic, myogenic, and osteogenic cells in the presence of lineage-specific induction factors. In conclusion, the data support the hypothesis that a human lipoaspirate contains multipotent cells and may represent an alternative stem cell source to bone marrow-derived MSCs.

INTRODUCTION

THE THERAPEUTIC POTENTIAL of multilineage stem cells for applications such as tissue engineering and gene therapy is enormous. Conceptually, there are two general types of stem cells potentially useful for these applications: embryonic stem cells (ESCs) and autologous stem cells. Although theoretically appealing because of their pluripotentiality, the practical use of ESCs is limited due to potential problems of

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cell regulation and ethical considerations. In contrast, autologous stem cells, by their nature, are immunocompatible and have no ethical issues related to their use. For the engineering of mesodermally derived tissues, autologous stem cells obtained from bone marrow have proven experimentally promising. Human bone marrow is derived from the embryonic mesoderm and is comprised of a population of hematopoietic stem cells (HSCs), supported by a mesenchymal stroma.[1–5] Although the proliferation and differentiation of HSCs have been well documented, less is known about the stromal component. The bone marrow stroma, in both animals and humans, is heterogenous in composition, containing several cell populations, including a stem cell population termed mesenchymal stem cells or MSCs.[6] Studies on MSCs have demonstrated their differentiation into adipocytes,[7,8] chondrocytes,[6,8–11] myoblasts,[12,13] and osteoblasts.[6,8,14–19] These cells represent a promising option for future tissue engineering strategies. However, traditional bone marrow procurement procedures may be painful, frequently requiring general or spinal anesthesia and may yield low numbers of MSCs upon processing (approximately 1 MSC per 10^5 adherent stromal cells[8,17,20]). From a practical standpoint, low stem cell numbers necessitate an *ex vivo* expansion step to obtain clinically significant cell numbers. Such a step is time consuming, expensive, and risks cell contamination and loss. An ideal source of autologous stem cells would, therefore, be both easy to obtain, result in minimal patient discomfort, yet be capable of yielding cell numbers substantial enough to obviate extensive expansion in culture.

Adipose tissue may represent such a source. Although it is known that many tissues contain lineage-committed progenitor cells for tissue maintenance and repair, several studies have demonstrated the presence of uncommitted MSCs within the connective tissue matrices of several organs in birds, mice, rats, and rabbits.[21–26] Furthermore, adipose tissue, like bone marrow, is derived from the embryonic mesoderm and contains a heterogenous stromal cell population.[27–31] These similarities, together with the identification of MSCs in several tissues, make plausible the concept that a stem cell population can be isolated from human adipose tissue. Therefore, in this study, we sought to determine if a population of multipotential stem cells could be isolated from human adipose tissue.

MATERIALS AND METHODS

Materials

All materials were purchased from Sigma (St. Louis, MO) unless otherwise stated. All tissue culture reagents were purchased from Life Technologies (New York, NY). Fetal bovine serum (FBS) and horse serum (HS) were purchased from Hyclone (Logan, UT) and Life Technologies, respectively.

Cell lines

Normal human osteoblasts (NHOsts), human skeletal muscle (SkM) cells, and population of MSCs derived from bone marrow were purchased from Clonetics (Walkersville, MD). The murine 3T3-L1 preadipocyte cell line[32] was obtained from ATCC (Rockville, MD). Human foreskin fibroblasts (HFFs) were obtained from Cascade Biologics (Portland, OR).

Isolation and culture of stem cells—PLA and MSCs

Human adipose tissue was obtained from elective liposuction procedures under local anesthesia (HSPC #98-08 011-02). In this procedure, a hollow blunt-tipped cannula was introduced into the subcutaneous space through small (~1 cm) incisions. The cannula was attached to gentle suction and moved through the adipose compartment, mechanically disrupting the fat tissue. A solution of saline and the vasoconstrictor epinephrine was infused into the adipose compartment to minimize blood loss and contamination of the tissue by peripheral blood cells. The raw lipoaspirate (~300 cc) was processed according to established methodologies to obtain a stromal vascular fraction (SVF).[33,34] To isolate the SVF, lipoaspirates were washed extensively with equal volumes of phosphate-buffered saline (PBS), and the ECM was digested at 37°C for 30 min with 0.075% collagenase. Enzyme activity was neutralized with Dulbecco's modified Eagle's medium (DMEM), containing 10% FBS and centrifuged at 1200 × *g* for 10 min to obtain a high-density

SVF pellet. The pellet was resuspended in 160 mM NH_4Cl and incubated at room temperature for 10 min to lyse contaminating red blood cells. The SVF was collected by centrifugation, as detailed above, filtered through a 100-μm nylon mesh to remove cellular debris and incubated overnight at 37°C/5% CO_2 in control medium (DMEM, 10% FBS, 1% antibiotic/antimycotic solution). Following incubation, the plates were washed extensively with PBS to remove residual nonadherent red blood cells. The resulting cell population was termed a processed lipoaspirate (PLA), to distinguish it from the SVF obtained from excised adipose tissue. PLA cells were maintained at 37°C/5% CO_2 in noninductive control medium. Cells did not require specific FBS sera lots for expansion and differentiation (data not shown). For immunofluorescence studies, a population of MSCs was obtained from human bone marrow aspirates according to the protocol of Rickard *et al.*[17] and maintained in control medium. To prevent spontaneous differentiation, cells were maintained at subconfluent levels.

Indirect immunofluorescence of PLA cells

PLA cells and MSCs obtained from human bone marrow aspirates were plated onto glass chamber slides and fixed for 15 min in 4% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.0). The cells were washed for 10 min in 100 mM glycine in PBS (PBS/glycine) and blocked for 1 h in immunofluorescent blocking buffer (IBB) containing 5% bovine serum albumin (BSA), 10% FBS, 1 × PBS, 0.1% Triton X-100). The cells were subsequently incubated for 1 h in IBB containing the following cell-specific monoclonal antibodies: (1) anti-smooth muscle actin (anti-SMA; Cedarlane Inc., Hornby, Ontario), to identify smooth muscle cells and pericytes[35–38]; (2) anti-Factor VIII (anti-FVIII; Calbiochem, San Diego, CA), to identify endothelial cells[39,40]; and (3) ASO2 (dianova, Hamburg, Germany), to identify fibroblasts and cells of mesenchymal origin.[41,42] The cells were washed extensively with PBS/glycine and incubated for 1 h in IBB containing an fluoroisothiocyanate (FITC)-conjugated secondary antibody. The cells were washed with PBS/glycine and mounted with a solution containing DAPI to detect nuclei (VectaShield, Vector Labs, Burlingame, CA).

Flow cytometry

PLA samples from 5 donors were cultured in control medium for 72 h prior to analysis. Flow cytometry was performed on a FACScan argon laser cytometer (Becton Dickson, San Jose, CA). Cells were harvested in 0.25% trypsin/EDTA and fixed for 30 min in ice-cold 2% formaldehyde. Following fixation, cells were washed in flow cytometry buffer (FCB; 1 × PBS, 2% FBS, 0.2% Tween-20). Cell aliquots (1×10^6 cells) were incubated in FCB containing monoclonal antibodies to FVIII, smooth muscle actin, or ASO2. In addition, cells were also incubated with FCB containing a monoclonal antibody to vimentin (anti-VIM; Biogenesis, Brentwood, NH), to identify mesenchymal cells.[43,44] To assess viability, duplicate samples were harvested, fixed for 30 min with ice-cold 1% paraformaldehyde, permeabilized with 0.05% Nonidet-40, and incubated with propidium iodide (PI) at a concentration of 25 μg/mL. Debris and dead cells were excluded by eliminating PI-positive events. All subsequent PLA samples were corrected accordingly.

Cumulative population doubling

PLA cells were maintained in control medium until 80% confluent. Cells were harvested at confluence and population doubling calculated using the formula $\log N_1/\log N_2$, where N_1 is the number of cells at confluence prior to passaging and N_2 is the number of cells seeded after passaging. Cumulative population doubling was determined in cultures maintained until passage 13 (approximately 165 days). The mean cumulative population doubling obtained from 3 donors was expressed as a function of passage number.

Cell senescence assay

Senescence was assessed using a β-gal actosidase (β-Gal) staining assay, in which β-Gal activity is detected in senescent cells at pH 6.0 but is absent in proliferating cells.[45] Cells from each culture passage (passage 1 to passage 15) were fixed for 5 min in 2% formaldehyde/glutaraldehyde and incubated in a β-Gal reaction buffer (containing 1 mg/ml X-Gal, 40 mM citric acid/sodium phosphate buffer (pH 6.0), 5 mM

TABLE 1. LINEAGE-SPECIFIC DIFFERENTIATION INDUCED BY MEDIA SUPPLEMENTATION

Medium	*Media*	*Serum*	*Supplementation*
Control	DMEM	10% FBS	none
Adipogenic (AM)	DMEM	10% FBS	0.5 mM isobutyl-methylxanthine (IBMX), 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin, 1% antibiotic/antimycotic
Osteogenic (OM)	DMDM	10% FBS	0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate, 10 mM β-glycerophosphate, 1% antibiotic/antimycotic
Chondrogenic (CM)	DMEM	1% FBS	6.25 μg/ml insulin, 10 ng/ml TGF-β1, 50 nM ascorbate-2-phosphate, 1% antibiotic/antimycotic
Myogenic (MM)	DMEM	10% FBS, 5% HS	0.1 μM dexamethasone, 50 μM hydrocortisone, 1% antibiotic/antimycotic

each of potassium ferrocyanide and potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$). Senescent cells (blue) were identified by light microscopy.

Confirmation of multilineage differentiation of PLA cells

PLA cells at passage 1 were analyzed for their capacity to differentiate toward the adipogenic, osteogenic, chondrogenic, and myogenic lineages. To induce differentiation, PLA cells were cultured with specific induction media, as detailed in Table 1. Each medium has been previously described and shown to induce multilineage differentiation of MSCs.[8,14,15,31,33] Differentiation was confirmed using the histological and immunohistological assays outlined in Table 2. A commercial source of bone marrow-derived MSCs and lineage-specific precursors were examined as positive controls. PLA cells maintained in control medium and HFFs were analyzed as negative controls.

Adipogenesis: Adipogenic differentiation was induced by culturing PLA cells for 2 weeks in adipogenic medium (AM) and assessed using an Oil Red O stain as an indicator of intracellular lipid accumulation.[46] Prior to staining, the cells were fixed for 60 min at room temperature in 4% formaldehyde/1% calcium and washed with 70% ethanol. The cells were incubated in 2% (wt/vol) Oil Red O reagent for 5 min at room temperature. Excess stain was removed by washing with 70% ethanol, followed by several changes of distilled water. The cells were counterstained for 2 min with hematoxylin.

Osteogenesis: Osteogenic differentiation was induced by culturing PLA cells for a minimum of 2 weeks in osteogenic medium (OM) and examined for alkaline phosphatase (AP) activity and ECM calcification by von Kossa staining. To detect AP activity, cells were incubated in OM for 2 weeks, rinsed with PBS, and stained with a 1% AP solution (1% naphthol ABSI phosphate, 1 mg/mL Fast Red TR) at 37°C for 30 min. For von Kossa staining, the cells were incubated in OM for 4 weeks and fixed with 4% paraformalde-

TABLE 2. DIFFERENTIATION MARKERS AND ASSAYS OF LINEAGE-SPECIFIC DIFFERENTIATION

Lineage	*Lineage-specific determinant*	*Histologic/immunohistochemical assay*
Adipogenic	Lipid accumulation	Oil Red O stain
Osteogenic	1. AP activity	1. AP stain
	2. Calcified matrix production	2. Von Kossa stain
Chondrogenic	1. Sulfated proteoglycan-rich matrix	1. Alcian Blue (pH 1.0) stain
	2. Collagen II synthesis	2. Collagen II-specific monoclonal antibody
Myogenic	1. Multinucleation	1. Phase contrast microscopy
	2. Skeletal muscle myosin heavy-chain and MyoD1 expression	2. Myosin- and MyoD1-specific monoclonal antibodies

hyde for 60 min at room temperature. The cells were rinsed with distilled water and then overlaid with a 1% (wt/vol) silver nitrate solution in the absence of light for 30 min. The cells were washed several times with distilled water and developed under UV light for 60 min. Finally, the cells were counter-stained with 0.1% eosin in ethanol.

Chondrogenesis: Chondrogenic differentiation was induced using the micromass culture technique.[47–49] Briefly, 10 μL of a concentrated PLA cell suspension (8×10^6 cells/mL) was plated into the center of each well and allowed to attach at 37°C for 2 h. Chondrogenic medium (CM) was gently overlaid so as not to detach the cell nodules, and cultures were maintained in CM for 2 weeks prior to analysis. Chondrogenesis was confirmed using the histologic stain Alcian Blue at acidic pH. PLA cell nodules were fixed with 4% paraformaldehyde for 15 min at room temperature and washed with several changes of PBS.

Studies have shown specific staining of sulfated proteoglycans, present in cartilagenous matrices, at pH levels of 1 and below.[50] In light of this, the cells were incubated for 30 min with 1% (wt/vol) Alcian Blue (Sigma A-3157) in 0.1 N HCl (pH 1.0) and washed with 0.1 N HCl for 5 min to remove excess stain. In addition to Alcian Blue staining, expression of the cartilage-specific collagen type II isoform was also determined. PLA cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Cells were incubated in 0.2 U/mL chondroitinase ABC for 40 min at 37°C to facilitate antibody access to collagen II. The eells were rinsed in PBS and endogenous peroxidase activity quenched by incubating for 10 min in 3% hydrogen peroxide in methanol. Following a wash in PBS, nonspecific sites were blocked by incubating cells for 1 h in blocking buffer (PBS, containing 10% horse serum). The cells were subsequently incubated for 1 h in blocking buffer containing a monoclonal antibody specific to human collagen type II (ICN Biomedical, Costa Mesa, CA). The cells were washed extensively in blocking buffer, and collagen type II was shown using a commercially available kit for the detection of monoclonal antibodies according to the manufacturer (VectaStain ABC kit, Vector Labs Inc., Burlingame, CA).

Myogenesis: Myogenic differentiation was induced by culturing PLA cells myogenic medium (MM) for 6 weeks and confirmed by immunohistochemical staining for the muscle-specific transcription factor MyoD1 and the myosin heavy chain. Cells were rinsed twice with PBS, fixed for 20 min with 4% paraformaldehyde, and washed several times with PBS. The cells were incubated with 3% hydrogen peroxide in PBS for 10 min to quench endogenous peroxidase enzyme activity, and nonspecific sites were blocked by incubation in blocking buffer (PBS, 10% HS, 0.1% Triton X-100) for an additional 60 min. The cells were washed three times for 5 min each in blocking buffer and incubated for 1 h in blocking buffer containing either a monoclonal antibody specific to skeletal muscle myosin heavy chain (Biomeda, Foster City, CA) or to MyoD1 (Dako Corp, Carpenteria, CA). The cells were washed extensively in blocking buffer and the monoclonal antibodies detected using the VectaStain ABC kit according to manufacturer's specifications. The cells were counterstained with hematoxylin for 3 min.

RESULTS

In this study, we examined the constituent cell types and multilineage potential of a putative mesodermal stem cell population obtained from human adipose tissue. Human adipose tissue was obtained by suction-assisted lipectomy (*i.e.*, liposuction), and the lipoaspirates were processed based on adapted methodologies,[34] to obtain a PLA cell population containing the putative stem cell fraction. Processing of 300 cc of liposuctioned tissue routinely yielded PLA samples of $2–6 \times 10^8$ cells. PLA cultures were maintained in DMEM supplemented with 10% FBS. Supplementation with FBS has been shown to be important for human and animal MSC attachment and proliferation *in vitro*.[16,51,52] However, studies suggest that proliferation and differentiation of human MSCs may be dependent upon FBS source and quality, making sera screening critical.[51,52] PLA cells expanded easily *in vitro* and exhibited a fibroblast-like morphology, consistent with that of MSCs obtained from bone marrow and a commercial source (Fig. 1A). PLA cells did not appear to require specific sera lots for expansion and multilineage differentiation. Ten FBS lots from three manufacturers were tested and did not appear to alter PLA cell morphology, proliferation rate, or their differentiative capacity *in vitro* (data not shown).

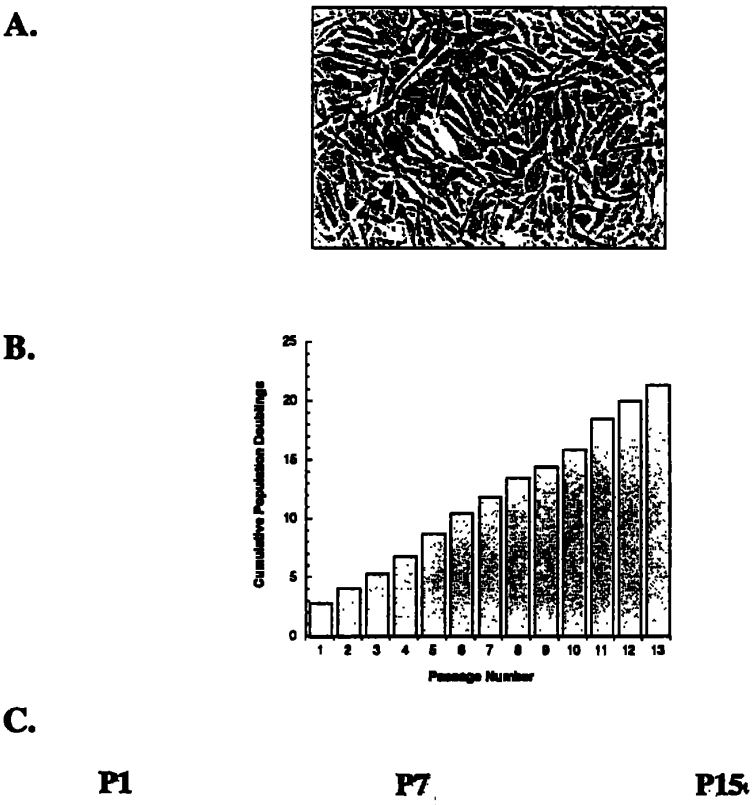

FIG. 1. Morphology, growth kinetics and senescence of PLA cells over long-term culture. (A) The morphology of a processed lipoaspirate or PLA obtained from liposuctioned adipose tissue is shown. (B) PLA cells, obtained from 3 donors, were cultured for an extended period and cumulative population doubling was measured and expressed as a function of passage number. (C) Senescence in PLA cultures was detected by staining cells at passages 1, 7, and 15 (P1, P7, and P15, respectively) for β-Gal expression at pH 6.0. Representative senescent cells are shown (arrows).

Growth kinetics and composition of the PLA

PLA cells, obtained from 20 donors and cultured under standard conditions (*i.e.*, 10% FBS), exhibited an average population doubling time of 60 h using several sera sources and lots (data not shown). Following isolation, an initial lag time of 5–7 days was observed in PLA cultures (data not shown). Cells then entered a proliferative phase, reaching confluence within 48 h. To examine long-term growth kinetics of PLA cultures, we measured cumulative population doublings with respect to passage number in multiple donors. Consistent with the observed lag time upon initial culture, PLA cells underwent an average of three population doublings prior to the first passage (Fig. 1B). An average of 1.5 population doublings was observed upon subsequent passages. A linear relationship between cumulative population doubling and passage number was observed, indicating a relatively constant population doubling rate over the range studied. Fur-

thermore, no appreciable decrease in cumulative population doublings was observed at later passages (P13 = 165 days in culture), suggesting that PLA cultures maintain their proliferative potential during extended culture periods.

In addition to cumulative population doubling, we also examined cell senescence in long-term PLA cultures using a β-Gal staining protocol, in which β-Gal expression is absent in proliferating cells but can be detected in senescent cells at a pH of 6.0.[45] Using this assay, PLA cultures were examined for senescence at each passage. PLA cultures at passage 1 exhibited no appreciable β-Gal staining (Fig. 1C, P1). An increase in β-Gal staining was observed at later passages (P7 and P15); however, the percentage of senescent cells remained below 5% through 10 passages and increased to 15% at passage 15 (data not shown). Taken together, the data indicate that PLA samples are relatively stable over long-term culture, maintaining a consistent population doubling rate and exhibiting low levels of senescence.

The SVF processed from excised adipose tissue is a heterogenous population including mast cells, endothelial cells, pericytes, fibroblasts, and lineage-committed progenitor cells, or preadipocytes.[29,33] These components may also be present, together with the putative stem cell fraction, in the PLA obtained from liposuctioned adipose tissue. However, no literature regarding this has been published. To characterize the PLA phenotypically, samples from several donors were examined by indirect immunofluorescence using antibodies specific to established cell-surface markers. A bone marrow stromal fraction obtained from human marrow aspirates was also examined as a control. To identify endothelial cells, PLA cells were incubated with a monoclonal antibody to FVIII.[39,40] Smooth muscle cells were identified using a monoclonal antibody to smooth muscle actin.[43,44] This antibody has also been shown to react with transitional pericytes (*i.e.*, pericytes of pre- and post-capillaries) and the contractile apparatus of pericytes committed to the smooth muscle lineage.[37,53] Low levels of endothelial cells, smooth muscle cells and pericytes were observed in the PLA (Fig. 2). In comparison, no staining for these markers was observed in processed bone marrow stromal samples. In addition to FVIII and smooth muscle actin, cells were also incubated with a monoclonal antibody (ASO2) specific to fibroblasts and mesenchymal cells.[41,42] The majority of the PLA and bone marrow stromal cells stained positively with ASO2, suggesting a mesenchymal origin (Fig. 1, ASO2 panels).

To determine PLA composition quantitatively, samples were analyzed by flow cytometry using the cell-surface markers described above. PLA samples were obtained and cultured for 72 h in control medium. Cell size and granularity were measured using forward- and side-scatter settings (Fig. 3A). The majority of the PLA sample was comprised of small, agranular cells. In addition, PLA cells were incubated with monoclonal antibodies to FVIII, smooth muscle actin, and ASO2 and a monoclonal antibody to vimentin, an intermediate filament protein found predominantly in cells of mesenchymal origin.[43,44] Viability was assessed using propidium iodide and samples were corrected for viability, nonspecific fluorescence, and autofluorescence. Data are shown from a representative patient (Fig. 3B). Cytometry data was collected from 5 donors, and the number of positive events for each cell-specific marker was expressed as a percentage of the total PLA cell number. Consistent with the immunofluorescent data, a fraction of the PLA cells expressed FVIII (FVIII-positive cells = 24.9% ± 8.2 of total PLA cell number) and SMA (SMA-positive cells = 29.2% ± 2.1 of total PLA cell number) (Fig. 3C), indicating that the PLA contains endothelial cells, smooth muscle cells, and, possibly, pericytes. Furthermore, the majority of the PLA cells stained positively for ASO2 (ASO2-positive cells = 85.0% ± 12.8 of total PLA cell number) and vimentin (VIM-positive cells = 63.2% ± 5.6 of total cell number), indicative of cells of mesenchymal origin. Taken together, the results suggest that the PLA is a relatively homogenous population of mesodermal or mesenchymal cells with low contamination by endothelial cells, pericytes, and smooth muscle cells.

PLA cells exhibit multilineage potential

To study the multilineage capacity of PLA cells, cells were differentiated toward the adipogenic, osteogenic, chondrogenic, and myogenic lineages using lineage-specific induction factors (Table 1). Human and animal bone marrow-derived MSCs have been shown to differentiate toward the adipogenic, osteogenic, and chondrogenic lineages with appropriate medium supplementation.[8,14,15,31,33] Following induction, differentiation was assessed using histology and immunohistochemistry (Table 2). Commercially available

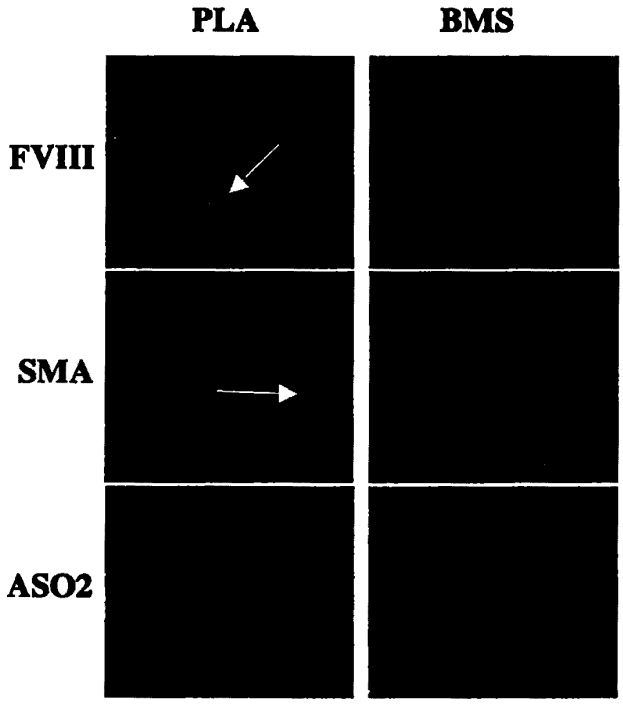

FIG. 2. Composition of the PLA: indirect immunofluorescence. PLA cells (PLA), in addition to bone marrow stromal cells (BMS), were processed for immunofluorescence using the following antibodies to cell type-specific markers: (1) anti-Factor VIII (FVIII); (2) anti-smooth muscle actin (SMA); and (3) ASO2 (ASO2). Factor VIII- and smooth muscle actin-expressing cells are shown (arrows).

MSCs and lineage-committed progenitor cells served as positive controls whereas PLA cells maintained in control medium and HFF cells were examined as negative controls.

Preadipocytes and MSCs treated with adipogenic induction medium, containing cAMP agonists and induction agents such as isobutyl-methylxanthine (IBMX), indomethacin, insulin, and dexamethasone, develop lipid-containing droplets that accumulate the lipid dye Oil Red-O.[8,54,55] To determine if PLA cells undergo adipogenesis, cells were cultured in medium containing these agents (adipogenic medium, AM) and stained with Oil Red-O. PLA cells cultured in AM were reproducibly induced toward the adipogenic lineage as early as 2 weeks post-induction (Fig. 4). A significant fraction of the cells contained multiple, intracellular lipid-filled droplets that accumulated Oil Red-O. The Oil Red O-containing PLA cells exhibited an expanded morphology with the majority of the intracellular volume (90–98%) occupied by lipid droplets, consistent with the phenotype of mature adipocytes. The mean level of adipogenic differentiation measured in 6 donors under 35 years of age was 42.4% ± 10.6% (% Oil Red O-positive cells/total PLA cell number; data not shown). Prolonged culture times (*i.e.*, 4 weeks) resulted in the detachment of differentiating cells from the culture plate and flotation to the surface (data not shown). The observed morphology and lipid accumulation of differentiated PLA cells were similar to that observed upon treatment of bone marrow-derived MSCs and the preadipocyte cell line 3T3-L1 in AM. No lipid droplets were observed in undifferentiated PLA cells or in HFF negative controls (data not shown). In contrast to MSCs, in which adipogenic differentiation dramatically decreases beyond the third culture passage,[56] the adipogenic potential of PLA cells was maintained over long-term culture (*i.e.*, passage 15 = 175 days culture) (data not shown). Taken together, the results indicate that PLA cells undergo adipogenic differentiation.

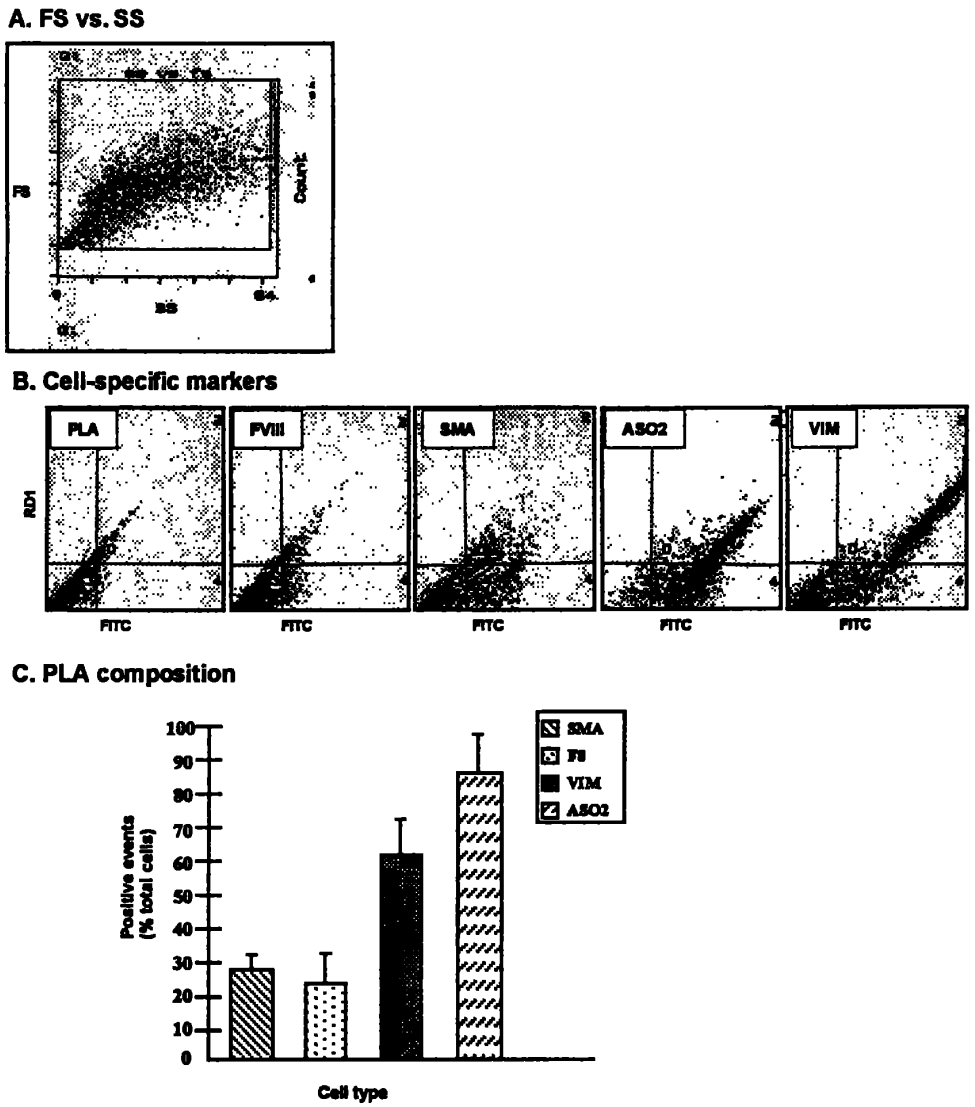

FIG. 3. Composition of the PLA: flow cytometry. (A) PLA samples were examined by flow cytometry using forward and side scatter (FS and SS, respectively). A representative PLA sample is shown. (B) The cell composition of a representative PLA sample from one donor (PLA) was determined by incubating the sample with the following monoclonal antibodies: anti-Factor VIII (FVIII), anti-smooth muscle actin (SMA), ASO2, and a monoclonal antibody to vimentin (VIM), an additional marker for cells of mesenchymal origin. (C) Flow cytometry data from 5 donors was collected and the mean number of positive events for each cell-specific marker is expressed as a percentage of total PLA cell number.

Differentiation of osteoprogenitor cells and marrow-derived MSCs into osteoblasts is induced *in vitro* by treating cells with low concentrations of ascorbic acid, β-glycerophosphate, and dexamethasone.[8,15,56] Early differentiation of these cells into immature osteoblasts is characterized by AP enzyme activity with human MSCs expressing AP as early as 4 days and maximum levels observed at 12 days post-induction.[57] To confirm their osteogenic capacity, PLA cells were treated with OM for 14 days, and the expression of AP was

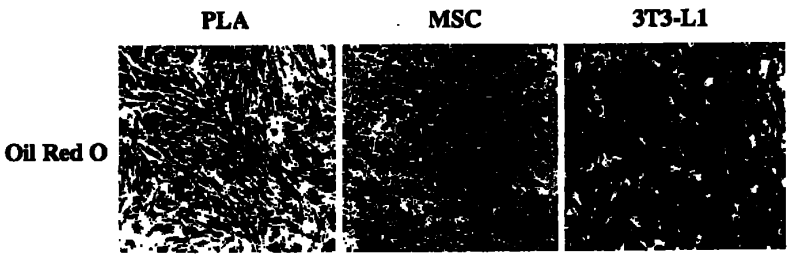

FIG. 4. PLA cells accumulate lipid-filled droplets upon treatment with adipogenic medium (AM). PLA cells (PLA), bone marrow-derived MSCs (MSC), and 3T3-L1 preadipocyte cells (3T3-L1) were cultured for 2 weeks in AM and stained with Oil Red O to identify lipid-filled intracellular vacuoles. Undifferentiated PLA cells maintained in control medium (−ve Control) were stained as a negative control.

examined. PLA cells cultured in OM formed an extensive network of dense, multilayered nodules that stained positively for AP (Fig. 5). The mean number of AP-positive staining cells measured in 6 donors was 50.2% ± 10.8% of total PLA cell number (data not shown). Expression of AP was also observed in both MSCs and NHOst-positive controls maintained in OM. In contrast, undifferentiated PLA cells and HFF negative controls (data not shown) did not show evidence of AP expressions. Although AP expression is dramatically upregulated in osteogenic tissues, its expression has been observed in several nonosteogenic cell types and tissues such as cartilage, liver, and kidney.[58–60] Therefore, AP expression is frequently used, in conjunction with other osteogenic-specific markers, as an indicator of osteogenesis. One such indicator is the formation of a calcified ECM. Mature osteoblasts secrete a collagen I-rich ECM that becomes calcified during the later stages of differentiation.[61] Therefore, to confirm osteogenic differentiation, calcification of the ECM matrix was assessed in PLA cells using a von Kossa stain. Calcification appears as black regions within the cell monolayer. Consistent with osteogenesis, several black regions, indicative of a calcified ECM, were observed in PLA cells treated for 4 weeks in OM. Calcification was also identified in MSC and NHOst-positive controls, whereas no calcification was observed in undifferentiated PLA cells or HFF cells (data not shown). The osteogenic potential of PLA cells was maintained over long-

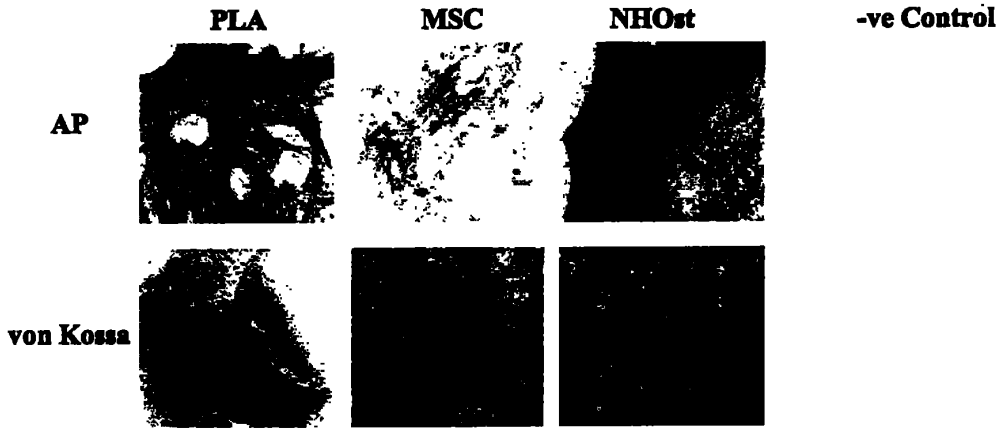

FIG. 5. PLA cells induced with osteogenic medium (OM) express AP and are associated with a calcified ECM. PLA cells (PLA), MSCs (MSC), and a human osteoblast cell line (NHOst) were cultured in OM to induce osteogenesis. Cells were stained at 2 weeks for AP activity (AP; red). The presence of a calcified extracellular matrix (black regions) was examined at 4 weeks (von Kossa). Undifferentiated PLA cells maintained in control medium were examined for AP expression and matrix calcification as a negative control (−ve Control).

term culture, with cells expressing AP as late as 175 days of culture (data not shown). Taken together, the expression of AP by PLA cells and the production of a calcified ECM strongly suggest that these adipose-derived cells can be induced toward the osteogenic lineage.

Chondrogenic differentiation can be induced *in vitro* using a micromass culture technique, in which cellular condensation (a critical first event of chondrogenesis) is duplicated.[47–49,62] Enhanced differentiation can be obtained by treating cells with dexamethasone and transforming growth factor-β1 (TGF-β1).[63] Marrow-derived MSCs, cultured with these agents under micromass conditions, form cell nodules associated with a well-organized ECM rich in collagen II and sulfated proteoglycans.[8,64] These sulfated proteoglycans can be specifically detected using the stain Alcian Blue under acidic conditions.[50] To assess the chondrogenic capacity of PLA cells, cells were cultured via micromass in CM, containing dexamethasone and TGF-β1. Micromass culture of PLA cells resulted in the formation of dense nodules consistent with chondrogenic differentiation. The PLA nodules were associated with an Alcian Blue-positive ECM, indicative of the presence of sulfated proteoglycans within the matrix (Fig. 6). Cartilaginous nodules were also observed upon micromass culture of MSC controls. To confirm the specificity of Alcian Blue for cartilaginous matrices, human cartilage and bone sections were stained with Alcian Blue under acidic conditions. As expected, human cartilage sections stained positively with Alcian Blue, whereas no staining was observed in bone sections (data not shown). In addition to the presence of sulfated proteoglycans within the ECM, both PLA cells and human cartilage sections expressed the collagen type II isoform, but no staining was observed in undifferentiated PLA cells. Consistent with adipogenic and osteogenic differentiation, PLA cells retained their chondrogenic differentiation potential after extended culture periods (*i.e.*, up to 175 days; data not shown). The above results suggest that PLA cells possess the capacity to differentiate toward the chondrogenic lineage.

Myogenesis is characterized by a period of myoblast proliferation, followed by the expression of muscle-specific proteins and fusion to form multinucleated myotubules. Early myogenic differentiation is char-

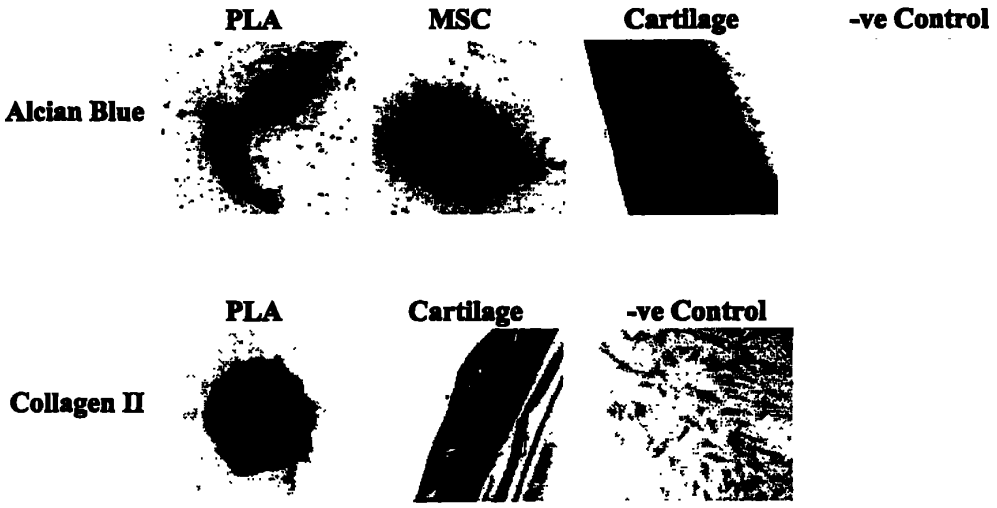

FIG. 6. PLA cells treated with chondrogenic medium (CM) are associated with a proteoglycan-rich matrix and express collagen type II. PLA cells (PLA) and MSCs (MSC) were cultured for 2 weeks in CM using the micromass technique to induce chondrogenesis. The cells were fixed and processed for the presence of sulfated proteoglycans with Alcian Blue under acidic conditions (Alcian Blue). Paraffin sections of human cartilage were used as a positive control (Cartilage) while undifferentiated PLAs maintained in control medium were processed as a negative control (−ve Control). In addition, the expression of collagen type II (Collagen II) was examined in PLA cells and human cartilage sections. PLA cells cultured in control medium (−ve Control) were stained with Alcian Blue and for collagen II expression as a negative control.

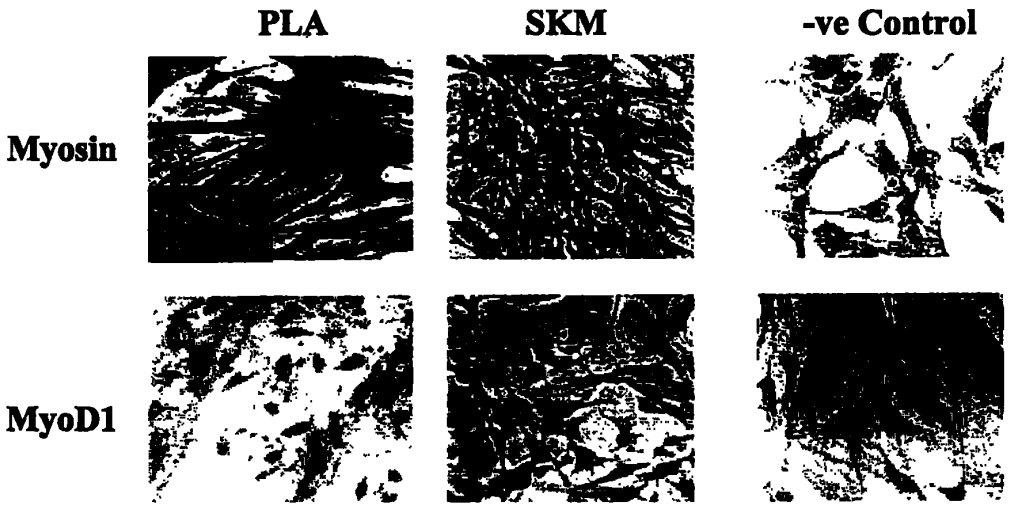

FIG. 7. PLA cells cultured in myogenic medium (MM) express the myosin heavy chain and MyoD1. PLA cells (PLA) were treated with MM and stained with antibodies specific to skeletal muscle myosin heavy chain (Myosin) or MyoD1 (MyoD1). A human skeletal muscle cell line (SKM) was examined as a positive control. In addition, the presence of multinucleated cells in PLA cultures is shown (PLA, inset box). Myosin and MyoD1 expression were also assessed in undifferentiated PLA cells (−ve Control) as a negative control.

acterized by the expression of several myogenic regulatory factors including myogenic determination factor 1 (MyoD1).[65–67] Terminally differentiated myoblasts can be characterized by the expression of myosin and the presence of multiple nuclei.[68] Proliferation and myogenic differentiation of muscle precursors and bone marrow-derived stem cells can be induced by dexamethasone and results in the expression of muscle-specific proteins.[14,69,70] Furthermore, addition of hydrocortisone is known to stimulate human myoblast proliferation, prior to their transition into differentiated myotubules.[71] To examine if PLA cells undergo myogenesis, cells were cultured for 6 weeks in the presence of dexamethasone and hydrocortisone, and incubated with antibodies specific to MyoD1 and myosin (heavy chain). Consistent with early myogenic differentiation, treatment of PLA cells with MM for 1 week induced the expression of MyoD1 (Fig. 7). PLA cells treated for longer time periods (6 weeks) stained positively for myosin. In addition to myosin expression, the presence of discrete 'patches' of large, elongated cells with multiple nuclei were also observed, suggesting that PLA cells underwent myoblast fusion (PLA panel, inset). Like PLA cells, MyoD1 and myosin heavy-chain expression were also detected in human skeletal muscle-positive control cells. Using myogenic medium, myogenic differentiation was not observed in MSC controls even at 6 weeks of induction (data not shown). These cells may be adversely affected by hydrocortisone and may require alternate conditions to induce differentiation. Myogenic differentiation levels in PLA cells averaged 12% (data not shown). Multinucleation, myosin heavy-chain, and MyoD1 expression were not observed in undifferentiated PLA cells nor in HFF negative controls (data not shown). The presence of multinucleated cells and the expression of both MyoD1 and myosin heavy chain suggest that PLA cells have the capacity to undergo myogenic differentiation.

DISCUSSION

In this paper, we report that a cellular fraction with multiple mesodermal lineage capabilities can be processed from human lipoaspirates. This cellular fraction, which we call a processed lipoaspirate (PLA),

is comprised of fibroblast-like cells that can be expanded easily *in vitro* without the need for specific sera lots or media supplementation. PLA samples, from several donors, maintained a linear growth rate with no appreciable senescence over extended culture periods. The PLA population was heterogeneous in nature, with the majority of the cells being mesenchymal in origin. However, contaminating endothelial, smooth muscle, and pericyte cell populations were identified. PLA cells also exhibited multilineage potential *in vitro*, differentiating toward the adipogenic, osteogenic, chondrogenic, and myogenic lineages when cultured in the presence of established lineage-specific differentiation factors. PLA differentiation results were consistent with those observed upon lineage-specific differentiation of bone marrow-derived MSCs and lineage-committed precursors.

Although the apparent multidifferentiative capacity of PLA cells suggests the presence of a stem cell population within human liposuctioned adipose tissue, it is not conclusive. Multilineage differentiation may also be due to the presence of: (1) multiple lineage-committed progenitor cells; (2) multipotent cells from other sources (*e.g.*, pericytes, marrow-derived MSCs from peripheral blood); or (3) a combination of the above.

The observed differentiation may be due to the presence of lineage-committed progenitor cells, such as preosteoblasts, premyoblasts, or preadipocytes within the PLA. Cellular fractions (*i.e.*, SVFs) obtained from excised adipose tissue are known to contain preadipocytes that differentiate into mature adipocytes.[29,30] It is possible that the observed adipogenic differentiation by PLA cells is simply the commitment of existing preadipocytes and not the differentiation of a multipotent cell. However, we do not believe this to be the case. As little as 0.02% of the SVF obtained from excised adipose tissue have been identified as preadipocytes capable of adipogenic differentiation.[29] If preadipocyte numbers within the PLA are comparable to those levels measured in the SVF from excised tissue, one would expect a relatively low level of adipogenesis. However, the degree of adipogenesis observed in the PLA is significant (~40% of the total PLA cell number) and may result from the differentiation of additional cell types (i.e. stem cells).

Damage to the underlying muscle during liposuction may introduce myogenic precursor cells or satellite cells into the PLA, resulting in the observed myogenic differentiation by these cells. Located between the sarcolemma and the external lamina of the muscle fiber, myogenic precursor cells in their undifferentiated state are quiescent and exhibit no distinguishing features, making their identification difficult. Several groups have attempted to identify unique markers for these precursors with limited success. Currently, the expression of the myogenic regulatory factors, MyoD1, and myogenin have been used to identify satellite cells during embryogenesis and in regenerating adult muscle in rodents.[72–76] In addition, MyoD1 expression has been identified in proliferating myoblasts prior to the onset of differentiation.[66] Although these markers have not been used to identify myogenic precursors in human subjects, MyoD1 is expressed during early myogenic differentiation in normal skeletal muscle and has been used to identify the skeletal muscle origin of rhabdosarcomas in humans.[77–79] The absence of MyoD1 expression in PLA cells maintained in noninductive CM (see Fig. 7), suggests that our observed myogenic differentiation is not due to the presence of myogenic precursors or proliferating myoblasts within the PLA. Consistent with this, the blunt contour of the liposuction cannula would make it extremely difficult to penetrate the fibrous fascial cavity surrounding the muscle and introduce these precursors into the adipose compartment.

Human adipose tissue is vascularized and, as such, contains potential systemic vascular 'conduits' for contamination by multipotent cells, such as pericytes and marrow-derived MSCs. Disruption of the blood supply during liposuction may result in the release of pericytes, known to possess multilineage potential both *in vivo* and *in vitro*.[80–82] Consistent with this, our immunofluorescent and flow cytometry data show that a small fraction of the PLA is comprised of cells that express smooth muscle actin, a component of transitional pericytes and pericytes committed to the smooth muscle lineage.[37] The multilineage differentiation observed in PLA may be, in part, due to the presence of pericytes. Disruption of the blood supply may also introduce MSCs into the PLA. However, the literature is conflicted as to the presence of these stem cells in the peripheral blood.[83,84] If the peripheral blood does indeed represent a source of MSCs, our observed multilineage differentiation by PLA cells may be due to the contamination of adipose tissue by these stem cells. However, MSCs are a small constituent of the bone marrow stroma in humans (~1 MSC per 10^5 adherent

stromal cells.[8,17,20] If these cells do exist in peripheral blood, they are likely to be in even smaller quantities than in the bone marrow and contamination levels of the PLA by these cells may be negligible.

These arguments provide support for the presence of a multipotent stem cell population within liposuctioned adipose tissue; however, definitive confirmation requires the isolation and characterization of multiple clones derived from a single cell. Preliminary data confirm that clonal PLA cell populations possess multilineage potential, capable of adipogenic, osteogenic, and chondrogenic differentiation.[85] Although promising, isolation and analysis of multiple PLA cell clones will be required to confirm the presence of a stem cell population within liposuctioned adipose tissue.

The future of engineering mesodermally derived tissues from stem cells is promising and the development of these strategies will likely require a readily available source of donor cells. Current research has demonstrated exciting results using bone marrow-derived MSCs. MSCs can differentiate into osteogenic and chondrogenic tissues *in vivo*,[86–89] and preliminary data suggest that these cells can be used to repair bony and cartilagenous defects.[12,88–92] We believe that PLA cells obtained from liposuctioned adipose tissue may represent another source of multilineage mesodermal stem cells. Like the bone marrow stroma, our data suggest that adipose tissue may contain a significant fraction of cells with multilineage capacity. These adipose-derived stem cells may be readily available in large quantities with minimal morbidity and discomfort associated with their harvest. The autologous nature of these stem cells, together with their putative multipotentiality and ease of procurement, may make these cells an excellent choice for many future tissue engineering strategies and cell-based therapies.

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