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脂肪由来幹細胞の臨床応用への展開
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脂肪組織由来多系統前駆細胞を用いた重症心不全治療細胞組織加工医薬品の開発

Adipose tissue-derived multi-lineage progenitor cells as promising tool for cardiac regenerative medicine

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Summary 頻回の虚血障害により残存心筋幹細胞が消失する虚血性心筋症など重症心不全においては，これまでの内科的治療も十分な効果を上げえず，PCI(percutaneous coronary intervention)による血流改善も限定的な効果しかない．これら治療抵抗性の重症心不全end-stageにあっては1年死亡率が75%とされ，新規治療法・医薬品の開発が待たれている．著者らは，大量に簡便・安全・容易に採取可能なヒト皮下脂肪組織から新規間葉系幹細胞として，脂肪組織由来多系統前駆細胞の単離・培養法を確立した．当該細胞から誘導した心筋芽様細胞が慢性心筋梗塞モデルラットに移植した結果，心機能と長期生存率を改善し，被投与細胞を慢性心筋梗塞モデルラット心筋組織内で心筋細胞への分化を組織学的に確認している．非臨床試験として，単回投与毒性試験(経左心室腔内投与・経静脈投与)を中枢・呼吸安全性薬理試験(GLP)下で実施した．毒性を認めず，特殊毒性試験としては，造腫瘍試験，軟寒天コロニー形成試験，核型分析試験をGLPにて終了している．薬理試験のうち安全性薬理コアバッテリー試験では，GLPが終了している．このように著者らは，自らが行ってきた研究の成果を1日でも早く社会に還元するため，当初から薬事開発をめざしている．

Key word 脂肪組織由来多系統前駆細胞(ADMPCs)，心筋再生，*in situ* stem cell therapy，薬事開発，非臨床試験

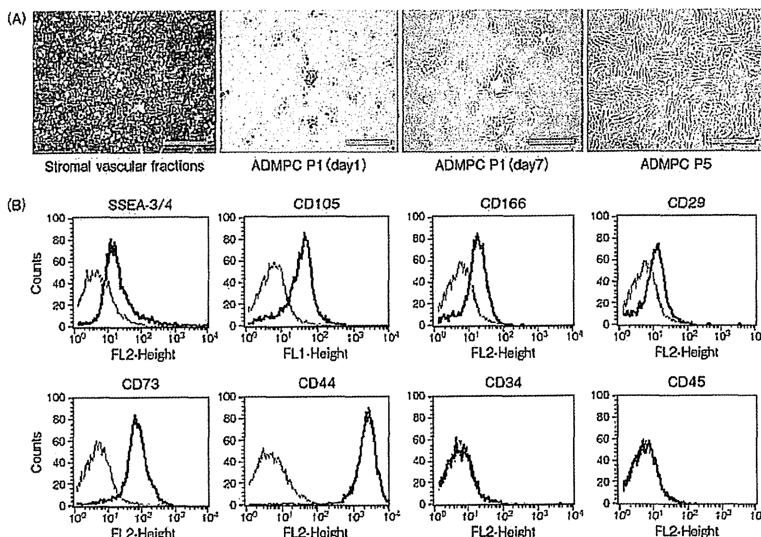

図 1 脂肪組織由来多系統前駆細胞の特性

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◎頻回の虚血障害により残存心筋幹細胞が消失する虚血性心筋症など重症心不全においては，これまでの内科的治療も十分な効果を上げえず，PCI(percutaneous coronary intervention)による血流改善も限定的な効果しかない．これら治療抵抗性の重症心不全end-stageにあっては1年死亡率が75%とされ，新規治療法・医薬品の開発が待たれている．著者らは，大量に簡便・安全・容易に採取可能なヒト皮下脂肪組織から新規間葉系幹細胞として，脂肪組織由来多系統前駆細胞の単離・培養法を確立した．当該細胞から誘導した心筋芽様細胞が慢性心筋梗塞モデルラットに移植した結果，心機能と長期生存率を改善し，被投与細胞を慢性心筋梗塞モデルラット心筋組織内で心筋細胞への分化を組織学的に確認している．非臨床試験として，単回投与毒性試験(経左心室腔内投与・経静脈投与)を中枢・呼吸安全性薬理試験(GLP)下で実施した．毒性を認めず，特殊毒性試験としては，造腫瘍試験，軟寒天コロニー形成試験，核型分析試験をGLPにて終了している．薬理試験のうち安全性薬理コアバッテリー試験では，GLPが終了している．このように著者らは，自らが行ってきた研究の成果を1日でも早く社会に還元するため，当初から薬事開発をめざしている．

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心筋梗塞に対しては，これまでも従前の治療法により多くの患者が救われてきた．しかし，頻回の虚血障害により残存心筋幹細胞が消失する虚血性心筋症など重症心不全においては，これまでの内科的治療も十分な効果を上げえず，PCI(percutaneous coronary intervention)による血流改善も限定的な効果しかない．これら治療抵抗性の重症心不全end-stageにあっては1年死亡率が75%とされ，新規治療法・医薬品の開発が待たれている．

重症心不全に対する心移植・心移植に代わる新規治療法として，補助人工心臓の開発が進められてきた．心臓移植では移植時年間5,000万円の費用，補助人工心臓においても初年度で3,000万円の費用と翌年以降1,000万円の維持費用がかかる．医療保険財政の逼迫が提起する費用対効果の問題に加え，心臓移植では絶対的ドナー不足によって，だれがレシピエントになるべきかという倫理的課題がある．補助人工心臓では，永久植込み型補助人工心臓(destination use)がその歴史30年によってさえ，いまだ開発途上にあるという問題が残っている．これら問題意識を背景に，著者らは心疾患に対する新規治療法のひとつとして再生医療研究を進めている．

心疾患を対象とする再生医療開発の現状

重症心不全に対する補助人工心臓併用細胞治療として大阪大学の骨格筋芽細胞シート技術が先鞭をつけ，テルモ株式会社が当該技術を引き継ぎ企業治験を開始している．京都府立医大では自己培養心筋幹細胞とbFGF含有ヒドロゲルの冠動脈バイパス術時同時移植によるEF 40%以上の中等度心不全に対する臨床研究が開始されている．間葉系組織のひとつである脂肪組織を用いる心不全治療としては，PRECISE試験とAPOLLO試験がヨーロッパの一部で行われている．これら細胞治療は，移植した細胞によるサイトカイン効果による血管系再構築と残存心筋幹細胞の活性化がその機序であると想定される．間葉系幹細胞を用いての心不全治療にあっては抗炎症効果を期待している場合もあろう．頻回に繰り返す心筋虚血の終末像としての虚血性心筋症では，血流が改善しても臓器幹細胞(心筋幹細胞)が枯渇するため，外部から心筋幹細胞あるいは前駆細胞を補充する必要がある．末期虚血性心筋症を対象として，有用性が予測できる再生医療研究はこれまで報告されていなかった．

脂肪組織由来細胞を用いる心筋再生医療

いままで実施されている体性幹細胞を用いる心筋再生細胞治療法は，骨格筋芽細胞移植であれば大腿筋肉から骨格筋肉組織を採取し，心筋幹細胞移植であれば心筋バイオプシーにて組織塊を得るため侵襲性が高く，同種への展開はない．死体からの採取を想定されることもあるが“墓地および埋葬に関する法律”などの規定により，日本国内では法的整備がなされないかぎり産業展開は望めない．

ヒト皮下脂肪組織は大量に簡便・安全・容易に採取可能で，倫理的に乗り越えるべきハードルは低い．自己から安全かつ簡易に採取できるのみならず，形成外科領域で手術時余剰医療廃棄物として大量に得ることができるため，現行法制度下でも自己由来細胞移植のみならず同種由来細胞移植を用いる再生医療へ展開可能な数少ない組織・細胞のひとつである．

一方，脂肪組織を採取する際のデメリットもある．欧米人と異なり，日本人は皮下脂肪採取に際して有害事象として内出血の頻度・量が多いことが知られている．とくに大量採取では疼痛を含め心負荷が過大となる可能性も否定できない．骨髄採取であってもドナーの心負荷増加による心不全悪化例があり，健常人でも死亡例の報告がある．皮下脂肪採取でもチューリップ針が腹壁を突き破ったことによる死亡例も知られており，採取にあたっては形成外科専門医などによって行われることが望ましい．

脂肪組織由来細胞に期待される心機能再生メカニズム

重症心不全は心臓のポンプ機能の低下を示す症候群であり，その発症機序と病理病態はバラエティーに富んでいる．したがって，重症心不全をきたす疾患の病理病態を要素分解し，それぞれに移植細胞に期待される薬効薬理作用を理解する必要がある．

心筋虚血を発症機序とする心不全の場合，①抗炎症作用，②血管網再生・構築，③抗線維化作用，④内因性心筋幹細胞の活性化，が期待される．心筋梗塞急性期にあっては，炎症に伴う心筋組織浮腫も虚血を増悪させ虚血再灌流障害も炎症機序の一環であるため，抗炎症作用が一義的に期待される．この点で，脂肪組織由来幹細胞をはじめとする間葉系幹細胞は抗炎症作用を有することが知られており，優位に立つ．

心筋梗塞後遠隔期(慢性期)にあっては，血管網再生・構築，抗線維化・線維退縮，加えて内因性心筋幹細胞活性化が求められる．脂肪組織をコラゲナーゼにて処理して得られるstromal vascular fractionは血管内皮細胞が豊富に含まれているため，血管網構築には有効と推測される．抗線維化・線維退縮に関しても脂肪組織由来幹細胞は，matrix metalloproteinase(MMPs)を発現しているため有効性が想定される．脂肪組織由来幹細胞を脂肪分化させると，急性心筋梗塞モデルラットでは有効であったとの報告がなされている[1)]が，arrhythmogenic rigit ventricular cardiomyopathyは心外膜直下の脂肪変性がその病態であり，また脂肪分化に従いMMPsの発現は低下するため，遠

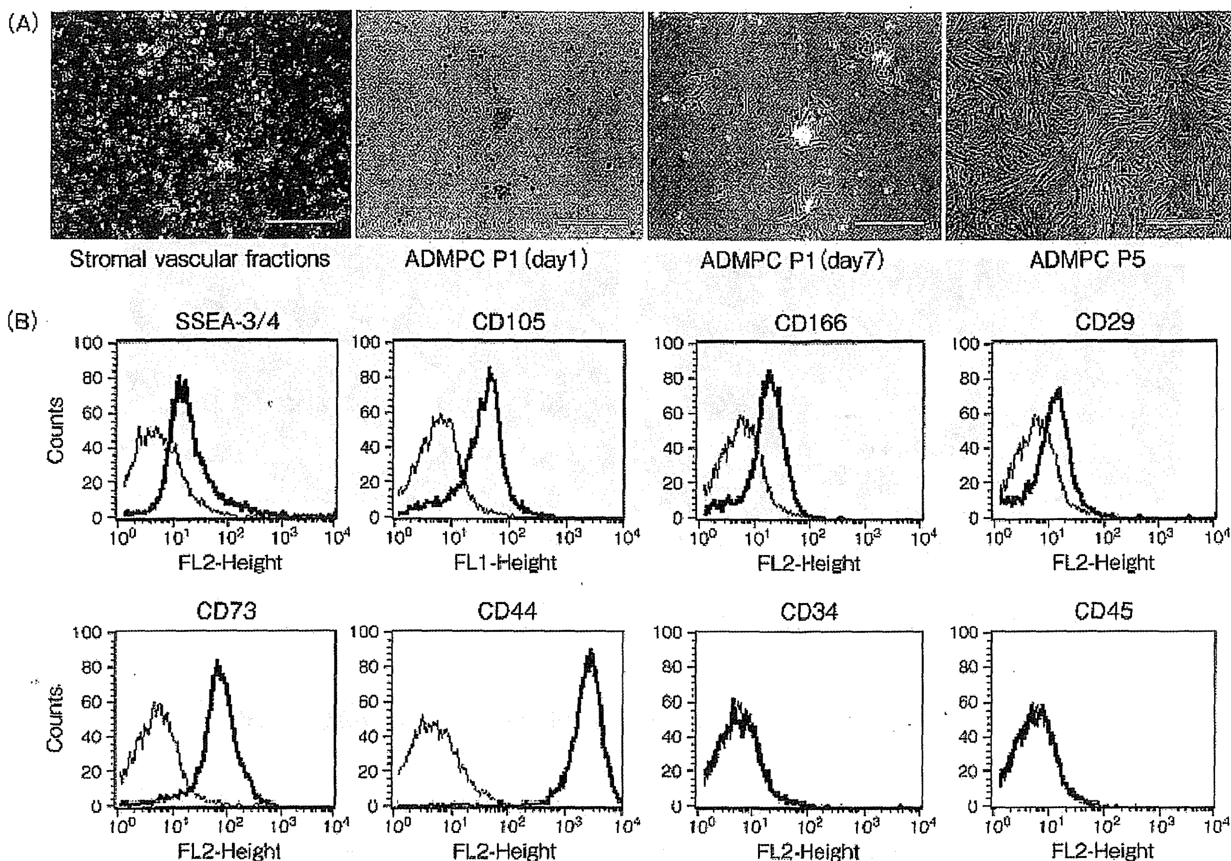

図 1　脂肪組織由来多系統前駆細胞の特性

A：SVF から EDTA 処理により剥離浮遊する細胞集団．小型単核細胞で核/細胞質比が大きく，ゴルジ体が未発達である．スケールバー：100 μm．

B：脂肪組織由来多系統前駆細胞の細胞表面マーカー．多くの間葉系幹細胞と同様に CD105/CD166/CD29/CD73/CD44 が陽性で CD34/CD45 陰性．加えて，SSEA-3/4 が陽性という特徴を有する．

隔期心筋梗塞に用いるには今後の検討をまたなければならない．

内因心筋幹細胞活性化においては，移植細胞による，いわゆるサイトカイン効果が期待され，著者らは cell-based cytokine delivery とよんでいる．移植細胞から分泌されるサイトカインには血管網を再生構築するもののみならず，HGF をはじめとする内因性残存心筋幹細胞を活性化させるものもある．この作用機序を基盤とし，ADSC をシート化して移植することで心筋梗塞モデルラットの心機能を改善させたとの報告がある[2]．頻回に心筋虚血を繰り返すと，内因性心筋幹細胞も消耗・枯渇してしまう．この病態が虚血性心筋症であり，細胞治療による有効性を期待するのであれば，心筋幹(前駆)細胞あるいは心筋細胞が移植される必要がある．

心筋虚血を発症機序としない心不全として，拡張型心筋症があげられる．これは心筋細胞・心筋幹細胞そのものの疾患であり，外部から心筋(幹)細胞の移植が治療法となる．遺伝性心疾患(ミトコンドリア脳筋症など)であれば，従前のサイトカイン効果を期待する再生細胞治療は無効である．

新規間葉系幹細胞としての脂肪組織由来多系統前駆細胞(ADMPCs)

ヒト脂肪組織由来多系統前駆細胞(human adipose tissue-derived multilineage progenitor cells：hADMPCs；図 1)は間葉系幹細胞のひとつであり，ヒト脂肪組織に由来し接着培養系においてEDTA 反応性の違いから獲得される細胞群で，自己複製能ならびに骨芽細胞，軟骨細胞および脂肪細胞への分化能を有する[3]．

これまでにヒト脂肪組織からはさまざまな stemness を有する間葉系幹細胞が見出され，そ

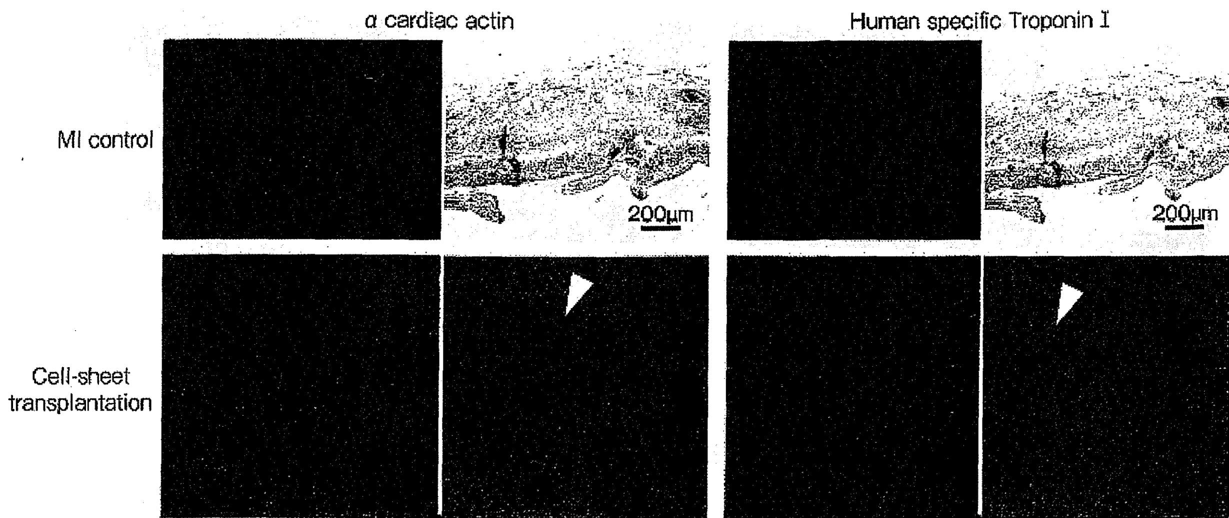

図 2　慢性心筋梗塞モデルラット心筋組織内における心筋細胞への分化
ヒト脂肪組織由来多系統前駆細胞が，慢性心筋梗塞モデル免疫不全ラット心筋組織内で心筋細胞へ分化することを組織学的に確認した．α-cardiac actin 陽性細胞のうち，心外膜側の1層(矢頭)がヒト特異的 troponin I 陽性である．

の採取方法が報告されている．1978年にはBjörntorp らが皮下脂肪に脂肪幹細胞(preadipocyte)の存在を見出し，その単離培養法の確立に成功している[4]．1999年には脂肪組織もその範疇に入る間葉系組織に“間葉系幹細胞”が存在することが報告されている[5]．2001年にはZuk らが，脂肪幹細胞は“間葉系幹細胞”としてのpotencyを有していることを報告し，脂肪組織由来幹細胞(adipose tissue derived stromal cells：ADSCs)とした[6]．著者らは，脂肪組織をコラゲナーゼ処理した後に得られるstromal vascular fractionのなかから新規の間葉系幹細胞として，ADSCsとは異なる細胞群(hADMPCs)の単離培養法を確立し，当該細胞から肝細胞・膵島細胞の分化誘導法を報告している[7-11]．

hADMPCsは，従前報告されている各種間葉系幹細胞と同様に骨芽細胞，軟骨細胞，脂肪細胞への分化能を有する[3]．多くの間葉系幹細胞と同様にCD90, CD105, CD166を発現するのみならず，SSEA-3/4を発現し，心筋指向核内転写因子であるIslet-1やNkx2.5を発現している．また，多くの間葉系幹細胞と同様にMHC class IIを発現せず，ウシ胎仔血清含有培地での培養でも拒絶反応が低い[3]．

脂肪組織由来多系統前駆細胞を用いる心筋再生

hADMPCsが心筋芽様細胞に誘導され，慢性心筋梗塞モデルラットへの移植で心機能と長期生存率が改善することを著者らは見出した．また，被投与細胞が慢性心筋梗塞モデルラット心筋組織内で心筋細胞に分化することを組織学的に確認している(図2)[12]．

齧歯類で有効性を確認しても，first-in-manへの根拠としては薄弱である．そこで著者らは，低分子化合物における非臨床試験項目(表1)を参考に非臨床試験の組み立てを行い，ヒト幹細胞臨床研究あるいは治験(薬事開発)をめざしている．

毒性試験のうち単回投与毒性試験(一般毒性試験)として，齧歯類(ヌードラット)を用いた安全性用量設定試験(non-GLP)にて安全性を担保する投与細胞用量を見出し，当該最大安全用量で単回投与毒性試験(経左心室腔内投与・経静脈投与)をGLP下で実施し，その結果毒性は認められなかった．hADMPCsから誘導する心筋芽様細胞にあっては投与後心筋細胞として生着機能することから，単回投与でも長期細胞に曝露された状態となり，蓄積毒性を考慮する必要はないと考えた．

特殊毒性試験としては細胞固有特性の評価として造腫瘍試験，軟寒天コロニー形成試験，核型分析試験をGLPにて終了し，当該細胞の遺伝毒性試

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表 1　低分子化合物で求められる非臨床試験項目

試験の種類	試験の内容
1．毒性試験	
1）一般毒性試験	
単回投与毒性試験	1回投与後の毒性(安全性用量設定試験含む)
反復投与毒性試験	反復投与後の毒性
2）特殊毒性試験	
癌原性試験	発癌性
抗原性試験	アレルギー反応
遺伝毒性試験	DNA，染色体に対する影響
生殖・発生毒性試験	生殖細胞の形成，受精・着床，胚・胎児の発育，妊娠・分娩，授乳などに対する毒性
その他	局所刺激試験，依存性試験，光毒性試験など
2．薬理試験	
1）安全性薬理試験	
コアバッテリー試験	生命機能(中枢神経系，心血管系，呼吸器系)に対する作用
フォローアップ試験	コアバッテリー試験で問題がある場合に，さらに詳細な追加検討
補足的安全性薬理試験	コアバッテリー試験以外の器官系(腎・泌尿器系，自律神経系，消化器系など)に対する作用
2）薬効薬理試験	効能・効果を裏づける試験(有効性用量設定試験含む)
3．薬物動態試験	
吸収	薬物吸収の程度・速度，生物学的利用能
分布	組織分布，蛋白結合など
代謝	代謝物，代謝酵素，酵素阻害など
排泄	排泄経路，排泄速度など
トキシコカイネティクス試験	毒性試験時の血中濃度
4．製剤学的試験	非臨床試験，治験に使用する薬剤の製剤化，品質評価など

験をほぼ終了している．

薬理試験のうち安全性薬理コアバッテリー試験では中枢・呼吸安全性薬理試験(GLP)が終了し，中枢毒性，呼吸毒性ともに認められなかった．安全性循環薬理試験については通常のコアバッテリー試験では不十分と想定されるため，不整脈観察を中心にフォローアップ試験項目の追加が必要となろう．薬効薬理試験は効能効果を裏づける試験であり，有効性用量設定試験を実施している．

薬物動態試験に相当する試験として体内動態試験(運命試験)がある．これまでの細胞の投与後体内動態は細胞を放射性同位元素標識しその分布を追うものであったが，半減期による追跡期間の限界と特異度の観点から限界があった．そこで著者らは，3頭の慢性心筋梗塞モデルブタへの細胞投与後6カ月経過観察し，30余臓器をリストアップし，それぞれについて肉眼的所見，組織学的病理所見を確認，異所性生着および慢性毒性試験として病的所見を認めないことを報告している[13]．加えて著者らは，*Alu*-PCR法を用いるヒト由来細胞の非ヒト動物体内動態追跡が可能であることを報告している[8]．

おわりに

心・血管領域を含め，脂肪組織を原材料とする再生細胞医療の臨床展開に向けた研究開発が数多く進められている．アカデミアの多くは厚生労働省の“ヒト幹細胞を用いる臨床研究に関する指針”に基づく臨床研究として研究を進めている．いまだ有用性が保証されていない再生医療にあっては，試行錯誤の幅が広い臨床研究というトラックを用いるのは首肯できる．しかし，医師法・医療法下での臨床研究は“医師が自ら”実施し，かつ院内で研究が貫徹することを想定しており，企業参入の余地はない．これは医療法15条2項問題といわれ，医師が医療法のもと外部委託できるポジティブリストに細胞単離あるいは培養委託が明示されていないため，法的に不安定かつ保険診療への出口がないことによる．薬事上の製造販売を受けなければ，細胞製剤の上市はできず，広く国民

に行き渡らない．

著者らは医療者として，自らが行ってきた研究の成果を1日でも早く社会に還元するため，当初から薬事開発をめざしている．

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Addition of Mesenchymal Stem Cells Enhances the Therapeutic Effects of Skeletal Myoblast Cell-Sheet Transplantation in a Rat Ischemic Cardiomyopathy Model

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Introduction: Functional skeletal myoblasts (SMBs) are transplanted into the heart effectively and safely as cell sheets, which induce functional recovery in myocardial infarction (MI) patients without lethal arrhythmia. However, their therapeutic effect is limited by ischemia. Mesenchymal stem cells (MSCs) have prosurvival/ proliferation and antiapoptotic effects on co-cultured cells *in vitro*. We hypothesized that adding MSCs to the SMB cell sheets might enhance SMB survival post-transplantation and improve their therapeutic effects.
Methods and Results: Cell sheets of primary SMBs of male Lewis rats (r-SMBs), primary MSCs of human female fat tissues (h-MSCs), and their co-cultures were generated using temperature-responsive dishes. The levels of candidate paracrine factors, rat hepatocyte growth factor and vascular endothelial growth factor, *in vitro* were significantly greater in the h-MSC/r-SMB co-cultures than in those containing r-SMBs only, by real-time PCR and enzyme-linked immunosorbent assay (ELISA). MI was generated by left-coronary artery occlusion in female athymic nude rats. Two weeks later, co-cultured r-SMB or h-MSC cell sheets were implanted or no treatment was performed ($n=10$ each). Eight weeks later, systolic and diastolic function parameters were improved in all three treatment groups compared to no treatment, with the greatest improvement in the co-cultured cell sheet transplantation group. Consistent results were found for capillary density, collagen accumulation, myocyte hypertrophy, Akt-signaling, STAT3 signaling, and survival of transplanted cells of rat origin, and were related to poly (ADP-ribose) polymerase-dependent signal transduction.
Conclusions: Adding MSCs to SMB cell sheets enhanced the sheets' angiogenesis-related paracrine mechanics and, consequently, functional recovery in a rat MI model, suggesting a possible strategy for clinical applications.

Introduction

A RECENT LARGE-SCALE clinical trial, in which autologous skeletal myoblasts (SMBs) were directly injected into the heart by needle, reported only modest therapeutic benefits and a substantial risk of ventricular arrhythmias, due at least partly to the delivery method.[1,2] The major drawbacks of SMB delivery by needle injection are poor cell survival in the heart, leading to insufficient paracrine effects, and mechanical myocardial injury, potentially causing lethal arrhythmia.[1–3] In contrast, cell-sheet techniques, which we developed, deliver SMBs more effectively with minimal myocardial injury, enhanced paracrine effects, and consequently better cardiac function than attained by needle injection.[4–8]

The mechanism by which damaged myocardium is restored by transplanted SMB cell sheets is complex, involving many pathways.[4–8] Recent reports show beneficial effects of SMB cell-sheet transplantation in several animal experimental models and patients with heart failure, which are primarily attributed to cytokine secretion from the transplanted cell sheets (i.e., a paracrine effect).[4–9]

However, SMB cell sheets attached to the surface of the infarcted myocardium are poorly supported by the vascular

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network of the native myocardium, which limits the survival of the SMBs and, consequently, their therapeutic effects.[7] Thus, conventional SMB cell-sheet transplantation might be insufficient to repair severely damaged myocardium, which has poor viability. Mesenchymal stem cells (MSCs) are used as feeder cells to support the survival, proliferation, and differentiation of co-cultured stem/progenitor cells *in vitro*.[10–12] Moreover, MSCs are advantageous for cellular therapy because they are multipotent, potentially immune privileged, and expand easily *ex vivo*. MSCs also proliferate rapidly and induce angiogenesis.[13,14]

We hypothesized that adding MSCs to the SMB cell sheets *in vitro* might enhance their survival and function after transplantation, which might enhance the benefits of SMB cell-sheet transplantation therapy. Here, we investigated whether co-culturing SMBs with MSCs would enhance the SMBs' cytokine production *in vitro*. We also examined the therapeutic effects on chronic ischemic heart failure of transplanting cell sheets created from co-cultured SMBs and MSCs, compared with SMB-only and MSC-only cell sheets.

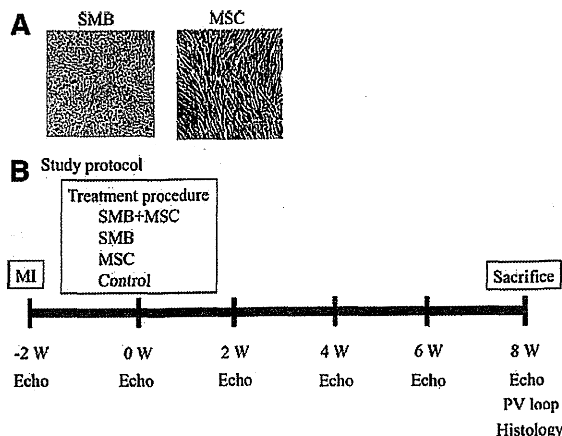

FIG. 1. **(A)** Morphology of SMB and MSC. **(B)** Study protocol used for the assessment of cardiac function and histology. Athymic nude rats (F344/NJcl-rnu/rnu) underwent induction of myocardial infarction by occluding the LAD permanently, followed by the treatment procedure 2 weeks later. Cardiac function was assessed by echocardiography just before 2, 4, 6, and 8 weeks after the treatment procedure. Eight weeks after the treatment procedure, invasive hemodynamic analysis and histological examination were performed following the sacrifice. SMB+MSC, co-culture of SMBs and MSCs; SMB, skeletal myoblast; MSC, derived mesenchymal stem cell; Echo, echocardiography; PV loop, invasive hemodynamic analysis. Color images available online at www.liebertpub.com/tea

Materials and Methods

This study was approved by the Institutional Ethics Committee of the Osaka University. Humane animal care was used in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research, and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Animal Resources and published by the National Institutes of Health (Publication No. 85-23, revised 1996). All procedures and evaluations, including assessments of cardiac parameters, were carried out in a blinded manner. The authors had full access to the data and take full responsibility for its integrity. All authors have read and agreed to the article as written.

Isolation of SMBs and adipose tissue-derived mesenchymal cells, and cell-sheet preparation

Primary skeletal myoblasts of rat origin (r-SMBs) were isolated from Lewis rats (3 weeks old, male; CLEA Japan, Inc.) and expanded *in vitro* as described previously[7,8]: more than 70% of the isolated cells were actin positive and 60–70% were desmin positive, as determined by flow cytometry (data not shown). To detect r-SMBs, we used GFP transgenic Lewis rats.[15] Primary human MSCs (h-MSCs) were isolated from female subcutaneous adipose tissue samples as described.[12] h-MSCs exhibit mesenchymal morphology (Fig. 1A). Cell sheets consisting of r-SMBs or h-MSCs were prepared using temperature-responsive culture dishes (UpCell®; CellSeed), as described.[12] Cell sheets containing both r-SMBs and h-MSCs were prepared by co-culturing these cells in temperature-responsive culture dishes.

Rat myocardial infarction model and cell-sheet implantation

A proximal site of the left anterior descending coronary artery (LCA) of athymic nude rats (F344/NJcl-rnu/rnu, 8-week-old, female, 120–130 g; CLEA Japan) was permanently occluded using a thoracotomy approach. The animals were then kept in temperature-controlled individual cages for 2 weeks to generate a subacute ischemic heart failure model.[7,8,12] The rats were then divided into 4 experimental groups ($n=10$ in each) as follows: (1) transplantation of triple-layer h-MSC cell sheets (7.5×10^5 cells per sheet), (2) transplantation of triple-layer r-SMB cell sheets (3.0×10^6 cells per sheet), (3) transplantation of triple-layer co-cultured r-SMB (3.0×10^6 cells per sheet) and h-MSC (7.5×10^5 cells per sheet) sheets, and (4) no treatment (control) (Fig. 1B). Thereafter, the rats were kept in individual cages for 4 weeks.

Echocardiography

Echocardiography was performed under general anesthesia using 1% isoflurane just before, and 2, 4, 6, and 8 weeks after the treatment procedure (SONOS 7500; Philips Medical Systems) (Fig. 1B). Left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), and end diastolic anterior wall thickness at the level of the papillary muscles were measured for at least three consecutive cardiac cycles, following the American Society for Echocardiology leading-edge method. Fractional shortening (FS) and ejection fraction (EF) were calculated as parameters of systolic function, as follows:

$$FS\ (\%) = (LVEDD - LVESD)/LVEDD$$

$$EF\ (\%) = [(LVEDD^3 - LVESD^3)/LVEDD^3].^4$$

Cardiac catheterization

To assess systolic and diastolic cardiac function, cardiac catheterization was performed under general anesthesia using 1% isoflurane, 8 weeks after the treatment procedure. A MicroTip catheter transducer (SPR-671; Millar Instruments, Inc.) and conductance catheters (Unique Medical

Co.) were placed longitudinally in the left ventricle (LV) from the apex and connected to an Integral 3-signal conditioner-processor (Unique Medical Co.). End-systolic pressure-volume relationships (ESPVR) were determined by transiently compressing the inferior vena cava. Data were recorded as a series of pressure–volume loops (~20), which were analyzed using Integral 3 software (Unique Medical Co.). The maximal and minimal rates of change in LV pressure (dP/dt max and dP/dt min, respectively) were obtained from steady-state beats using custom-made software. We assessed the early active part of the relaxation using the relaxation time constant (τ), which was determined from the LV pressure decay curve. After the hemodynamic assessment, the heart was removed for further biochemical and histological analyses.

Real-time quantitative PCR

Total RNA was extracted from cultured cell sheets or cardiac muscle tissue 8 weeks post-transplantation using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). Subsequently, real-time PCR assays were performed using an ABI PRISM 7700 machine.[4,7,8] Hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin growth factor (IGF), and thymosin β were assayed using rat-specific primers and probes (Applied Biosystems). The average copy number of gene transcripts for each sample was normalized to that for GAPDH.

Survival of grafted donor cells

The presence of grafted male cells in the female heart was quantitatively assessed by real-time PCR for the Y chromosome-specific gene *sry*. Four weeks after cell-sheet transplantation, genomic DNA was extracted from the entire LV walls using the QIAmp genomic DNA purification system (Qiagen). The signals for the autosomal single-copy gene were normalized to the amount of total DNA.[7] The primers were *sry*: forward, 5′ GCCTCAGGACATATTAATCTCTGGAG-3′; reverse, 5′-GCTGATCTCTGAATTCTGCATGC-3′.

Protein analysis

Enzyme-linked immunosorbent assay (ELISA) kits were used to measure proteins, such as HGF (Institute of Immunology) and VEGF (Quantikine; R&D) of rat origin, secreted from the cultured cell sheets *in vitro*, according to the manufacturers' suggested protocols. Values were calibrated for the extracted total proteins ($n=5$ in each group). The ELISA kits were also used to quantitatively analyze HGF (r-HGF) and VEGF (r-VEGF) of rat origin in heart tissue lysates ($n=5$ in each group).

Cytokine/chemokine multiplex immunology assay

The amount of each protein secreted from the cultured cell sheets *in vitro* was measured by Milliplex Rat Cytokine/Chemokine Panel Premixed 32Plex (Millipore), according to the manufacturer's instructions.[4] In this procedure, we applied human SMBs (h-SMBs) isolated and cultured from the patient (age 53 years, male) and expand *in vitro* as described previously.[6]

Histological analyses

Eight weeks after cell-sheet implantation, the hearts were dissected, fixed in 4% paraformaldehyde, and embedded in either optimum cutting temperature compound for 5-μm-thick cryosections or paraffin for 5-μm-thick sections ($n=5$ in each group) (Fig. 1). The paraffin-embedded sections were used for routine hematoxylin–eosin (HE) staining to assess the myocardial structure. Masson's trichrome staining was performed to assess cardiac fibrosis in the remote myocardium. The fibrotic cardiac area was calculated as the percentage of myocardial area. The data were collected from 10 individual views per heart at a magnification of ×200. The heart sections were also stained with an antibody to von Willebrand Factor (vWF) to assess capillary density, which was calculated as the number of positively stained capillary vessels that were 5–10 μm in diameter in 10 randomly selected fields in the peri-infarct area, per heart. To determine the extent of apoptosis, sections from frozen tissue samples were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) with an *in situ* apoptosis detection kit (Apoptag; Chemicon). Image J software was used for quantitative morphometric analysis.

To detect r-SMBs, we used GFP transgenic Lewis rats.[15] Cryosections were stained with an anti-HGF antibody (1:50 dilution; LifeSpan BioSciences). To detect h-MSCs and differentiation of the transplanted cell sheet, sections were stained with an antibody to human leukocyte antigen (1:50 dilution; Dako). The secondary antibody was Alexa Fluor 555 goat anti-mouse (1:200 dilution; Molecular Probes). Cell nuclei were counterstained with 6-diamidino-2-phenylindole (DAPI; Invitrogen). The images were examined by fluorescence microscopy (Keyence).

Western blotting

Tissue homogenates from LV samples in the cell-sheet transplanted site ($n=3$ in each group, on day 1) were prepared using lysis buffer (100 mM Tris pH 7.4, 20% SDS, 10 mM EDTA. 10 mM NaF, 2 mM sodium orthovanadate). The equivalent total protein was loaded onto SDS-polyacrylamide gel electrophoresis gels. Antibodies obtained from Cell Signaling were antiphosphorylated STAT3 (#9145), antiphosphorylated Akt (#4051), anti-Bcl_2 (#2876), and anti-poly (ADP-ribose) polymerase (PARP) (#9542). The labeled membrane was stripped and then re-probed with anti-STAT3 (#9132), anti-AKT (#9272), and anti-cleaved PARP (#9545) antibodies. Blots were scanned, and quantitative analysis was performed using Image J software. The relative proportion of the phosphorylated STAT3 was referred to that of the STAT3. The relative proportion of the phosphorylated Akt was referred to that of the Akt. The relative proportion of the PARP, cleaved PARP, Bcl_2 was referred to that of the control group.

Statistical analysis

Continuous variables are expressed as the mean ± SD. The significance of differences was determined using a two-tailed multiple *t*-test with Bonferroni correction following repeated-measures analysis of variance for individual differences. A *p*-value less than 0.05 was considered to be statistically significant. All statistical calculations were performed using the SPSS software (version 11.0; SPSS, Inc.).

Results

Production and release of cytokines/chemokines by cell sheets

Both h-SMBs and h-MSCs, as analyzed by cytokine antibody array, released abundant angiogenic factors *in vitro*, with distance profiles (Fig. 2A). Co-cultures of h-SMBs and h-MSCs showed significantly enhanced levels of HGF, VEGF, Leptin, and PECAM-1, but not of follistatin, G-CSF, IL-8, or PDGF-BB from the h-SMBs.

The seeding ratio of 4:1 r-SMBs:h-MSCs elicited the greatest *in vitro* mRNA expression of rat HGF and VEGF by real-time PCR (Fig. 2B). The mRNA levels of SMB-derived r-HGF and r-VEGF, analyzed by real-time PCR using rat-specific primers, were significantly greater in the co-cultured cell sheets than r-SMB-only ones (Fig. 2C), whereas the mRNA levels of IGF-1, bFGF, SDF-1, and TMSB4 were essentially the same (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). No mRNAs for cytokines of rat origin were detected in h-MSC-only cell sheets. Rat HGF and VEGF in the culture supernatants, analyzed by ELISA with rat-specific primary antibodies, were significantly higher in the co-culture supernatants than the r-SMB-only ones, and no rat cytokines were detected in the h-MSC-only supernatants (Fig. 2D).

Cardiac functional recovery after cell-sheet transplantation

The effects of cell-sheet transplantation on cardiac function were assessed in a rat chronic ischemic heart-failure model. Two weeks after permanent occlusion of the LCA, the LV developed echocardiographic features typical of chronic ischemic heart failure, including decreased FS, EF, and anterior wall thickness, and increased end-diastolic and systolic diameter (EDD and ESD, respectively). Following myocardial

A Cytokine/Chemokine multiplex immunology assay

	SMB+MSC	SMB	MSC	Medium only
Follistatin (pg/ml)	276±35*,†	350±56†	127±8.0	3.3
G-CSF (pg/ml)	333±44*,†	487±89†	7.7±0.7	1
HGF (pg/ml)	1190±256*,†	167±28†	695±44	3.8
IL-8 (pg/ml)	805±36*,†	1079±138†	579±29	0.6
Leptin (pg/ml)	1335±690*,†	262±40	195±13	59
PDGF-BB (pg/ml)	51±11*	25±2†	51±3	3.3
PECAM-1 (pg/ml)	438±70*	239±71†	386±16	64
VEGF (pg/ml)	5673±812*,†	1402±272	2019±184	1.9

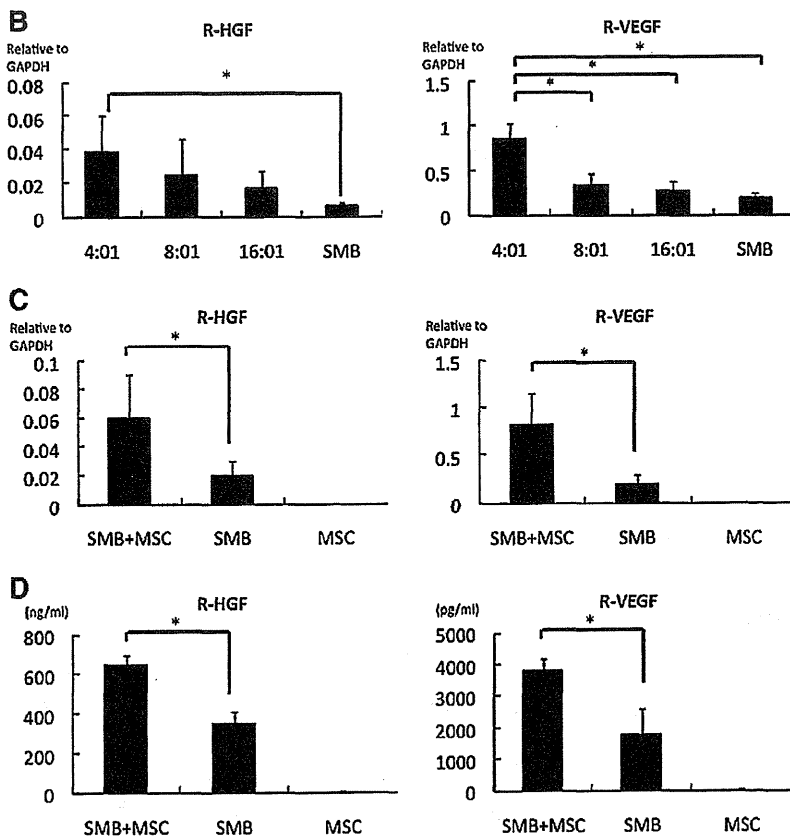

FIG. 2. Production and release of angiogenic factors by cell sheets. **(A)** Cytokine/chemokine multiplex immunology assay results from cultured cell sheets *in vitro*, prepared from human SMBs, human MSCs (h-MSCs), or both. SMB+MSC showed significantly enhanced the release of HGF, VEGF, leptin, and PECAM-1. $N=4$ in each group. $^*p<0.05$ versus SMB. $^{\dagger}p<0.05$ versus MSC. **(B)** Optimal seeding ratio of rat SMBs (r-SMBs) to h-MSCs. The *in vitro* mRNA levels of rat HGF and VEGF, analyzed by real-time PCR, were highest at 4:1 r-SMBs:h-MSCs. $N=4$ in each group. $^*p<0.05$. **(C)** mRNA levels in cultured cell sheets determined by real-time PCR using rat-specific primers. The SMB+MSC sheets expressed significantly more HGF and VEGF than the SMB-only ones. $N=5$ in each group. $^*p<0.05$. **(D)** Secretion of cytokines into the culture medium determined by enzyme-linked immunosorbent assay (ELISA) kits. The SMB+MSC sheets secreted significantly more HGF and VEGF than the SMB-only sheets. $N=5$ in each group. $^*p<0.05$. G-CSF, granulocyte-colony stimulating factor; HGF, hepatocyte growth factor; IL, interleukin; PDGF, platelet-derived growth factor; PECAM, platelet/endothelial cell adhesion molecule; VEGF, vascular endothelial growth factor. Error bars = SD.

infarction (MI), FS, EF, and anterior wall thickness showed steady reductions, whereas EDD/ESD showed steady increases, suggesting progressive LV remodeling.

Following either SMB-only or MSC-only cell-sheet transplantation, the heart showed mild recovery, including increases in FS, EF, and anterior wall thickness. At 2, 4, 6, and 8 weeks after treatment, FS, EF, and anterior wall thickness were significantly greater following SMB-only or MSC-only cell-sheet transplantation than the control, and significantly better recovery was obtained using the co-cultured cell sheets than either single cell-type sheet (Fig. 3A).

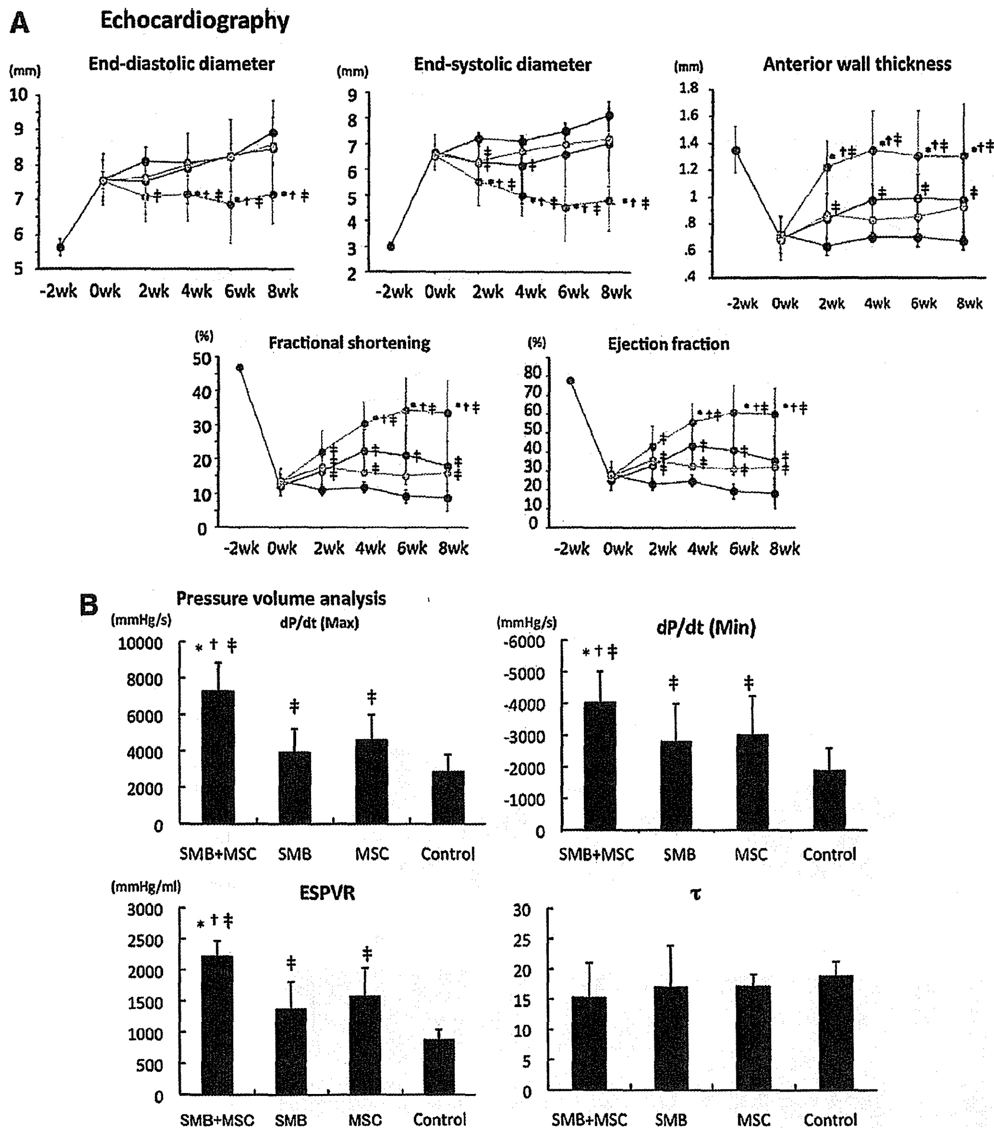

FIG. 3. Cardiac functional recovery after cell-sheet transplantation. **(A)** Echocardiographic analysis. Fractional shortening, ejection fraction, and anterior wall thickness were significantly improved 2, 4, 6, and 8 weeks after cell-sheet transplantation in the SMB+MSC sheet group, compared with the other three groups. Left ventricular end-diastolic and end-systolic diameters in the SMB+MSC sheet group were significantly decreased 4, 6, and 8 weeks after cell-sheet transplantation, compared with the other three groups ($N=10$ in each group. SMB+MSC group, green line; SMB group, blue line; MSC group, pink line; control group, red line). **(B)** Hemodynamic measurements determined by cardiac catheterization ($n=10$ in each group). Max. and min. dP/dt and ESPVR significantly improved in the SMB+MSC group, compared with the other three groups. Max. dP/dt, maximal rate of change in left ventricular pressure; min. dP/dt, minimal rate of change in left ventricular pressure; ESPVR, end-systolic pressure-volume relationship; EDPVR, end-diastolic pressure-volume relationship; τ, active part of relaxation shown by the relaxation time constant. $N=10$ in each group. $^{*}p<0.05$ versus SMB-only cell sheet. $^{\dagger}p<0.05$ versus MSC-only cell sheet. $^{\ddagger}p<0.05$ versus control. n.s., not significant. Error bars=SD. Color images available online at www.liebertpub.com/tea

Assessment by LV catheter showed a similar trend. Eight weeks after transplantation, the maximal and minimal rate of change in LV pressure (max. dP/dt and min. dP/dt, respectively) and end-systolic pressure-volume relationship (ESPVR) were significantly greater following either single-cell-type cell-sheet transplantation than the control, but τ was significantly different. After the co-culture cell-sheet transplantation, the max. dP/dt, min. dP/dt, and ESPVR improved further, with no significant difference in EDPVR or τ (Fig. 3B).

Reverse remodeling after co-culture cell-sheet transplantation

The LV structure was better maintained after SMB-only or MSC-only cell-sheet transplantation, compared to the control, in which the LV cavity was severely enlarged with a thin anterior wall, as assessed by HE staining (Fig. 4A). The LV structure was even better maintained after the co-culture cell-sheet transplantation. In the control, abundant collagen accumulations were observed in the infarct area, and diffuse fibrotic changes were induced in the remote area, whereas collagen accumulation was attenuated in both the remote area with the single cell-type sheet transplants, as assessed by Masson's trichrome staining (Fig. 4B, C). Fibrotic changes in the remote area were further attenuated by transplantation of the co-cultured cell sheet (Fig. 4D).

A greater number of vWF-positive blood vessels was detected in the peri-infarcted myocardium following the transplantation of either single-cell-type cell sheet, compared to the control (Fig. 5A), and even more vWF-positive blood vessels were seen with transplantation of the co-cultured cell sheet. The capillary density in the peri-infarcted myocardium, which was semi-quantitatively assessed in 10 randomly selected individual fields, was significantly greater following the transplantation of either single-cell-type cell sheet, compared to the control (Fig. 5B), and it was further increased after the co-cultured cell-sheet transplantation.

Major intercellular signaling molecules relevant to angiogenesis and cell survival were analyzed by western blotting. The ratio of p-STAT3 over total STAT3 was greatly increased after co-cultured cell-sheet transplantation (Fig. 5C).

Survival of transplanted cells in the heart

Four weeks after the cell-sheet transplantation, significantly more transplanted rat cells survived in co-cultured sheets than SMB-only sheets, as analyzed by PCR assays for the Y-chromosome-specific *Sry* gene (Fig. 6A).

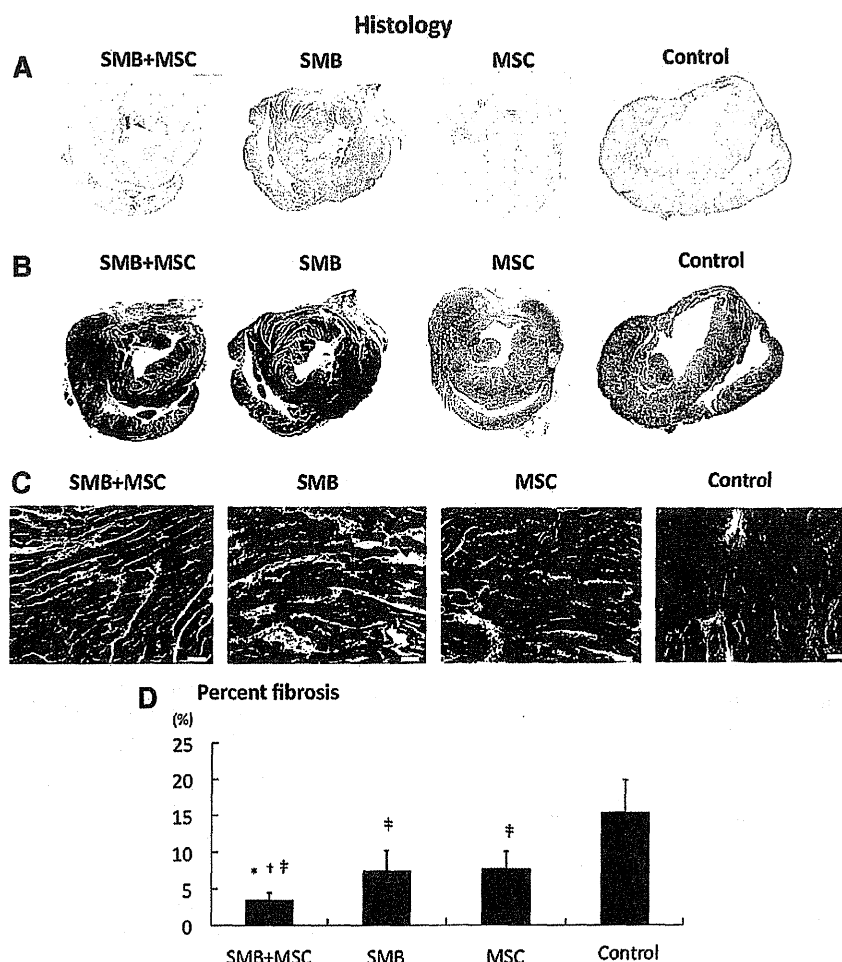

FIG. 4. Histological reverse remodeling after cell-sheet transplantation. **(A)** Macroscopic (×40) views of the heart stained by hematoxylin–eosin. **(B)** Macroscopic (×40) views of the heart stained by Masson's trichrome. **(C)** Microscopic (×200) representative Masson's trichrome staining at the remote myocardium (white bar = 40 μm). **(D)** Quantification of percent fibrosis at the remote area. Significant suppression of fibrosis was found after SMB + MSC sheet transplantation compared with the other three groups. $N = 5$ in each group. *$p < 0.05$ versus SMB. †$p < 0.05$ versus MSC. ‡$p < 0.05$ versus control. Error bars = SD. Color images available online at www.liebertpub.com/tea

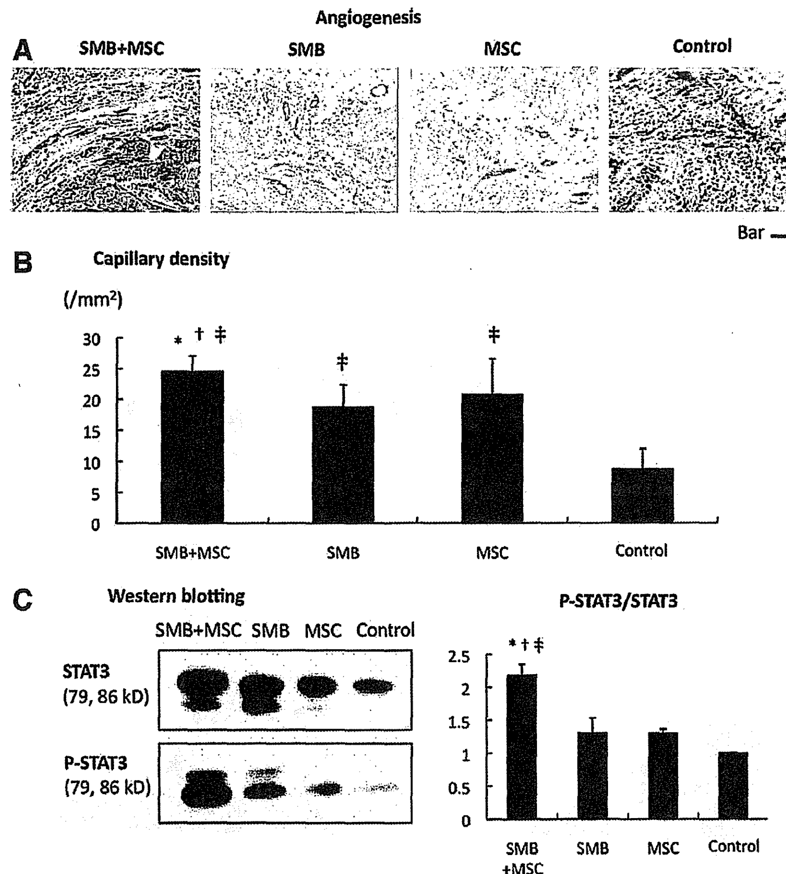

FIG. 5. Angiogenesis. **(A)** Microscopic (×100) views of sections of the peri-infarct border-zone region stained with anti-von Willebrand factor antibody (factor VIII) in the four groups (bar = 50 μm). **(B)** Capillary density: the SMB + MSC group showed significant improvement in capillary density as assessed by immunostaining for von-Willebrand factor-positive blood vessels. $N = 5$ in each group. **(C)** Western blotting showing enhanced STAT3 phosphorylation over total STAT3 in the SMB + MSC sheet group. $N = 3$ in each group. $^{*}p < 0.05$ versus SMB. $^{\dagger}p < 0.05$ versus MSC. $^{\ddagger}p < 0.05$ versus control. Error bars = SD. STAT3, signal transducer and activator of transcription 3. Color images available online at www.liebertpub.com/tea

The percentage of TUNEL-positive myocytes was significantly lower following the transplantation of the co-cultured cell sheet compared to the control (Fig. 6B).

Akt-1 and Bcl-2 were highly expressed in the heart following transplantation of the SMB-only or co-cultured cell sheet, compared with the control, as analyzed by real-time quantitative PCR using rat-specific primers (Fig. 6C).

Notably, among apoptosis-signaling molecules, Bcl_2 and cleaved PARP were increased 1 day after the co-culture cell-sheet transplantation. There was no significant difference in the ratio of phosphorylation of Akt over Akt (Fig. 6D).

Upregulation of cardioprotective factors in the myocardium after cell-sheet transplantation

The mRNA expression of cardioprotective factors, such as HGF, VEGF, IGF-1, and bFGF, in the infarct and infarct-remote areas of the myocardium was analyzed by real-time PCR using rat-specific primers, which detected factors released by transplanted SMB or the native myocardium. The expression of these factors was not significantly different after transplantation of either single-cell-type cell sheet or no treatment, except for HGF expression in the infarct area, which was significantly greater after the SMB-only sheet transplantation (Fig. 7A, B). In contrast, following transplantation of the co-cultured cell sheet, the HGF and VEGF levels in the infarct area were significantly greater than after transplantation of either single cell-type cell sheet or control, although the levels of IGF-1 and bFGF were unchanged (Fig. 2A). The intramyocardial protein levels of HGF and VEGF, analyzed by ELISA, were significantly greater after transplantation of the co-cultured cell sheet than of either single-cell-type cell sheet or no treatment (Fig. 7C).

Immunoconfocal microscopy showed that HGF was found in the transplanted SMBs from the co-cultured cell sheet (Fig. 8A).

MSCs differentiate into new vessels in situ

The differentiation capacity of the transplanted h-MSCs was assessed by immunoconfocal microscopy. As expected, no human-derived cells were seen in either the r-SMB-only transplantation group or the control group. However, human vWF-positive staining was observed in the host vessels in both the co-cultured cell-sheet group and the h-MSC-only cell-sheet transplantation group. Thus, the h-MSCs could differentiate into vessel walls *in vivo* (Fig. 8B).

Discussion

Here, we demonstrated that SMB cell sheets abundantly synthesized and extracellularly released multiple cytokines and chemokines, and adding MSCs enhanced the SMB cell

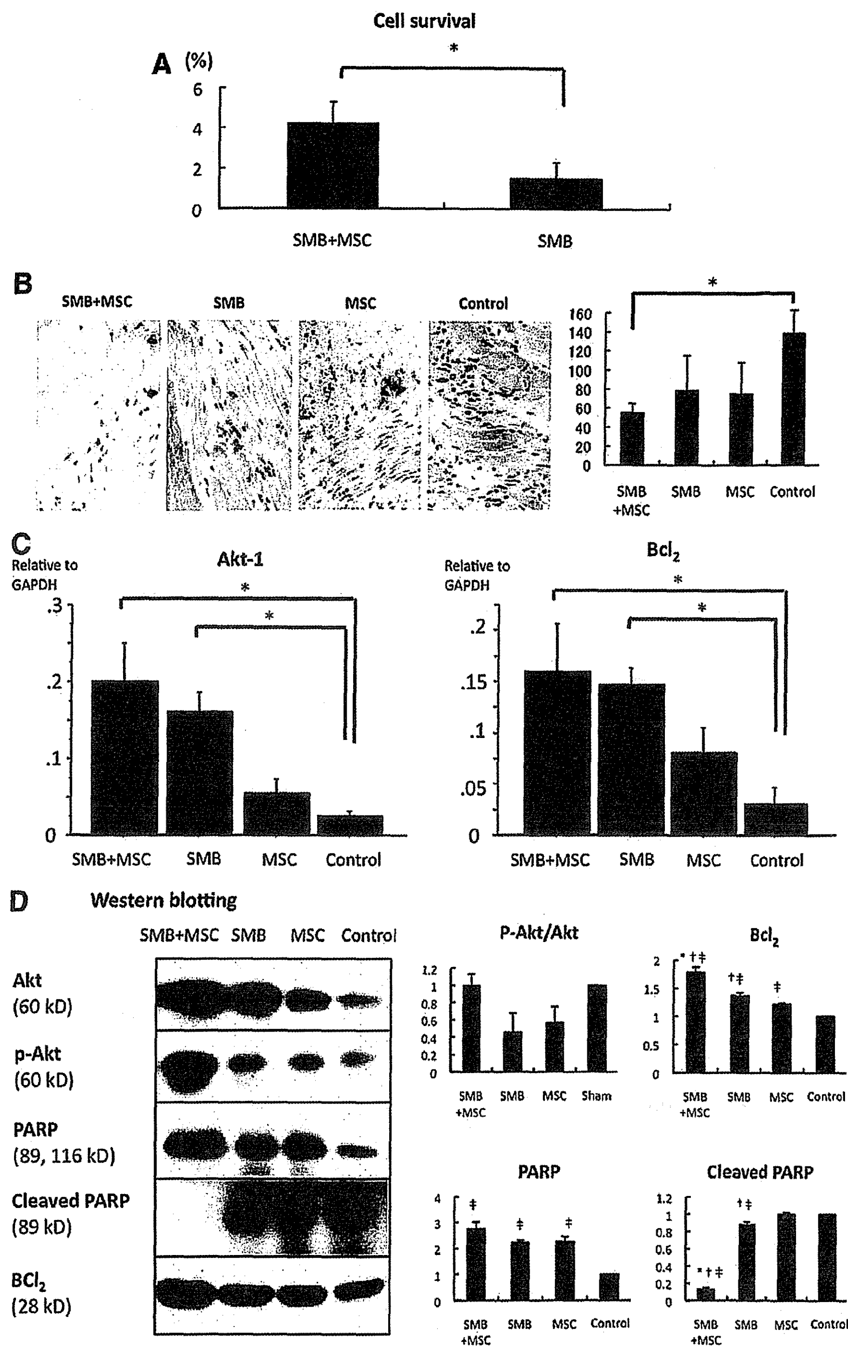

FIG. 6. Cell survival. **(A)** Survival of transplanted cells of rat origin was significantly greater in the SMB+MSC sheet group than in the SMB sheet group. $N = 4$ in each group. $^{*}p < 0.05$. **(B)** The number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive myocytes was significantly lower in SMB+MSC group than in control. $N = 4$ in each group. $^{*}p < 0.05$. **(C)** Expressions of mRNA in the transplanted infarct area of hearts were determined by real-time PCR using rat-specific primers. The expressions of Akt-1 and Bcl_2 mRNA were significantly increased in the SMB+MSC sheet group compared with the other groups. $N = 4$ in each group. $^{*}p < 0.05$. **(D)** Western blotting showed that Bcl_2 was much more enhanced, and cleaved PARP was significantly downregulated in the SMB+MSC group. There was no significant difference in the ratio of phosphorylation of Akt over Akt. $N = 3$ in each group. $^{*}p < 0.05$ versus SMB. $^{\dagger}p < 0.05$ versus MSC. $^{\ddagger}p < 0.05$ versus control. Error bars = SD. Color images available online at www.liebertpub.com/tea

sheets' release of HGF and VEGF but not of IGF-1, bFGF, or SDF-1, *in vitro*. The transplantation of SMB-only cell sheets into the chronically ischemic failing rat heart resulted in reversed LV remodeling, including increased capillaries, attenuated collagen accumulation, and prolonged cell survival, which increased global functional recovery, mediated by the paracrine effects of upregulated HGF and VEGF in the myocardium.

Recent studies, including ours,[3–9] have suggested that a paracrine effect mediated by cytokines secreted from the transplanted cell sheets is a likely mechanism for the therapeutic effects on the myocardium, which was a focus of the present study. Here, we added h-MSCs to the cell sheets to enhance the potential performance of the transplanted r-SMB sheets. Our *in vitro* findings, that h-MSCs enhanced rat mRNA levels and the secretion of cytokines such as r-HGF

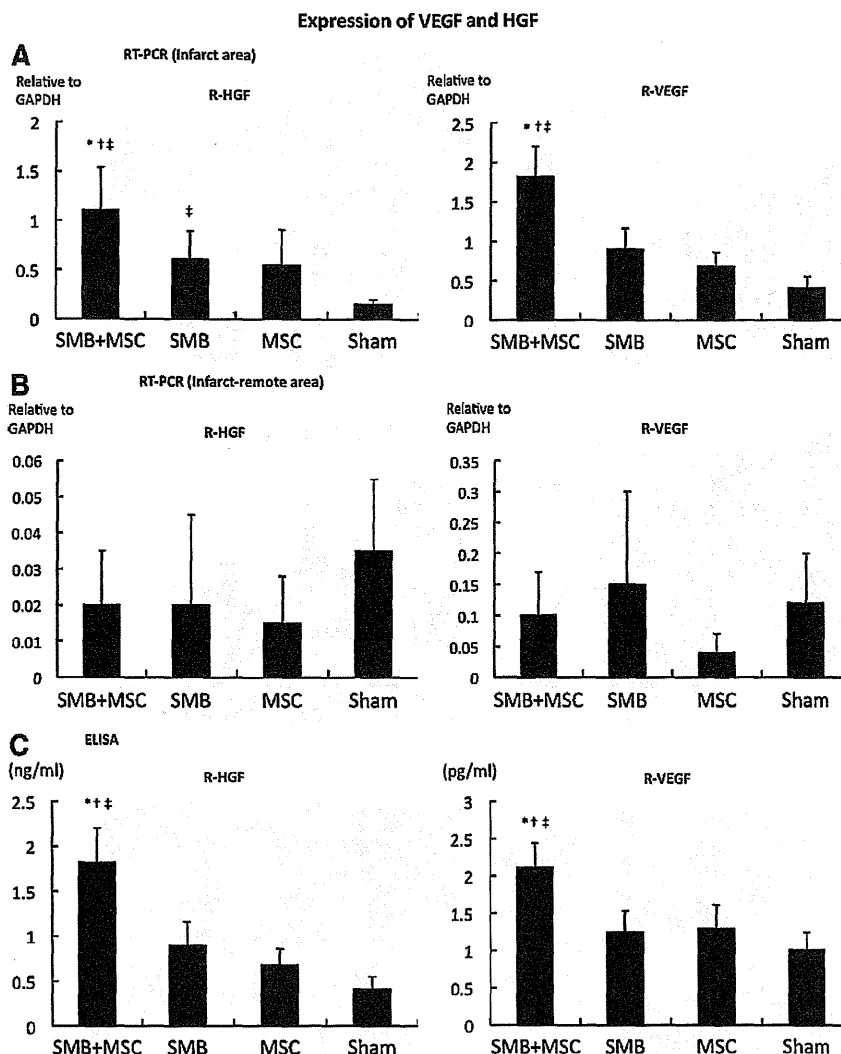

FIG. 7. Expression of VEGF and HGF is higher at the infarct area. **(A, B)** Levels of mRNA in the transplanted infarct and infarct-remote heart areas by real-time PCR using rat-specific primers. The HGF and VEGF mRNA expressions within the transplanted infarct area of the hearts were significantly increased in the SMB + MSC sheet group compared with the other groups. $N = 4$ in each group. $^{*}p < 0.05$ versus SMB. $^{\dagger}p < 0.05$ versus MSC. $^{\ddagger}p < 0.05$ versus sham. **(C)** Intramyocardial protein levels of HGF and VEGF, analyzed by ELISA, were significantly greater in the heart in the SMB + MSC sheet group compared with the other groups. $^{*}p < 0.05$ versus SMB. $^{\dagger}p < 0.05$ versus MSC. $^{\ddagger}p < 0.05$ versus control. Error bars = SD.

and r-VEGF from r-SMBs, suggested that transplanted co-cultured cell sheets would secrete r-HGF and r-VEGF *in vivo*. Although the exact mechanisms by which "feeder layers" support cell growth have not been elucidated, it is possible that h-MSCs enhance the r-SMBs directly (via cellular interaction) or indirectly (via secreted cytokines from the h-MSCs).[16] A more comprehensive examination aimed at differentiating these effects might help reveal how feeder layers work.

HGF and VEGF participate in many complex molecular and cellular mechanisms, and their signaling pathways have been intensively investigated *in vivo*.[3,9] SMBs or MSCs act as the natural supplier of both HGF and VEGF and provide feasible and safe sources for cell therapy in clinical applications. Indeed, SMBs and bone marrow-derived mesenchymal stem cell sheets can secrete growth factors (e.g., HGF and VEGF) into the myocardium and accelerate neovascularization in the damaged area.[5–8] More recent reports have revealed that angiogenesis induced by HGF or VEGF, an antifibrotic effect promoted by HGF, or the migration and survival of SMBs supported by VEGF,[17] could be beneficial to an impaired heart.[7,8] In addition, our data from a cytokine/chemokine multiplex immunology assay indicate that leptin may also be beneficial (e.g., by inducing angiogenesis though the Jak/STAT pathway).[18] Other cytokines may also contribute to the improvement of cardiac function by single-cell-type cell sheets in as-yet-undiscovered ways.

The mechanism by which the implanted cell sheet attenuates ventricular remodeling and improves cardiac function seems to depend on the cell sheet being placed over the scarred area of the myocardium and leads to repair of the anterior wall thickness, reduction of LV wall stress, and the improvement of ejection performance.[3] Previous studies indicated that the surviving myocardium and implanted cell sheet attenuate complex cellular and molecular events, including hypertrophy, fibrosis, apoptosis of the myocardium, and the pathological accumulation of extracellular matrix.[9] Similarly, the greater cellularity observed after cell-sheet

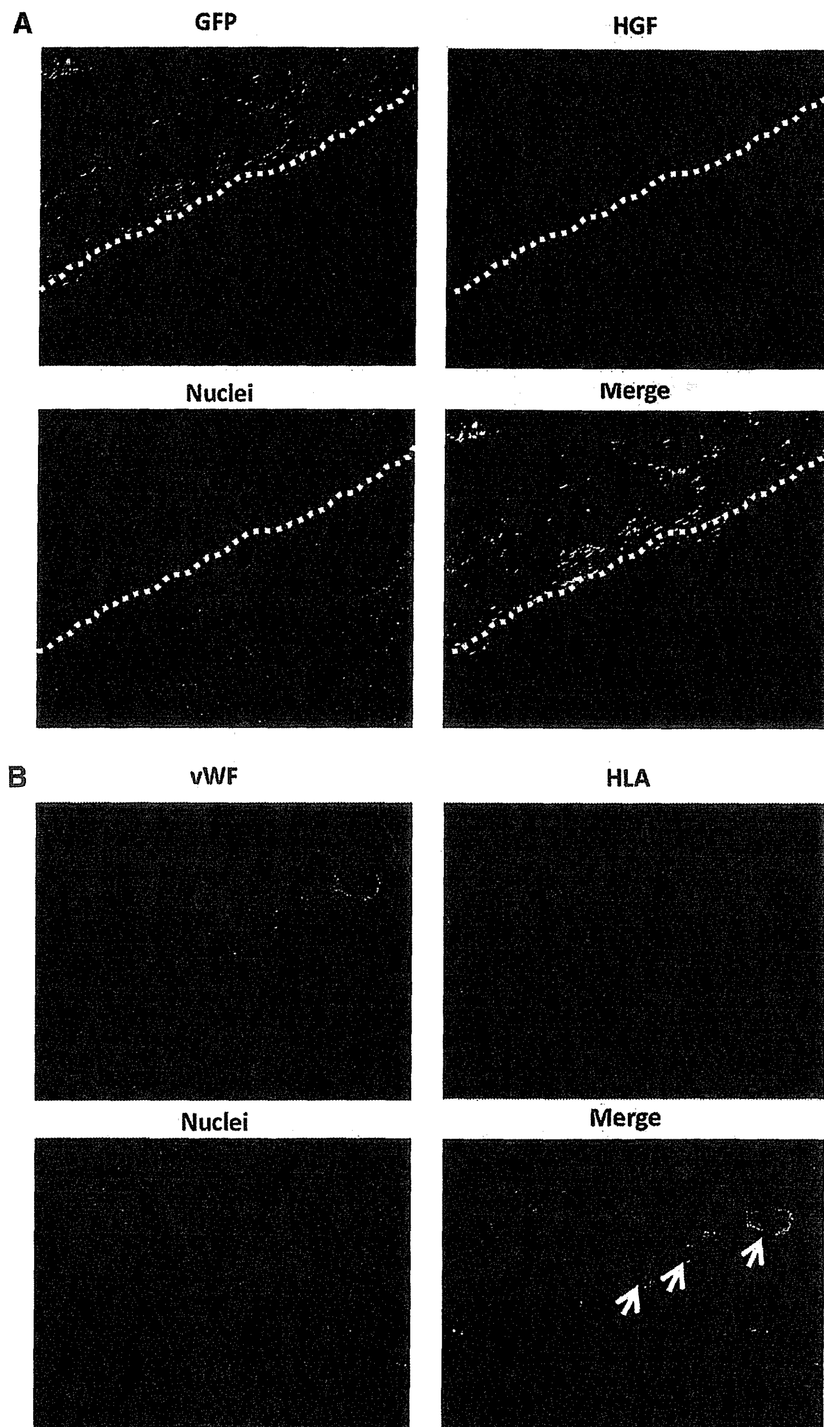

FIG. 8. Characterization of transplanted cells. **(A)** Cryosections were stained with an antibody to HGF to detect the distribution of SMB and HGF in the heart. HGF expressions and GFP-positive cells were found in the myocardium after transplantation of the SMB + MSC sheet. White broken line shows the border between the transplanted cell sheet and the host heart. Green indicates GFP; red, HGF; blue, nuclei. **(B)** Cryosections were stained with antibodies to human leukocyte antigen (HLA) and to von Willebrand factor (vWF). Human vWF-positive (white arrows) staining was observed in the host vessels in the h-MSC-transplanted group. Green indicates vWF; red, HLA; blue, nuclei. Color images available online at www.liebertpub.com/tea

treatment might have resulted from released SDF-1, which is related to cell migration, adhesion, and proliferation, by the transplanted cell sheet[19,20]

In this study, we performed additional investigations on the paracrine mechanism from a new perspective, by analyzing signaling pathways within the myocardium following cell-sheet transplantation because the signals induced by released paracrine mediators presumably activate phosphorylation cascades of signaling molecules. We found that STAT3 and Akt phosphorylations were significantly increased, and cleaved PARP was significantly downregulated, 24 h after the co-cultured cell-sheet implantation. Together

with our findings that vascular density was significantly enhanced, and myocardial apoptosis and fibrosis was significantly attenuated in the co-cultured group, it is possible that the co-cultured cell-sheet transplantation induced angiogenesis partially through the Jak/STAT signaling pathway[18] and that it prolonged cell survival by preventing apoptosis through PI-3K/Akt-mediated signaling, which is partially modulated by HGF.[21]

Although we emphasized combining SMBs with h-MSCs, some investigators have focused on different combinations of various cell sources. Sekine *et al.*[22] reported that cardiomyocytes co-cultured with endothelial cells induce greater numbers of capillaries, due to increased secretion of angiogenic growth factors.[22] Another report showed that a dermal fibroblast sheet co-cultured with endothelial progenitor cells was more effective than either single cell-type sheet for improving damaged heart function, accompanied by the inhibition of fibrotic tissue formation and the acceleration of neovascularization in the infarcted myocardium.[23] Thus, the paracrine effect may be improved by combining different cell sources; however, further investigation focused on determining the optimal combinations of cell sources is needed.

Regarding h-MSCs as a cell source, bone marrow-derived or adipose tissue-derived stem cells are reported to differentiate into mature endothelial cells and participate in blood vessel formation in the recipient heart.[24] The presence of endothelial capillary networks improves the survival and organization of implanted cells by maintaining a minimum intercapillary distance to provide oxygen and nutrients. Therefore, the presence of endothelial capillary networks may be partially correlated with cardiac function.

For future tissue engineering for cardiac therapy, the creation of thick cell-dense constructs with functional vessels may be essential. Capillary formation occurs via two basic vessel-constructing processes: angiogenesis, that is, the formation of new capillaries via sprouting or intussusception from pre-existing vessels, and vasculogenesis, which occurs in the developing embryo.[25] Here, the morphology of the vessel formation within myocardial tissues, including the diameter, composition, and fragility of vessel walls, suggested that improper vascularization may occur under pathological conditions. It is likely that not only biological factors but also physical stimuli such as flow and shear stress are required to mimic the *in vivo* environment and enable the formation of mature vascular networks.

A potential limitation of this study is that the exact number of transplanted cells was different in each group *in vivo*. Clinically, open-chest surgery is unlikely to gain easy acceptance except in certain situations; however, less invasive methods (e.g., intracoronary catheter-based procedures) might be technically difficult for carefully placing the cell sheets. Additionally, further studies that include longer timeframe than 8 weeks are needed to examine a longer term restoration of heart function post-MI. It is likely that the source of HGF is the transplanted SMB; however, it is unclear whether the source of other therapeutic cytokines is the transplanted cells, such as SMBs, MSCs, or both, or native cardiac cells.

In conclusion, we found that h-MSCs enhanced the paracrine effects of r-SMB sheets, thus enhancing angiogenesis, lowering fibrosis, inhibiting cellular hypertrophy, improving cardiac function, and prolonging cell survival in MI model rats. These observations of improved effects from this co-cultured cell sheet may lead to new regeneration therapies for heart failure following advanced cardiomyopathy that are superior to the conventional SMB-only cell-sheet technique.

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Disclosure Statement

T.S. is a consultant for CellSeed, Inc. T.O. is an Advisory Board Member in CellSeed, Inc., and the inventor/developer designated on the patent for temperature-responsive culture surfaces.

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in situ reprogrammed spermine treated-adipose tissue-derived multi-lineage progenitor cells improve left ventricular dysfunction in a swine chronic myocardial infarction model

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Abstract

Background: Spermine, known as one of polyamines, has been reported to make embryonic stem cells differentiate into cardiac lineage. In this study, we examined whether spermine could commit human adipose tissue–derived multi-lineage progenitor cells (hADMPC) into cardiac lineage and whether the spermine treated-hADMPCs would differentiate into cardiomyocytes-like cells and improve left ventricular dysfunction in a swine chronic myocardial infarction model. ***Methods and Results:*** After 24h-treatment with spermine, hADMPC showed the augmentation of cardiac marker-expressions; nkx2.5, islet-1, alpha-cardiac actin and cardiac troponin I (11.2-, 27.5-, 43.6- and 19.1-fold to hADMPC *per se*, respectively). To examine the effect of spermine treated-hADMPC on left ventricular dysfunction, swine chronic MI model were built up by first ballooning and reperfusion to first diagonal branch and second one to left ascending coronary artery (#6) 1 week-later. Four week-later second one, the swine (immunization with CyA 0.6mg i.m./kg/day) received transplantation of spermine treated-hADMPC ($1x10^5$, $3x10^5$, $1x10^6$ and $3x10^6$ cells/kg) or lactic Ringer's solution via intracoronary (#6), and echocardiogram was examined at 0, 4, 8 and 12 weeks after transplantation. Follow-up showed rescue of function in the transplanted, and the most effective dose was $3x10^5$ cells/kg (EF; 33.4%, 47.0%, 51.5% and 52.9% at 0, 4, 8 and 12 week-after transplantation, respectively). Histologically, the spermine treated-hADMPC were engrafted into the scarred myocardium and reprogrammed into human specific troponin I and alpha-cardiac actin positive cells *in situ* 12 week-after transplantation. ***Conclusion:*** The transplantation of spermine treated-hADMPC is a potentially effective therapeutic strategy for future cardiac tissue regeneration.

Keywords: adipose tissue, spermine, cardiocytoc differentiation, myocardial infarction

Introduction

Spermine, known as one of polyamines, has been reported to make embryonic stem cells differentiate into cardiac lineage. In this study, we examined whether spermine could commit human adipose tissue–derived multi-lineage progenitor cells (hADMPC) into cardiac lineage and whether the spermine treated-hADMPC would differentiate into cardiomyocytes-like cells and improve left ventricular dysfunction in a swine chronic myocardial infarction model.

Materials and Methods

Adipose tissue

Adipose tissue samples were resected from 5 human subjects during plastic surgery (all females, age, 20-60 years) as excess discards. Ten to 50 grams of subcutaneous adipose tissue were collected from each subject. All subjects provided informed consent. The protocol was approved by the Review Board for Human Research of Kobe University Graduate School of Medicine, Osaka University Graduate School of Medicine and Foundation for Biomedical Research and Innovation.