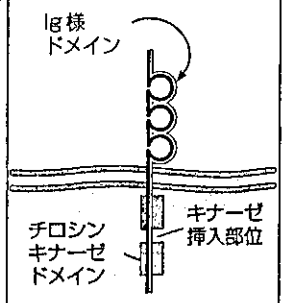
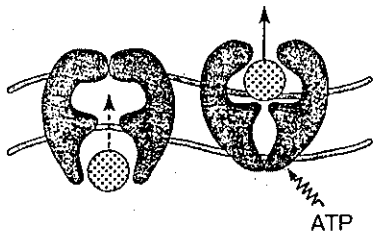
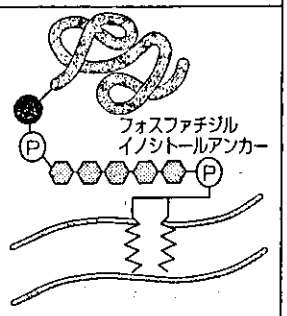
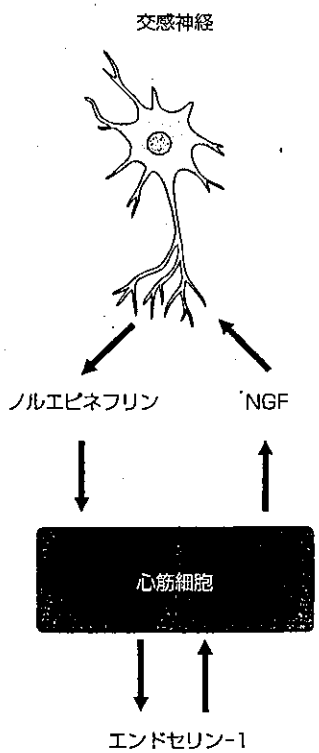
表❶ c-kit/MRD 1/Sca-1 の構造・分布および機能

	c-kit	MRD1	Sca-1
構造			
分布	メラノサイト 肥満細胞 生殖細胞 幹細胞	肝細胞，胆管細胞 刷子縁細胞 腎尿細管細胞，癌細胞 脳血管内皮細胞 幹細胞	血管壁 腎皮質細胞 胸腺，脾臓 Tリンパ球 幹細胞
機能	増殖 遊走 分化 分泌	膜に存在する排出ポンプ アポトーシスの抑制	細胞接着 シグナル伝達 T細胞活性化



図❷ 心臓と交感神経

交感神経の刺激は心臓に心拍数上昇，房室伝導の上昇，陽性変力作用などをもたらす．心臓を支配する交感神経は主として星状神経節の神経細胞の支配を受ける．われわれの近年の解析では，心臓への交感神経支配は胎性後期に心筋細胞から分泌される神経成長因子（nerve growth factor：NGF）を指標にして星状神経節細胞から軸索が伸長してくる．このときに心筋細胞がオートクリンに分泌するエンドセリン-1が心筋細胞自身に作用してET-Aレセプター，Gi$\beta\gamma$を介して経路でNGFを分泌させることが明らかとなった(図❷)．このエンドセリン-1/NGF経路が存在しない場合には心筋細胞に交感神経が伸長せず，さらに星状神経節の交感神経細胞体自身がアポトーシスを起こすことも明らかとなった．上述の問題点をクリアするために，再神経支配に関しても今後の研究が待たれる．

おわりに

上述のように骨髄間葉系幹細胞やES細胞などを用いた心筋細胞の分化誘導が可能になり，また，シート状にした再生心筋の作成も可能となってきた．しかし器官レベルで機能するためにはさらにクリアすべき問題が山積しており，これらが解決されなければ治療に利用できる心筋は作成できない．

しかし近年の進歩はめざましいものがあり，これらがクリアできたとき，再生心筋の臨床応用の具現化も可能であると確信している．

文 献

1) Jiang Y, Jahagirdar BN, Reinhardt RL *et al* : Plur-ipotency of mesenchymal stem cells derived from adult marrow. *Nature* **418** : 41-49, 2002
2) Beltrami AP, Barlucchi L, Torella D *et al* : Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* **114** : 763-776, 2003
3) Oh H, Bradfute SB, Gallardo TD *et al* : Cardiac progenitor cells from adult myocardium : homing, differentiation, and fusion after infarction. *Proc Natl Acad Sci USA* **100** : 12313-12318, 2003
4) Jackson KA, Majika SM, Wang H *et al* : Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest* **107** : 1395-1402, 2001
5) Goodell MA, Rosenzweig M, Kim H *et al* : Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple spiecies. *Nat Med* **3** : 1337-1345, 1997
6) Shimizu T, Yamamoto M, Akutsu T *et al* : Electrically communicating three-dimensional cardiac tissue mimic fabricated by layered cultured cardiomyocyte sheets. *J Biomed Mater Res* **60** : 110-117, 2002

6．間葉系幹細胞を用いた心筋再生

板橋　裕史*・福田　恵一*1)
Itabashi　Yuji　Fukuda　Keiichi

*慶應義塾大学医学部循環器内科　1)講師

Summary　各種の心疾患の終末像である重症心不全に対する唯一の治療法は，心臓移植しか存在しないが，ドナー不足や免疫抑制療法の副作用等の問題点は克服されていない。近年の研究により，骨髄細胞より心筋細胞が分化誘導されることが報告されてきており，さらにその由来として，骨髄間質細胞中の多能性幹細胞が注目されている。これらの分化誘導された心筋細胞を心臓へ移植したり，分化を促進するサイトカインを投与したりすることによって，心機能を改善させようとする試みがなされている。

はじめに

心筋梗塞や拡張型心筋症などの広範囲に及ぶ，心筋障害を原因とした各種の心疾患の最終病像は，心筋細胞の喪失や機能低下に伴う収縮力不足による心不全である。種々の薬物療法では充分な心機能の改善が望めない程の重篤な心不全では，現時点では，心臓移植以外に確立された治療法は存在しない。しかし，米国においても数多くの心臓移植対象患者がいるものの，移植の恩恵にあずかれるのはその中の極一部にとどまっており，心臓移植療法においても慢性的なドナー不足が深刻な問題となっている。また，心臓移植には免疫拒絶，感染症，発癌などの解決せねばならない問題も存在する。移植に変わる治療方法の開発が切望される中で，各種の多能性幹細胞を用いた再生医学に大きな期待が寄せられている。しかし，心筋細胞は生体内で最終分化した細胞であり，それ自体が増殖しない細胞であると考えられ，今日に至るまで心筋細胞の再生は極めて困難であるとされてきた。近年，骨髄細胞や胚性幹細胞（embryonic stem cell：ES 細胞）をはじめとした，各種の幹細胞から心筋細胞を分化誘導する報告が相次いでなされるようになり，中でも我々は，骨髄間葉系幹細胞を用いることにより心筋細胞が再生されることを報告している。本稿では，心筋梗塞による心不全への治療として期待される，様々な幹細胞を用いた心筋再生，再生心筋細胞移植の実際

《略語一覧》
ES 細胞（embryonic stem cell；胚性幹細胞）

について，今後の展望を踏まえ述べる。

1．心筋細胞再生のための治療戦略

心筋細胞は生後2週間くらいまで分裂増殖し，その後は細胞分裂を行わず，個々の細胞が増大する事によって心臓全体が大きくなり成人の心臓が形成される。骨格筋細胞の研究において，MyoDという転写因子が発生の段階で発現し，骨格筋細胞の形成に重要な役割を果たしていることが判明している[1]。この遺伝子は，他の細胞に導入し強制発現させると，その細胞が骨格筋細胞に形質転換するという性質を持つことから，マスター遺伝子と呼ばれた。MyoDの発見以来，心筋細胞におけるマスター遺伝子を単離する試みがなされたが，その結果，マスター遺伝子こそ単離されはしなかったものの，心筋細胞の発生に重要な遺伝子として*Nkx-2.5*，*GATA4*，*MEF2C*，*eHAND*，*TEF-1*などの心筋組織の形成において重要な役割を果たす遺伝子が次々に発見され[2~6]，心筋細胞の発生メカニズムの解明へ大きく貢献した。これらの遺伝子の導入により生理的な心筋細胞発生分化のプログラムを再現できれば，非心筋細胞の心筋細胞への分化誘導が実現できるのではないかと期待されている。

他の心筋再生の方法として，細胞の分裂増殖をつかさどる遺伝子を導入することにより，一度最終分化に至り増殖する能力を失った心筋細胞に，再び細胞分裂能を獲得させるという試みがある。細胞の分裂増殖にはサイクリン，サイクリン依存性キナーゼ（cyclin-dependent kinase：CDK）などの細胞周期調節遺伝子，癌抑制遺伝子などの細胞周期にかかわる遺伝子が関与しているが，これまで細胞周期調節遺伝子や発癌ウィルスの腫瘍抗原（SV40 large T抗原など）を心筋細胞に遺伝子導入する事で，分裂増殖可能な心筋細胞を作成する研究がなされている[7]。

そして第3の方法として，各種の多能性幹細胞から心筋細胞を分化誘導させる方法が挙げられるが，その際に用いられる幹細胞としては，体性幹細胞としての骨髄間葉系幹細胞とES細胞に期待が寄せられている。ES細胞の増殖能は著しく，心筋細胞へ効率的に分化誘導を行う技術が確立されれば，細胞供給源として極めて有用となる可能性を秘めている。しかし，ES細胞を作成する際に倫理的な問題が生じること，移植後の拒絶を回避するためには，宿主と同一の遺伝子を持ったES細胞を用いる必要があるが，そうしたES細胞を作成するのにあたり，表現形に異常をきたさずに核移植を行う手法が確立されるまでには，まだしばらく時間を要するであろうことなどが，現在の障害となっている。こうした背景の中で，宿主由来の間葉系幹細胞を用いて心筋細胞を再生させる手法は倫理的な問題がなく，拒絶の心配もないことから臨床への応用を考えた際に理想的な方法といえる（表1）。

2．心筋細胞の再生

1）体性幹細胞を用いた心筋細胞の再生

近年の研究により，成人の組織においても様々な臓器において，未分化幹細胞が残存していることが明らかになった。間葉系の幹細胞としては，骨髄中の幹細胞が注目されている。骨髄の細胞は，将来血球に分化する造血幹細胞とこれに由来する各種の血球系細胞，そしてこの血球系細胞を支持するための骨髄間質細胞より構成される。骨髄間質細胞は多彩なサイトカイン・細胞増殖因子等を分泌し，また造血幹細胞が生存・機能していける微小環境を構築し，血球系細胞の再生・増殖・

《略語一覧》
CDK（cyclin-dependent kinase；サイクリン依存性キナーゼ）

表1　心筋細胞再生のための戦略

心筋細胞再生法	方法	課題
心筋細胞特異的転写因子の遺伝子導入	マスター遺伝子の導入 非心筋細胞 → 心筋細胞	心筋細胞分化過程の解明 心筋のマスター遺伝子の単離 遺伝子導入のためのベクターの開発
心筋細胞の細胞分裂能獲得	細胞周期調節遺伝子 心筋細胞 → 分裂可能な心筋細胞	心筋細胞の終末分化機序の解明 遺伝子導入のためのベクターの開発
未分化幹細胞から心筋細胞への分化誘導	分化誘導 未分化幹細胞（骨髄間葉系幹細胞） → 心筋細胞	未分化幹細胞の単離 効率的な分化誘導法の確立 （分化に必須な細胞増殖因子などの同定）

分化を補佐するという機能を持つ。最近の研究によれば，骨髄間質細胞中には間葉系の多能性幹細胞が含まれており，骨芽細胞,，軟骨芽細胞，脂肪細胞，骨格筋細胞，靱帯，腱などの中胚葉系の様々な細胞に分化することが報告されている。

我々はマウス骨髄の初代培養を行い，付着系の細胞である骨髄間質細胞を分離した後，これを長期培養することで不死化した細胞株を作成した。この多クローン細胞株にDNAの脱メチル化剤である5-アザシチジンを添加し，さらに2週間程度培養を続けると少ない確率ではあるが，自己拍動する細胞が得られた。こうして得られた細胞株のなかで自己拍動する割合の高いクローンをcardiomyogenesis（CMG）細胞株として樹立した。CMG細胞は5-アザシチジンにより最終的に分化誘導を行うと約30%の細胞が自己拍動を開始するようになった[8]。

2）骨髄由来の心筋細胞の特徴

CMG細胞は最終分化誘導を行う前には線維芽細胞様の形態を示している（図1）。5-アザシチジンによる最終分化により形態は著しく変化し，分化誘導1週目頃より一部の細胞の細胞質が大きくなり，円形あるいは棒状を呈する様になる。分化誘導後2週になると，こうした細胞は互いに連結しあい，筋管細胞様となる。3週以後には，多くの細胞が縦に一列に並び，同期して収縮する。分化誘導4週以後には，培養皿の上の連結される細胞はすべて同期して収縮し心筋組織様になる。

心筋細胞は横紋筋であるが，CMG細胞も電子顕微鏡で観察すると典型的な横紋構造が観察され，また心房性ナトリウム利尿ペプチドを含有する心房顆粒と思われる高密度顆粒を多数認めた。遺伝子レベルでの解析では，CMG細胞は心筋細胞特異的とされる心房性ナトリウム利尿ペプチド（artial natriuretic polypeptide：ANP）および脳

《略語一覧》
CMG (cardiomyogenesis)　　ANP (artial natriuretic polypeptide；心房性ナトリウム利尿ペプチド)

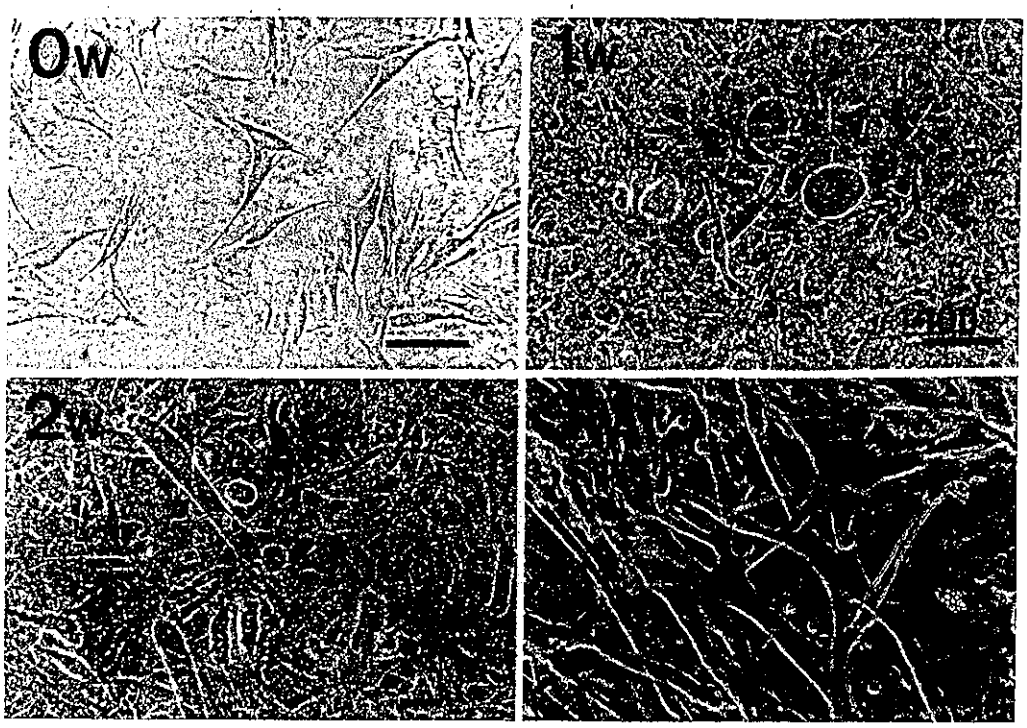


図1　最終分化誘導における CMG 細胞の位相差顕微鏡写真

5 - アザシチヂンによる，最終分化誘導後の CMG 細胞像。(左上) 5 - アザシチヂン投与前の CMG 細胞。線維芽細胞に似た様相を呈する。(右上) 5 - アザシチヂン投与後 1 週間後の CMG 細胞。(左下) 5 - アザシチヂン投与後 2 週間後の CMG 細胞。棒状に形態変化した細胞が認められる。(右下) 5 - アザシチヂン投与後 3 週間後の CMG 細胞。ネットワークを形成した細胞が同期した自律収縮を示す。　(パネル内の線は 100 μ m を示す。)

性ナトリウム利尿ペプチド (brain natriuretic peptide：BNP) を発現していた。

心筋細胞は筋肉が収縮するための蛋白として α - アクチン，ミオシン重鎖，ミオシン軽鎖などを持っているが，これらの収縮蛋白にはいくつかのアイソフォームが存在しているが，それは胎児 (胎仔) 期と成人 (成獣) 期，あるいは心房と心室などで筋肉の収縮速度，エネルギー効率などが異なり，それぞれに都合の良い条件を整えるためと考えられている。生体内の心筋細胞と CMG 細胞の収縮蛋白のアイソフォームを表 2 に示した。

心筋細胞に分化した CMG 細胞の場合，上記の様にアイソフォームの発現様式は α - アクチンの場合 Skeletal 型＞ Cardiac 型，ミオシン重鎖の場合 β 型＞ α 型であった。ミオシン軽鎖では 2v 型が発現しているのに対し，2a 型の発現はみられなかった。また，CMG 細胞では分化誘導後にはナトリウム利尿ペプチドである ANP，BNP の発現が観察された。心筋収縮蛋白質の発現様式より判断すると，CMG 細胞の心筋細胞としての表現型は，胎仔型心室筋細胞の形質をもつと考えられた。

心筋細胞は自律神経の作用により，心拍数の変動，心収縮力の調整，興奮伝播速度の調節が行われる。交感神経と副交感神経は神経終末よりノルエピネフリンとアセチルコリンを分泌する。一方，心筋側はノルエピネフリンの α 1，β 1，β 2 受容体，アセチルコリンのムスカリン型 M1,

《略語一覧》
BNP (brain natriuretic peptide；脳性ナトリウム利尿ペプチド)

表2　心筋収縮蛋白のアイソフォームから見たCMG細胞の表現型

	心房筋		心室筋			CMG細胞
	胎仔型	成獣型	胎仔型	新生仔型	成獣型	
α-アクチン	skeletal	cardiac	skeletal > cardiac	skeletal	cardiac	skeletal > cardiac
ミオシン重鎖	α型>β型	α型	β型>α型	α型>β型	α型	β型>α型
ミオシン軽鎖	2a	2a	2v	2v	2v	2v

表3　CMG細胞由来再生心筋細胞の受容体発現と受容体刺激による効果

受容体の種類	α受容体			β受容体		ムスカリン受容体	
受容体のサブクラス	α 1A	α 1B	α 1D	β 1	β 2	M1	M2
発現時期	最終分化誘導前より(漸増)	最終分化誘導前より(不変)	最終分化誘導前より(漸減)	最終分化誘導後1週より	最終分化誘導後1週より	最終分化誘導後1週より	最終分化誘導後1週より
シグナル伝達の確認	ERK活性化	ERK活性化	ERK活性化	cAMP上昇	cAMP上昇	IP3上昇	IP3上昇
確認出来た作用	肥大作用			心拍数上昇，心収縮力増強			

M2受容体を発現し，神経とシナプスを形成する。CMG細胞ではノルエピネフリンのα1受容体を最終分化前より発現し，ノルエピネフリンのβ1，β2受容体，アセチルコリンM1，M2受容体を心筋細胞の形質獲得後には発現していた。また，これらの受容体は細胞内シグナル伝達機構も有していた[9]（表3）。

3）サイトカインを用いた心筋細胞再生の促進

骨髄由来の幹細胞が，心筋細胞に分化することが判明してから，様々なサイトカインを用いて心筋再生を促進する試みがなされてきた。なかでも顆粒球コロニー増殖因子（granulocyte-colony-stimulating factor：G-CSF）は，骨髄から末梢血中への幹細胞の動員を促進することによって，障害心筋領域での心筋再生が増加する作用があると期待された。G-CSFが心筋再生に及ぼす影響を確認するために，我々は共同研究により致死量の放射線照射を行い，宿主の骨髄細胞を死滅させたマウスにEGFP（enhanced green fluorescence protein）を発現する全骨髄細胞を移植した後に心筋梗塞を作成し，G-CSFの投与の有無により骨髄細胞から再生される心筋細胞がどのように変化するのかを調査した。その結果，心筋梗塞作成後10日間G-CSFを投与したマウスでは，梗塞巣周辺においてEGFPを発現する心筋細胞，すなわち骨髄由来の再生心筋細胞が166倍に増加していた。また，骨髄中の幹細胞から心筋細胞が分化誘導されることが報告されて以来，造血幹細胞と骨髄間質細胞のどちらが再生心筋細胞の由来となっているかが，しばしば議論されてきた。我々は，再生心

《略語一覧》
G-CSF (granulocyte-colony-stimulating factor；顆粒球コロニー増殖因子)
EGFP (enhanced green fluorescence protein)

筋細胞へと分化した多能性幹細胞がいずれの由来であるかを調べるために放射線照射を行い，骨髄細胞を死滅させたマウスの骨髄中に，心筋細胞に分化した際にEGFPを発現するように遺伝子導入を行ったCMG細胞(骨髄間質細胞由来)を移植し，心筋梗塞を作成した後にG-CFSを投与したモデルでは，心筋梗塞巣の周囲に多くのEGFP陽性心筋細胞が認められた。これらの結果からG-CSFは心筋梗塞巣において，骨髄幹細胞からの心筋再生を促進し，心機能を改善する効果があること，これらの再生心筋細胞は造血幹細胞ではなく骨髄間質細胞より分化している可能性が高いことが示された[10]。

おわりに

成人の組織内に存在する多能性幹細胞の分化能が研究されるに従い，これらは組織再生の有力な材料であり，これまで不可能と考えられていた心筋組織の再生が実現の可能性を帯びてきた。しかし，心筋の再生に関してはまだ動物モデルのみの報告も少なくなく，ヒトに臨床応用するにはまだ解決すべき課題も多く残されている。

循環器領域における再生医療の分野でも，心筋再生治療に関する研究は着実に進んでおり，実際に骨髄細胞，筋芽細胞などを用いた臨床試験が欧米では次々に行われている。これらの臨床試験においては心機能が改善されたなど，おおむね良好な結果が報告されているようである。しかし，これらの細胞移植研究において，心機能改善の機序に関してはリモデリングの防止や血管新生の促進など，さまざまなメカニズムが考えられており，心筋再生自体が心機能の改善に，どの程度貢献しているのかは未だ不明である。

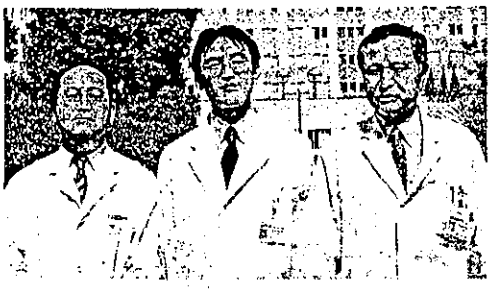
原疾患を問わず，重篤な心不全に陥った心臓をよみがえらせるためには，心筋組織の再生が不可欠である。今後，各種の多能性幹細胞から心筋が再生される機序がより詳細に解明され，臨床応用にたどり着く日が来ることが切望されている。

文　献

1) Runicki MA, et al : MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75 : 1351-1359, 1993
2) Komuro I, Izumo S : Csx : a murine homeobox-conteining gene specifically expressed in the developing heart. Proc Natl Acad Sci USA 90:8145-8149, 1993
3) Lints TJ, et al : Nkx-2.5 : a novel murine homeobox gene expressed in early heart progenitor cell and their myogenic descendans. Development 119 : 969, 1993
4) Kuo CT, et al:GATA4 transcription factor is required for ventral mrophogenesis and tube formation. Genes Dev 11 : 1061-1072, 1997
5) Lin Q, Schwarz J, Bucana C, Olson EN : Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. Science 276: 1404-1407, 1997
6) Firulli AB, et al : Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. Nat Genet 18 : 266-270, 1998
7) Leferovich JM, et al : Heart regeneration in adult MRL mice. Proc Natl Acad Sci USA 98 : 9830-9835, 2001
8) Makino S, et al : Cardiomyocytes can be generated from marrow stromal cells *in vitro*. J Clin Invest 103 : 697-705, 1999
9) Hakuno D, et al:Bone Marrow-Derived Regenerated Cardiomyocytes (CMG Cells) Express Functional Adrenergic and Muscarinic Receptors. Circulation 105 : 380-386, 2002
10) Kawada H, et al : Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. Blood 104 : 3581-3587, 2004

Direct cell-cell interaction of cardiomyocytes is key for bone marrow stromal cells to go into cardiac lineage in vitro

Shinya Fukuhara, MD,[a] Shinji Tomita, MD, PhD,[b] Seiji Yamashiro, MD, MSc,[c] Takayuki Morisaki, MD, PhD,[d] Chikao Yutani, MD, PhD,[a] Soichiro Kitamura, MD, PhD,[e] and Takeshi Nakatani, MD, PhD[b]



Tomita, Fukuhara, Nakatani *(left to right)*

From the Department of Pathology, National Cardiovascular Center, Osaka[a]; the Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, Osaka[b]; the Department of General Medicine, Saga Medical School, Saga[c]; the Department of Bioscience, National Cardiovascular Center, Research Institute, Osaka[d]; and the Department of Cardiovascular Surgery, National Cardiovascular Center, Osaka, Japan.[e]

Supported in part by Health Sciences Research Grants (Research for Cardiovascular Diseases [13C-1] and Research on the Human Genome, Tissue Engineering Food Biotechnology [12-007]) from the Ministry of Health, Labor, and Welfare, and by Grant-in-Aid for Scientific Research (A) and for Exploratory Research from the Japan Society for the Promotion of Science.

Read at the Eighty-second Annual Meeting of The American Association for Thoracic Surgery, Washington, DC, May 5-8, 2002.

Received for publication June 5, 2002; revisions requested Aug 5, 2002; revisions received Sept 1, 2002; accepted for publication Oct 10, 2002.

Address for reprints: Shinji Tomita, MD, Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka, 565-8565, Japan (E-mail: shinjitomita@hotmail.com).

J Thorac Cardiovasc Surg 2003;125:1470-80

0022-5223/2003 $30.00 + 0

doi:10.1016/S0022-5223(02)73610-6

Objectives: Cardiac environmental factors are thought to be powerful inducers in cardiomyogenic differentiation. In this study we simulated the cardiac environment using coculture and evaluated the cardiomyogenic differentiation in bone marrow stromal cells.

Methods: In group 1 only bone marrow stromal cells derived from transgenic mice expressing green fluorescent protein (GFP-BMCs) were cultured (n = 5). In group 2 cardiomyocytes from neonatal rats were grown on inserts, which we applied to culture dishes seeded with GFP-BMCs (n = 5). In group 3 GFP-BMCs were cocultured with cardiomyocytes on the same dishes (n = 5). We cultured these cells for 7 days and evaluated the synchronous contraction and the cardiomyogenic differentiation of GFP-BMCs by means of immunostaining.

Results: In groups 1 and 2 GFP-BMCs protein did not show any myogenic phenotypes for 7 days. In contrast, in group 3 some GFP-BMCs were incorporated in parallel with cardiomyocytes and revealed myotube-like formation on day 1. On day 2, some GFP-BMCs started to contract synchronously with cardiomyocytes. Myosin heavy chain–positive GFP-BMCs were recognized in 2.49% ± 0.87% of the total GFP-BMCs on day 5 ($P < .0001$). Cardiac-specific troponin I–positive GFP-BMCs were in 1.86% ± 0.53% of the total cells on day 5 ($P < .0001$). Atrial natriuretic peptide was also seen in GFP-BMCs, and connexin 43 was detected between GFP-BMCs and cardiomyocytes.

Conclusions: Direct cell-cell interaction with cardiomyocytes was important for bone marrow stromal cells to differentiate into cardiomyocytes. This coculture was useful for simulating the cardiac environment in vitro for the research of cell transplantation in the heart.

CSP

Bone marrow cell may be a candidate for cell-based therapy for regenerating many kinds of tissue such as liver, neuron, fat, and tendon,[1] and we previously reported that transplantation of bone marrow stromal cells (BMCs) induced myogenesis and angiogenesis in damaged hearts and improved impaired function.[2,3]

A part of the transplanted bone marrow cells differentiated into cardiomyocytes without any artificial manipulation in vivo.[2,4-6] Even xenogeneic stem cells went to site-specific differentiation in the body of another species.[7,8] These data suggested that environmental factors were natural inducers of differentiation. The heart might have the capacity to regenerate itself when it is damaged.[9] The effects are very difficult to investigate, however, because of their in vivo nature. We hypothesized that direct attachment between BMCs and cardiomyocytes was one of the environmental inducers.

In this study we simulated the cardiac environment with coculture composed of green fluorescent protein mouse-BMCs (GFP-BMCs) and rat cardiomyocytes and

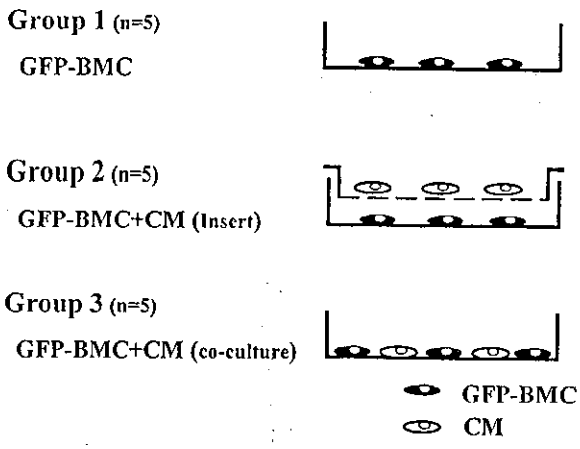


Figure 1. Experimental culture system with rat cardiomyocytes *(CM)* and GFP-BMCs.

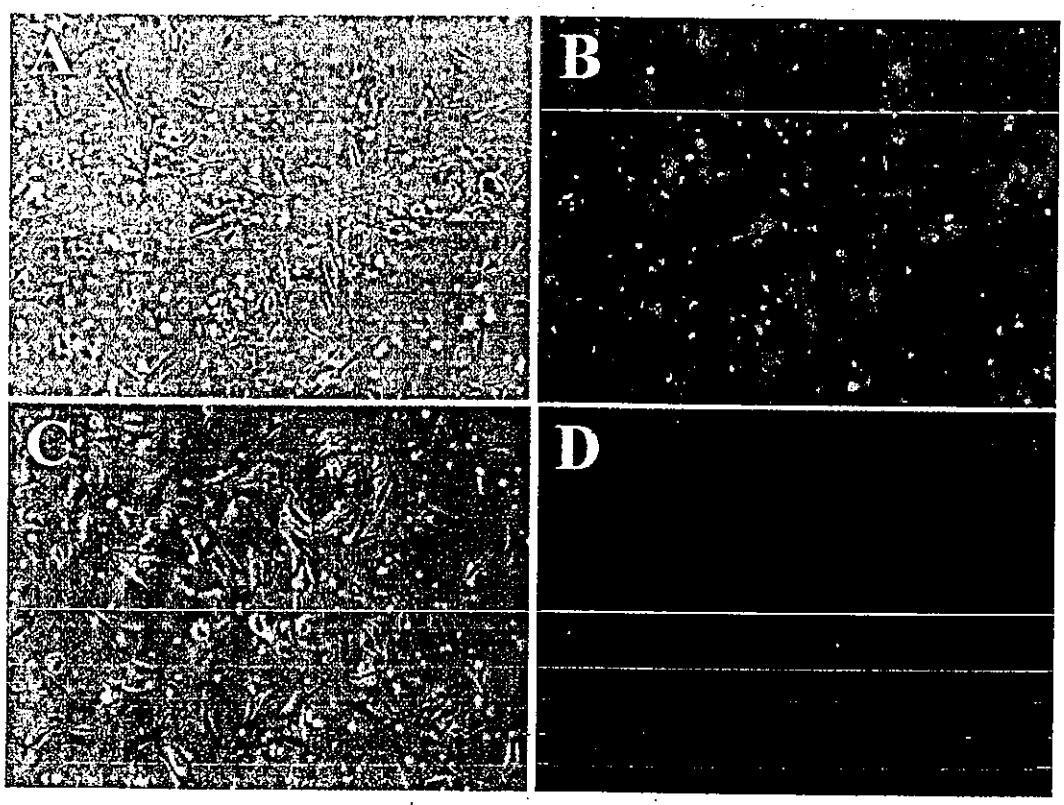


Figure 2. The morphology of GFP-BMCs in passage 2 (A and B) and rat cardiomyocytes (*CM*; C and D) in vitro. A, These cells were spindle, oval, wedge, or sheet shaped. B, All cells expressed green under fluorescent microscopy. (Original magnification 200×.) D, None of the cells were visible under fluorescent microscopy. (Original magnification 200×.)

report, for the first time to our knowledge, that BMCs differentiate into cardiomyocytes in a coculture and cell-cell attachment is one of the environmental factors of differentiation.

Materials and Methods

Subjects

Animals were studied on the basis of the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Labora-

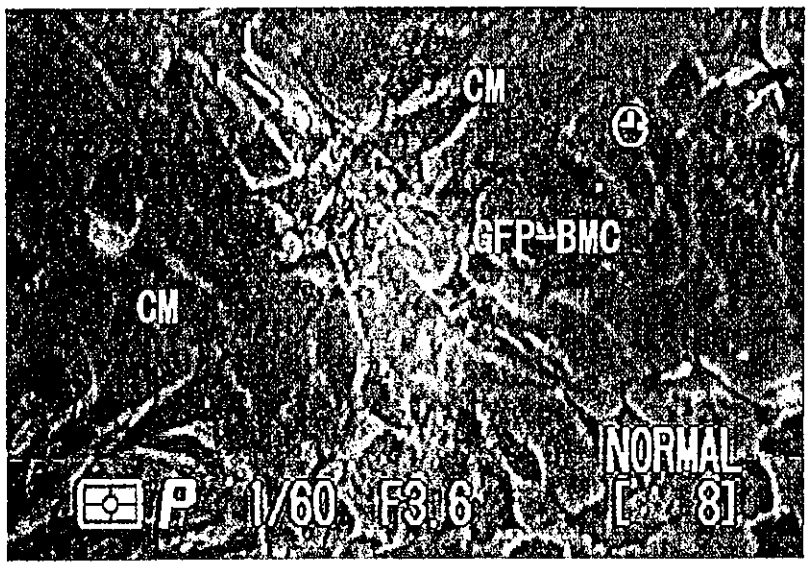


Figure 3. GFP-BMCs with rat cardiomyocytes *(CM)* on day 2 after coculture in group 3. GFP-BMCs were spindle shaped, attached to cardiomyocytes, and contracted synchronously with cardiomyocytes. (Original magnification 200×.)

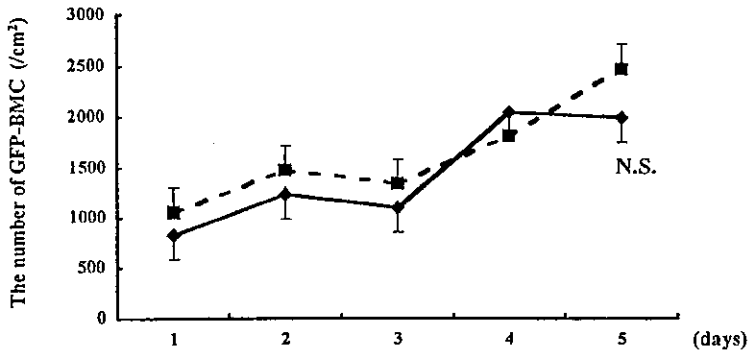


Figure 4. The proliferation of GFP-BMCs in groups 1 *(solid line)* and 3 *(dotted line)* is shown. There was no difference between groups 1 and 3 *(NS).*

tory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996, and approved by the Institutional Animal Care and Use Committee at the National Cardiovascular Center Research Institute. Pregnant Sprague-Dawley rats were purchased from a licensed vendor. Transgenic mice expressing green fluorescent protein (C57BL/6Tg14[act-EGFP]OsbY01: GFP mouse) were kindly provided by Dr M. Okabe.[10] Animals were housed in an air-conditioned room, with free access to food and water at all times.

BMCs From GFP Mice

A GFP mouse was anesthetized with diethylethanol. After achievement of general anesthesia, the femora and tibiae were collected.[4,11] After removing connective tissue around the bone, both ends of the bone were cut. Bone marrow plugs were flushed with a 27-gauge needle and a syringe filled with complete medium (Iscove modified Dulbecco medium with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin). Cells were introduced into 100-mm dishes and incubated at 37°C in 5% carbon dioxide and 95% air. Three days later, the medium was changed, and the nonadherent cells were discarded. Medium was completely replaced every 3 days. Passage was done when confluency exceeded 70%. BMCs in passages 2 or 3 were used in this study. We operationally called these cells stromal cells.

Neonatal Rat Cardiomyocytes

Cardiomyocytes were isolated from 1-day-old newborn Sprague-Dawley rats.[12] In brief, neonatal rats were anesthetized with diethylethanol and killed by means of decapitation, and their hearts were rapidly removed and placed into dishes on ice. After the atria

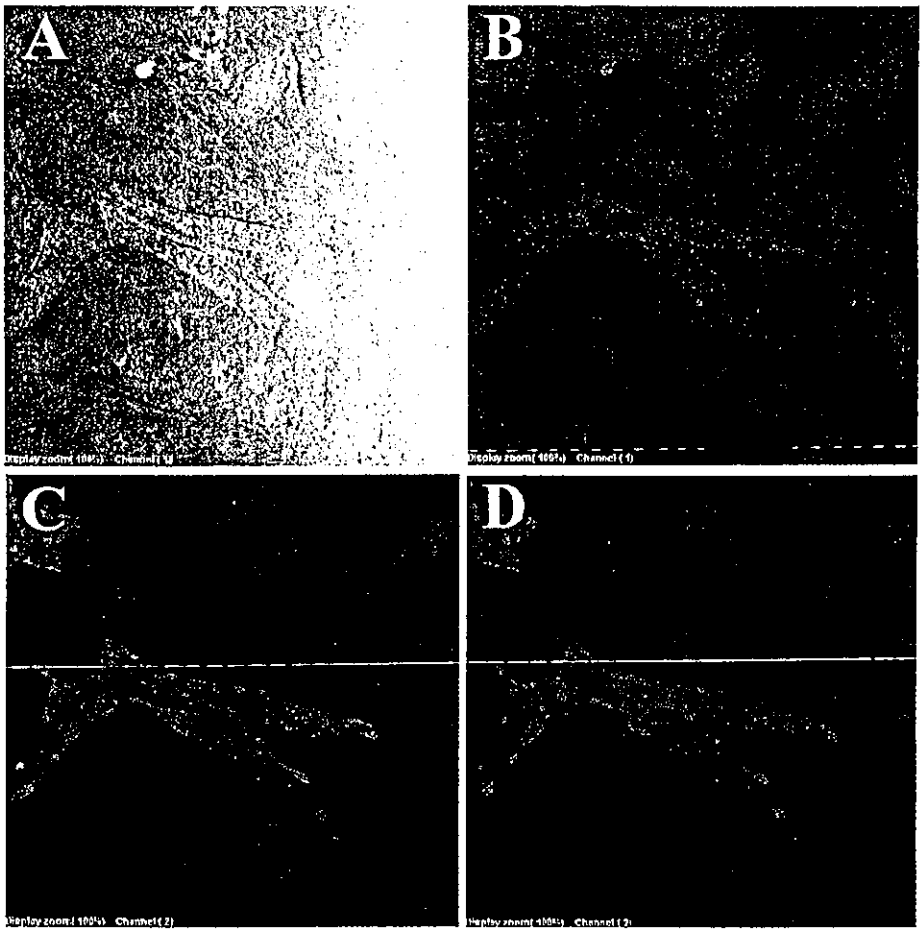


Figure 5. Myogenic differentiation of GFP-BMCs. Cells were stained with a first antibody against MHC. A was photographed during the contrast phase. B and C were photographed during the fluorescent phase (B, green, excitation at 515-540 nm; C, red, excitation at 574-640 nm). D was the double-labeled cell superposition of B and C. Positive cells showed a cross-striated pattern. Combined green and red fluorescence represented myogenic cells derived from BMCs. (Original magnification 400×.)

and the great vessels were discarded, hearts were minced into 1-mm^3 pieces with fine scissors, transferred to a sterile tube, and washed once in cold phosphate-buffered saline solution (PBS; NaCl, 136.9 mmol/L; KCl, 2.7 mmol/L; Na_2HPO_4, 8.1 mmol/L; and KH_2PO_4, 1.5 mmol/L [pH 7.3]) to remove any blood and clots. The minced tissue was digested in a PBS solution supplemented with 0.5% trypsin, 0.1% collagenase, and 0.02% glucose for 2 minutes at 37°C. The cell suspension was transferred into a tube containing 20 mL of complete medium and centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in complete medium and plated on 35-mm dishes (Falcon; Becton, Dickinson and Company, Franklin Lakes, NJ) at a density of $1.25 \times 10^4/cm^2$ and cultured at 37°C in 5% carbon dioxide and 95% air.

Experimental Culture Systems

GFP-BMCs and cardiomyocytes were prepared as described above, and 1×10^5 cells/dish were plated as follows (Figure 1). In group 1 only GFP-BMCs were plated on 35-mm dishes (Falcon) as control specimens (n = 5). In group 2 cardiomyocytes were plated onto cell culture inserts (Falcon), which we applied to 35-mm dishes seeded with GFP-BMCs 2 days later (n = 5). In group 3, cardiomyocytes were plated on 35-mm dishes, followed by additional plating of GFP-BMCs 2 days later to make up a coculture (n = 5). They were incubated at 37°C in 5% carbon dioxide and 95% air until further processing. All the dishes were then evaluated for 1 week with a fluorescent microscope (Nikon TE300, Nihon Kogaku, Tokyo, Japan) equipped with a heated plate (37°C), a digital video camera, and a confocal microscope (Olympus Fluoview, Tokyo, Japan).

Immunohistochemistry

The cultured cells were immunohistochemically stained. In brief, the cells were washed with PBS and fixed with 4% paraformaldehyde for 5 minutes at room temperature, whereas the dishes for staining against anticonnexin 43 were fixed for 10 minutes at 4°C. A mouse monoclonal antibody against myosin heavy chain

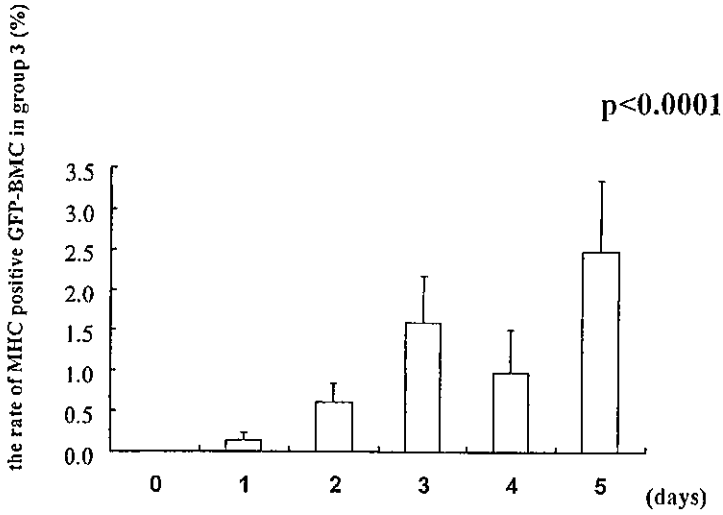


Figure 6. The percentage of MHC–positive BMCs from day 0 through day 5 in group 3. The *bar* represents mean and SE. MHC-positive GFP-BMCs appeared from day 1. There was a significant difference among days in group 3 ($P < .0001$). As the days passed, the expression of MHC-slow significantly increased to 2.5% on day 5.

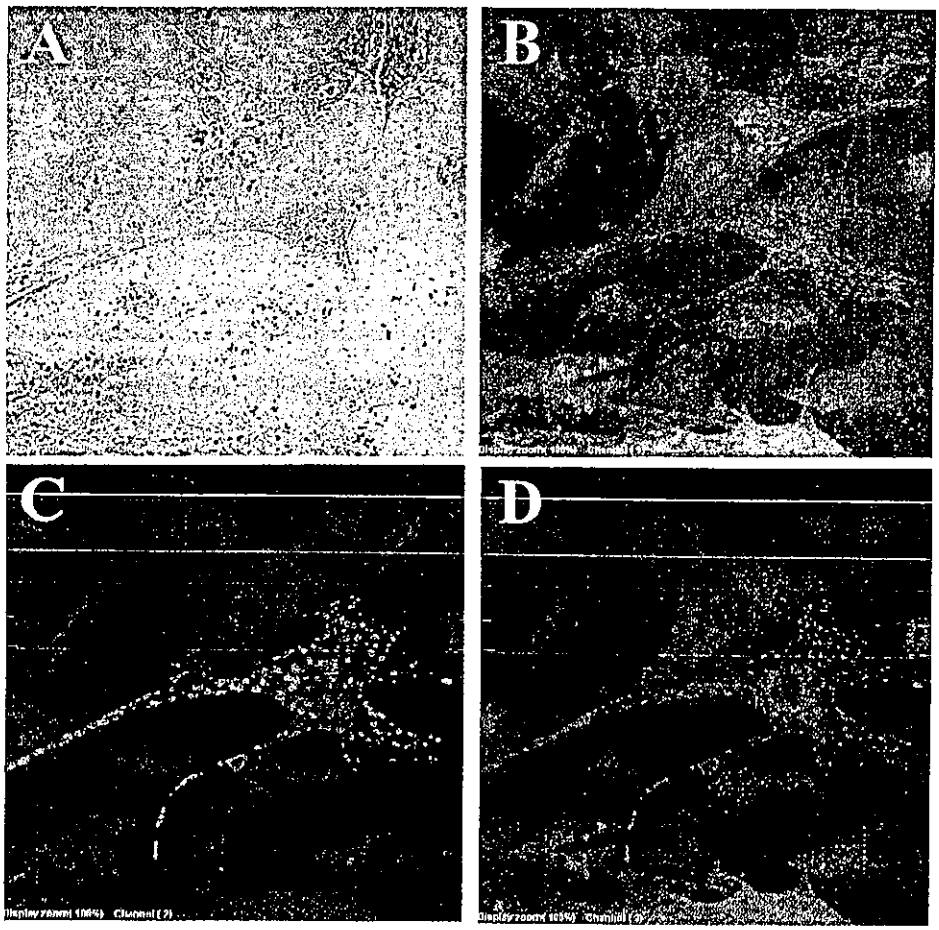


Figure 7. ANP-positive BMCs. Cells were stained with a first antibody against ANP. The phases (A-D) were the same microscopic conditions seen in Figure 5. (Original magnification 400×.)

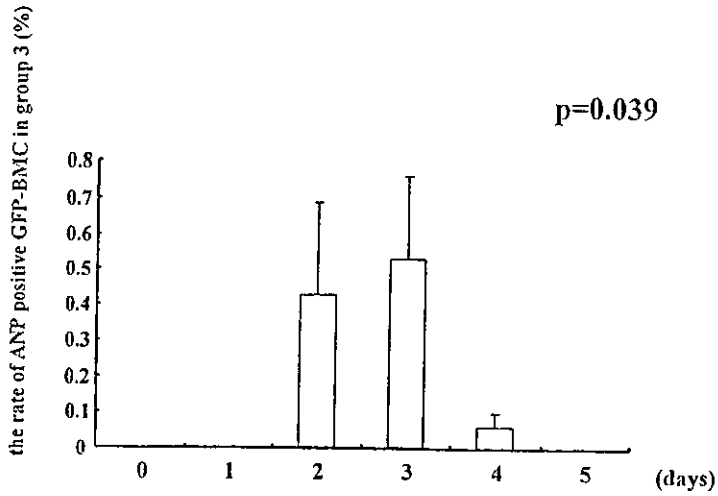


Figure 8. The percentage of ANP-positive BMCs in group 3. The *bar* represents mean and SE. Positive cells appeared on days 2, 3, and 4. Among days, the difference in the percentages were recognized as significant ($P = .039$).

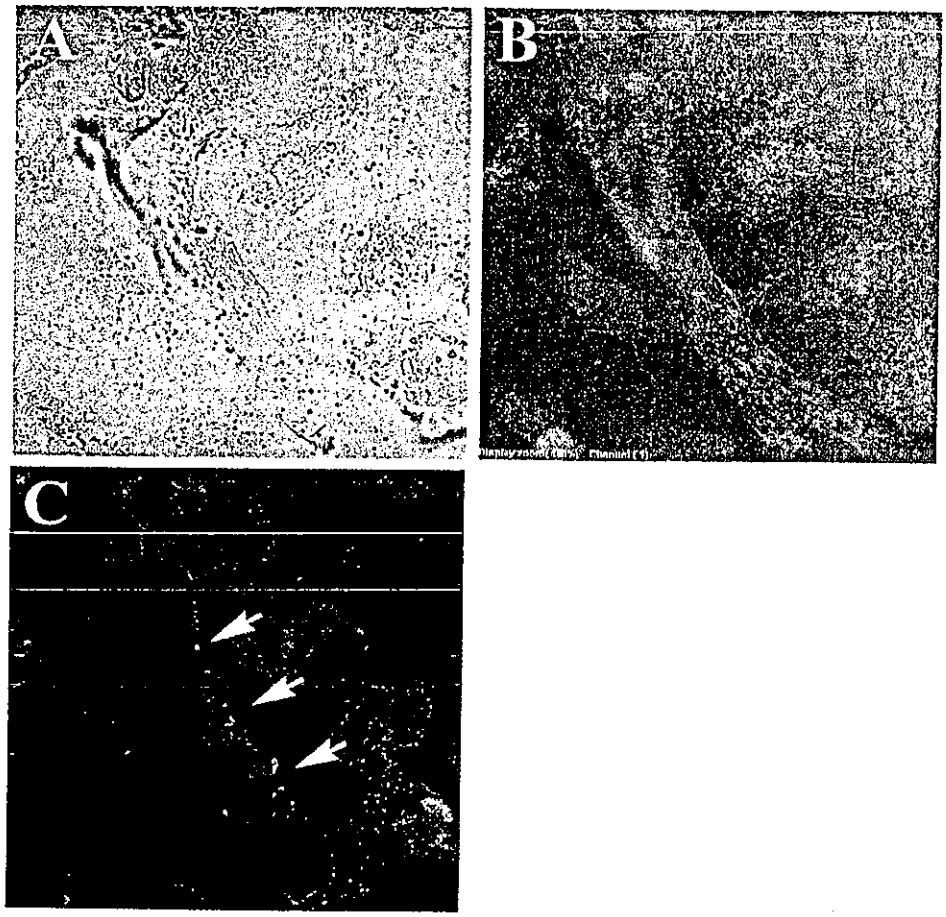


Figure 9. Connexin 43–positive BMCs. Cells were stained with a first antibody against connexin 43. Phases A and B were the same microscopic conditions as in Figure 5. Connexin 43 was detected at the margin of BMCs in phase C. (Original magnification 600×.)

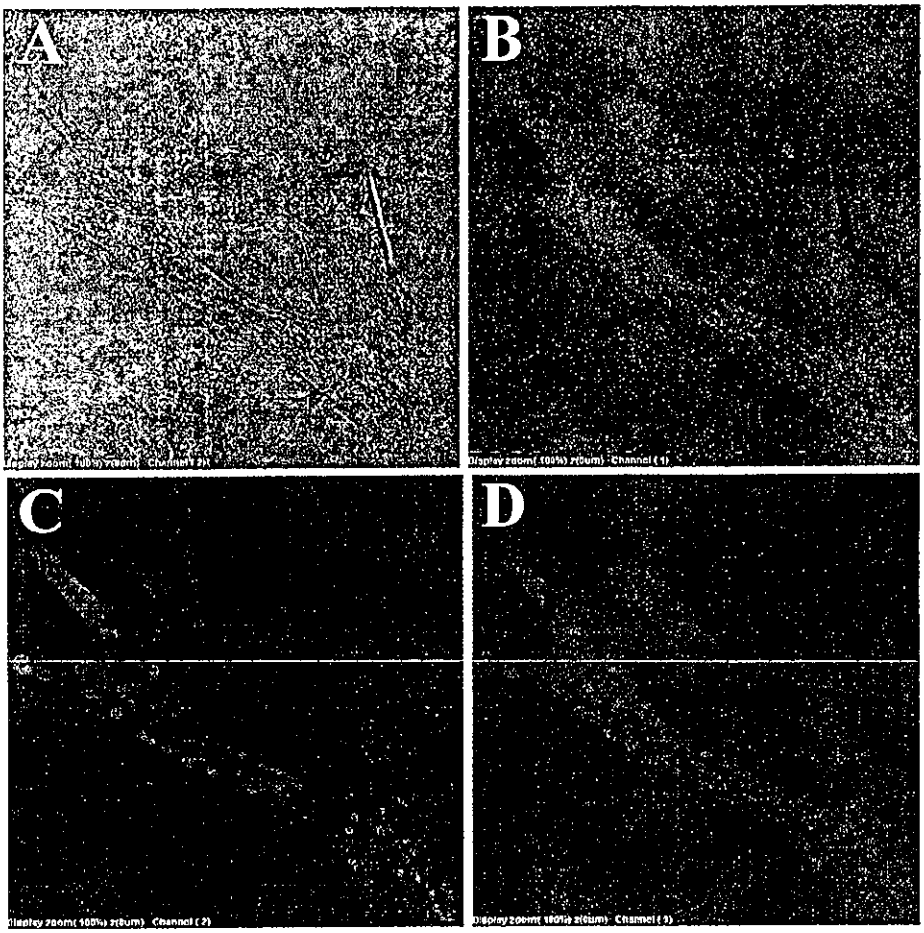


Figure 10. TnI–positive BMCs. Cells were stained with a first antibody against TnI. The phases (A-D) were the same microscopic conditions as in Figure 5. Some BMCs stained positively and showed myofibrils lengthwise along the cell. The expression of TnI corresponded to green fluorescence derived from BMCs. (Original magnification 400×.)

(MHC)-slow (Sigma, St Louis, Mo) diluted 1:1000 was used to evaluate the differentiation of striated muscle. A rabbit monoclonal antibody against atrial natriuretic peptide (ANP; Protos Biotech Corp, New York, NY) diluted 1:1000 was used to determine the cardiac-specific expression. Connexin 43 was detected by using a rabbit polyclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) diluted 1:1000 and a mouse monoclonal antibody against cardiac-specific troponin I (TnI; Hytest, 4C2, Euro City, Finland) diluted 1:200 to detect mature cardiomyocytes. The dishes were incubated with the first antibodies at 4°C overnight. The culture dishes were washed with PBS 3 times to remove unbound antibodies. The primary antibodies anti-MHC-slow and anti-TnI were detected with a goat anti-mouse IgG antibody (Alexa Fluor 568, Molecular Probes, Wako, Osaka, Japan), and anti-ANP and anti-connexin 43 were detected with a goat anti-rabbit IgG antibody (Alexa fluor 568, Molecular Probes, Wako, Osaka, Japan). After incubation, the culture dishes were rinsed with PBS. The cells were then evaluated and photographed with a Fluoview FV300 confocal laser scanning microscope equipped with a z-stepping system (Olympus, Tokyo, Japan).

Quantitative Analysis

The percentage of positively stained cells was determined by using a fluorescent microscope, and the structure of the differentiated GFP-BMCs was observed in detail by means of confocal microscopy. In briefly, the total cell number was counted in the bright field. GFP-BMCs were detected with a band beam splitter for simultaneous excitation at 515 to 540 nm and counted. Alexa dye, which conjugated the cells, was visualized with a band beam splitter for simultaneous excitation at 574 to 640 nm. The percentage of positively stained cells was calculated in 4 randomly selected fields of 5 culture dishes from the initial plating (day 0) through the seventh day (day 7).

Statistical Analysis

Statistical analysis was performed with StatView 5.0 software (SAS Institute, Inc, Cary, NC). All values are expressed as means ± SE. Comparison of the growth rate between 2 distinct groups was analyzed by using the Mann-Whitney *U* test. Comparison of the data among days in each group was performed with the Kruskal-Wallis test.

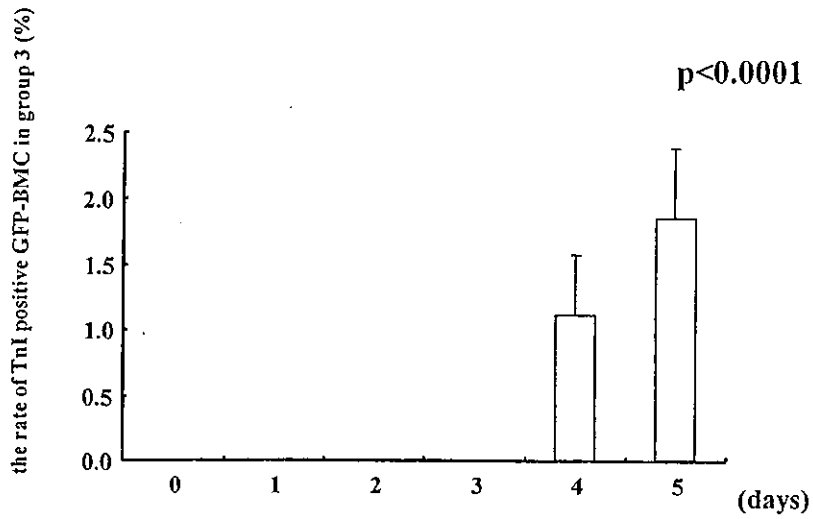


Figure 11. The percentage of cardiac-specific TnI–positive BMCs in group 3. The *bar* represents mean and SE. Positive cells appeared on day 4 and increased to 1.9% on day 5. The differences of the positive BMCs were recognized to be significant among days ($P < .0001$).

Results

Morphologic Changes of GFP-BMCs and Cardiomyocytes

Although GFP-BMCs were cultured and passaged, nonadherent cells were eliminated, and spindle-shaped cells formed colonies and proliferated rapidly. All of the GFP-BMCs but red blood cells expressed bright green fluorescence (Figure 2). Contracting cardiomyocytes were identified on day 1. Contracting frequency per minute of beating cardiomyocytes was 60 to 70. On day 2, a few neonatal cardiomyocytes connected each other, and the beating rate was 70 to 80 per minute. None of the cardiomyocytes was visible under the fluorescent condition (Figure 2). In group 1 the shapes of these cells varied (ie, spindle, oval, wedge, or sheet), and they did not show any contraction. In group 2 GFP-BMCs did not contract. The shape and proliferation of GFP-BMCs were not different from those in group 1. In group 3, however, on day 1, part of the spindle-shaped GFP-BMCs attached in parallel to the colony of contracting cardiomyocytes (12.5% ± 1.8%), whereas flattened GFP-BMCs covered the cardiomyocyte layer at random. On day 2, we found that GFP-BMCs attached to nonfluorescent contracting cells (cardiomyocytes) started to contract synchronously with cardiomyocytes (5.6% ± 2.3%, Figure 3). The beating rate was almost 60 to 80 per minute. On day 5, GFP-BMCs began forming colonies and maintained synchronous contraction (15.6% ± 4.2%). As time passed, the contracting cells communicated, and almost all the fields contracted synchronously. The proliferation of the GFP-BMCs between groups 1 and 3 was not different in this study (Figure 4). After day 6, the cultured cells were peeled off, and we could not evaluate them immunocytologically.

Phenotypic Changes of GFP-BMCs

In groups 1 and 2 GFP-BMCs did not express any type of myogenic or gap junction proteins. In contrast, in group 3 GFP-BMCs started to express MHC at 0.14% ± 0.09% of the total GFP-BMCs on day 1. The MHC-positive BMCs increased day by day and were recognized at 2.49% ± 0.87% on day 5 (Figures 5 and 6). The double-labeled cells indicated that striated muscles originated from GFP-BMCs, and almost all of these cells had dinuclei. The ANP-positive BMCs were detected mainly on days 2 and 3 (0.78% ± 0.56%, Figures 7 and 8). As the days passed, the ANP-positive BMCs decreased on day 4 and disappeared on day 5. Connexin 43 was identified between GFP-BMCs and unlabeled cardiomyocytes from day 2 through day 5 (Figure 9). The cardiac-specific TnI-positive BMCs appeared at 1.11% ± 0.42% on day 4 and increased to 1.86% ± 0.53% on day 5 (Figures 10 and 11). The results are summarized in Table 1. Groups 1 and 2 did not show any myogenic differentiation of GFP-BMCs. In contrast, GFP-BMCs in group 3 expressed MHC first, followed by the expression of connexin 43 and ANP. Finally, GFP-BMCs expressed TnI. Some GFP-BMCs stained positive against myogenic proteins attached directly to cardiomyocytes and some attached to cardiomyocytes through nonmyogenic cells.

Discussion

The evidence of cardiomyogenic differentiation of bone marrow cells in vivo suggests the existence of environmental factors.[2,4-6] However, these factors are not well known because they are in vivo phenomena, making investigation difficult. Possible factors might include cell-cell interaction,

Table 1. The trend of time-dependent expression of proteins

		0	1	2	3	4	5
Group 1		—	—	—	—	—	—
Group 2		—	—	—	—	—	—
Group 3	MHC	—	+	+	+	+	+
	connexin43	—	—	+	+	+	+
	ANP	—	—	+	+	+	—
	TnI	—	—	—	—	+	+

MHC: Myosin heavy chain slow. ANP: Atrial natriuretic peptide. TnI: Troponin I. +: some cells expressed protein. −: negative.

electrical and mechanical stimulation, and unknown growth factors.

We hypothesized the cell-cell interaction was a cardiogenic inducer for stem cells and set up a coculture to simulate the in vivo phenomena by using GFP-BMCs and cardiomyocytes, which have 2 advantages. First, GFP-BMCs are visible because the cells are alive. Contraction is a typical characteristic of myogenic cells, which is only seen in living cells. We can see the interaction between GFP-positive cells and GFP-negative cells dynamically. Second, GFP-BMCs facilitated 100% labeling efficiency, which enabled us to differentiate GFP-BMCs from cardiomyocytes. In other words it was possible to perform quantitative analysis of GFP-BMCs without false-negative and false-positive contamination. The cultured GFP-BMCs maintained green fluorescence strongly for at least 8 weeks. GFP-BMCs proliferated as C57 mouse-derived BMCs.

This study also has a characteristic: the simulation of xenogeneic cell transplantation from mice to rats in vitro. Even if xenogeneic cell transplantation has several issues, it might provide commercial availability in the future if immunologic problems are solved.[7]

Reinecke and colleagues[13] reported that some skeletal myoblasts contracted synchronously with adjacent cardiomyocytes in vitro. However, skeletal myoblasts did not differentiate into cardiomyocytes. Makino and associates[14] and ourselves[2] reported that BMCs were induced into cardiomyogenic cells with chemicals. In contrast, in this study we did not use any chemicals and only cocultured with cardiomyocytes. We showed here that multinuclei GFP-BMCs differentiated into cardiomyogenic cells. GFP-BMCs started to contract synchronously with cardiomyocytes. Isoproterenol (25 nmol/L) increased the heart rate of the GFP-BMCs and the cardiomyocytes from 80 to 100 per minute (unpublished data). This mechanism could also happen in vivo.[4,7]

There are some possible reasons why groups 1 and 2 did not show the cardiac differentiation of GFP-BMCs. Although BMCs have the capacity for cardiac differentiation, they might need some triggers, such as 5-azacytidine.[2,14] Furthermore, unknown soluble inducers might not exist or might exist only at low concentrations. We regarded the direct attachment with cardiomyocytes as one of the important triggers for the cardiogenic differentiation of GFP-BMCs.

Our results indicated that GFP-BMCs cocultured with cardiomyocytes expressed myogenic protein as the first step, gap junction protein and ANP as the second step, and TnI as the final step. In contrast to increasing MHC and TnI values, ANP vanished on day 5. ANP is important for proliferation in embryonal cardiac development.[15] Cardiomyogenic (CMG) cells from bone marrow stroma also expressed ANP.[16] The myogenic cells might have lost ANP as a result of ventricular phenotypic change.

Although we observed the differentiation of GFP-BMCs, the percentage of differentiated cells was low. We considered the possible reasons. We did not purify specific cell types, such as CD34, in this study. Therefore, cultured cells were heterogeneously populated, and only a very small percentage of the BMCs were pluripotent stem cells, whereas most others were lineage-destined progenitor cells.

In this study we evaluated this coculture for only 1 week because the cultured cells were detached from the bottom of dishes as a result of overconfluency. Given the time-dependent increase of MHC- and TnI-positive cells, the percentage of cardiomyogenic cells from GFP-BMCs might increase if a longer culture is possible. We simulated BMC transplantation into the normal myocardium in this study. Stem cells are thought to be subtle in the normal tissue. On the other hand, injury, including ischemia, might trigger these cells to be active.

Cell fusion was suggested as an explanation for stem cell plasticity.[17,18] In contrast, another group[19] was against the fusion theory because single euploid multipotent adult progenitor cells differentiated into cells of 3 germ layers in vitro. They showed a high frequency of chimerism in comparison with the results of the previous study.[17] Some in vivo studies have reported a robust (30%-50%) level of transdifferentiation.[20] Although we cannot exclude the possibility of cell fusion, our conversion rate (2.5%) was much higher than the frequency of spontaneous fusion (2-11 clones out of 10^6 BMCs; 0.0002%-0.0011%).[17] The mech-

anism of differentiation of stem cells should be investigated more deeply in future studies.

We used neonatal cardiomyocytes because we wanted to see contractions not seen in adult cardiomyocytes in vitro. The combination of adult cardiomyocytes and GFP-BMCs might be evaluated later.

The present study provides the first demonstration, to our knowledge, of the cardiomyogenic differentiation of BMCs without any chemicals in vitro. Using this coculture, we might be able to identify specific substances regulating cardiac development in the future.

We thank Ms K. Hattori for her help in breeding the GFP mice.

References

1. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999;284:143-7.
2. Tomita S, Li RK, Weisel RD, Mickle DA, Kim EJ, Sakai T, et al. Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation.* 1999;100(suppl 19):II247-56.
3. Tomita S, Mickle DA, Weisel RD, Jia ZQ, Tumiati LC, Allidina Y, et al. Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. *J Thorac Cardiovasc Surg.* 2002;123:1132-40.
4. Wang JS, Shum-Tim D, Galipeau J, Chedrawy E, Eliopoulos N, Chiu RC. Marrow stromal cells for cellular cardiomyoplasty: feasibility and potential clinical advantages. *J Thorac Cardiovasc Surg.* 2000;120: 999-1006.
5. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, et al. Bone marrow cells regenerate infarcted myocardium. *Nature.* 2001; 410:701-5.
6. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest.* 2001;107:1395-402.
7. Saito T, Kuang JQ, Bittira B, Al Khaldi A, Chiu RC. Xenotransplant cardiac chimera: immune tolerance of adult stem cells. *Ann Thorac Surg.* 2002;74:19-24.
8. Liechty KW, MacKenzie TC, Shaaban AF, Radu A, Moseley AM, Deans R, et al. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. *Nat Med.* 2000;6:1282-6.
9. Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, et al. Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med.* 2001;344:1750-7.
10. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. "Green mice" as a source of ubiquitous green cells. *FEBS Lett.* 1997;407: 313-9.
11. Wakitani S, Saito T, Caplan AI. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve.* 1995;18:1417-26.
12. Sakai T, Li RK, Weisel RD, Mickle DA, Jia ZQ, Tomita S, et al. Fetal cell transplantation: a comparison of three cell types. *J Thorac Cardiovasc Surg.* 1999;118:715-24.
13. Reinecke H, MacDonald GH, Hauschka SD, Murry CE. Electromechanical coupling between skeletal and cardiac muscle. Implications for infarct repair. *J Cell Biol.* 2000:149:731-40.
14. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest.* 1999;103:697-705.
15. Koide M, Akins RE, Harayama H, Yasui K, Yokota M, Tuan RS. Atrial natriuretic peptide accelerates proliferation of chick embryonic cardiomyocytes in vitro. *Differentiation.* 1996;61:1-11.
16. Fukuda K. Development of regenerative cardiomyocytes from mesenchymal stem cells for cardiovascular tissue engineering. *Artif Organs.* 2001;25:187-93.
17. Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature.* 2002;416:542-5.
18. Ying QL, Nichols J, Evans EP, Smith AG. Changing potency by spontaneous fusion. *Nature.* 2002;416:545-8.
19. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature.* 2002;418:41-9.
20. Lagasse E, Connors H, Al Dhalimy M, Reitsma M, Dohse M, Osborne L, et al. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat Med.* 2000;6:1229-34.

Discussion

Dr Frank W. Sellke *(Boston, Mass).* How do you know that it is due to direct cell-to-cell contact or interaction and that there is not some substance secreted that causes this effect?

Dr Tomita. Of course, from only the observation under the microscope, we do not know about that in detail. Some unknown soluble factors might go through gap junctions, and we speculated another mechanism of induction. We saw some TnI–positive cells derived from GFP-BMCs attached to GFP-negative cells, which were TnI negative. This observation suggested that some BMCs differentiated to cardiomyocytes by means of mechanical stretching. Therefore, there are several inducers in this system.

Dr Henry M. Spotnitz *(New York, NY).* What do you think the mediators are of this effect that are passing through the gap junctions?

Dr Tomita. Thus far I have no concrete evidence.

Dr Spotnitz. You are sure that these cells are being transformed and that they are not really myocytes?

Dr Tomita. Do you mean that the phenomenon is due to fusion?

Dr Spotnitz. Yes.

Dr Tomita. There were landmark articles regarding fusion between embryonic stem cells and BMCs published in the journal *Nature* in April. They include a warning that reported differentiation might be due to fusion.But in this study we just cultured cardiomyocytes and BMCs and not embryonic stem cells. Of course there are some possibilities, but embryonic stem cells are very energetic and immature. They are easy to communicate, and in the in vivo situation we put BMCs in the adult heart. They are not embryonic stem cells. Therefore, it is a different story.

Dr Marcio Scorsin *(Curitiba, Brazil).* I have some doubts concerning the fate of transplanted BMCs into a myocardial infarction scar. It is widely accepted that those cells might have a milieu-dependent differentiation (becoming cardiomyocytes) in normal myocardium. However, if you inject those cells into a myocardial scar, according to some studies, they would produce angiogenesis and differentiate into fibroblasts instead of cardiomyocytes. My question is whether you think that it is important to differentiate BMCs before transplantation.

Dr Tomita. For the in vivo study, it is not necessary to convert all BMCs into cardiomyocytes. For example, if you put BMCs into the scar, they might go in like myofibroblasts, but the myofibroblasts are also important to prevent extension of the scar. I saw some TnI–positive cells from transplanted BMCs in the scar tissue in the previous study. I agree to the hypothesis that fibroblasts are strong inducers for BMCs to transform to fibroblasts.

In terms of the strategy for the differentiation with BMCs, I do not know which is stronger for the differentiation, either the in vitro condition or the in vivo condition. However, when we think about the cell process for the clinical reality, it might be difficult to control preferable cell types in vitro under GMP regulation. Therefore, it might be more practical to manipulate cells in the in vivo environment.

Dr Marc J. H. Hendrikx *(Hasselt, Belgium).* If you did not use a coculture but just differentiated your BMCs by using 5-azacytidine, would you get the same expression of cardiac markers? Do you have any ideas about that?

Dr Tomita. We reported the BMC differentiation using 5-azacytidine in the journal *Circulation* in 1999, but in this study I just cultured in the cardiac environmental setting. Therefore, I did not use 5-azacytidine in this study. In the next step, we are considering using 5-azacytidine. It might increase the number of induced cardio-specific cells in the coculture system.

Artificial Organs
28(1):40–44, Blackwell Publishing, Inc.

Review Article

Cell-based Therapy to Regenerate Myocardium: from Bench to Bedside

Shinji Tomita

Cardiothoracic Surgical Unit, Green Lane Hospital, Auckland, New Zealand

Abstract: The field of cell-based therapy to regenerate myocardium has been expanding rapidly, with significant advances being made in both the laboratory and the clinical area. In this article we review this field, including our experiences and discuss remaining issues and possibilities for future clinical applications. **Key Words:** Heart—Cell-based therapy—Exogenous—Endogenous—Clinical application—Regenerative Medicine.

Although heart transplantation is an effective treatment for end-stage heart failure, the shortage of donors is a major limiting factor (1–3). While heart transplantation currently remains the best choice, other alternatives, such as mechanical support and drug therapy, are being investigated.

In 1992, research into cell transplantation began, based on the hypothesis that skeletal myoblast transplantation might regenerate myocardium and improve cardiac function (4). Since then, a variety of cell types have been investigated enthusiastically (5–8).

EXOGENOUS CELL TRANSPLANTATION

Bone marrow cells have many advantages. The technique of bone marrow aspiration is an established procedure in hematology. There are no immunological or ethical issues because of the autologous source.

Bone marrow comprises two major systems: one hematopoietic and the other mesenchymal. Bone, cartilage and fat derive from "mesenchymal stem cells". However, the lack of a universal way to identify "mesenchymal stem cells" represents a major obstacle.

In 1999, Makino et al. (9) and our group (6) reported that cardiomyocytes could be generated from bone marrow cells. Bittner et al. reported that intravenously-transplanted bone marrow cells could also differentiate into cardiomyocytes (10).

Other groups have tried to purify adult stem cells of several phenotypes, including cardiomyocytes, endothelial cells and smooth muscle cells. Orlic et al. injected Lin-neg, c-kit-pos-cells to regenerate the infarcted myocardium (11). Jackson et al. used side population (SP) cells (12). Researchers are attempting to manipulate stem cells in vitro and in vivo. However, currently nobody has established the best way to expand human cardiomyocytes or cardiomyoblasts to a number that would be sufficient for clinical application.

Skeletal myoblasts have several advantages compared to bone marrow-derived stem cells. Principally, they can form striated muscle tissue in the diseased area, and current efforts strive to demonstrate improved cardiac function as the result of the electrical and mechanical connection between host and donor cells. However, the first clinical trials from Menasche's group in France have raised a critical issue, namely arrhythmia (13). Four out of ten patients had ventricular tachycardia and required an automatic intracardiac defibrillator (AICD). They

Received September 2003.
Address correspondence and reprint requests to Dr. Shinji Tomita, Cardiothoracic Surgical Unit, Green Lane Hospital, Green Lane West, Auckland 1003, New Zealand. E-mail: shinjitomita@hotmail.com