標準書に記載しGMPで管理することを考慮すべきであろう。

有効性と安全性は臨床試験でしか評価ができないが（安全性の一部は非臨床試験で評価する）、品質は臨床試験前に評価が可能であるからこそ、品質の確保は重要である。試験物、あるいは製品の品質管理を行わなくてはならないのは、研究開発段階での品質の役割としてお被験者保護（患者保護）の観点（倫理的妥当性）からの品質確保のためであり、承認申請・製造販売に向けた品質の知識・データの取得の観点から、臨床試験の質を高め、結果を正当に評価するためでもある。再生医療製品にあっても、品質についての考え方が変わるわけではない。細胞製剤の特性を考慮しつつ、安定した品質の再生医療製品が提供されるべきである。

D. 考察

脂肪組織由来多系統前駆細胞（Adipose tissue-Derived Multi-lineage Progenitor Cells; ADMPC）は、脂肪組織をコラーゲン分解酵素で処理して得られたStromal Vascular Fractionを播種し、24時間後にEDTAへの反応性の差から単離される、小径で核/細胞質比が大きい細集団であって、未分化マーカである核内転写因子*GATA-4*や*Isl-1*が発現、多系統分化能を有している。細胞質が未発達で、ミトコンドリアが分化細胞に比して少なく、かつER-Golgi系が未発達であるために糖鎖が少ない。SSEA-3陽性で、しかも増殖能を有するという細胞特性をもつ。

われわれは、ADMPCが肝臓内に投与すると肝細胞様に分化生着し、高脂血症のモデル動物である渡辺遺伝性高脂血症ウサギのコレステロール値を低下させることを見出し、報告してきた。この知見に関しては、細胞投与による炎症に伴うIL-6血中濃度上昇が、肝細胞のLDL受容体をup-regulationさせるためではないか、との反駁をうけていた。

本研究では、酵素（βガラクトシダーゼ）が完全欠損しているGM1-gangliosidosisモデルマウスに健常マウス由来ADMPCを投与し、血中酵素活性を測定するとの試験系を採用した。細胞投与に伴う炎症などにより酵素血中濃度が上昇するとは考えられず、健常マウス由来ADMPCが生着してβガラクトシダーゼを分泌したと結論つけるのが合理的である。また、肝臓内に投与したADMPCが肝細胞様に分化生着していた。これら、Stem cell（ADMPC）が肝臓内で肝細胞へと分化誘導されているとの知見から、"*in situ* reprogramming"との概念を提唱した。Terminal differentiated cellを*in vitro*にて多能性幹細胞化する"reprogramming"、遺伝子導入等で*in vitro*にて直接目的細胞へと分化させる"direct reprogramming"に加えて新しい概念であり、治療へは*in situ* stem cell therapyとして展開することとなる。

一方で、GM1-gangliosidosisモデルマウスホモ接合体へADMPCを投与した群では、非投与群に比較して血中βガラクトシダーゼ活性の上昇を確認したものの、ヘテロ接合体に比較しても低値である。ヘテロ接合体は無症状であることから、治療効果が期待できると想定しているが、頻回投与など投与プロトコールの検討が必要かもしれない。

加えて、本研究「ライソゾーム病に対する細胞医薬品の開発にむけたConfidence-in-

Mechanism (CIM) 取得のための基礎研究」において、研究期間終了後の細胞組織利用医薬品としての展開を見据えると、開発初期からのマーケティングおよびプロダクト・マネージメントが必須であろう。

第1にマーケティング分析を行う。マーケティングは、Research → Segmentation, Targeting and Positioning → Marketing Mix → Implementation → Controlより成る。Researchにおいてはヒト脂肪組織由来多系統前駆細胞の環境分析（マクロ・ミクロ）からSWOT分析を実施し、それをSegmentation, Targeting and Positioningに生かし、当該製品たるヒト脂肪組織由来多系統前駆細胞浮遊細胞製剤が市場展開性を有するか検討・検証を行う。当該製品に市場展開性があると判断されれば、Marketing Mixとして、4P（product, price, place and promotion）を代表的手法として検証することとなる。これらをもとに、実施（Implementation）にてマーケティング組織を構築し、年間計画・収益性あるいは戦略のコントロールを行う。ヒト脂肪組織由来多系統前駆細胞は、いまだ製品として上市されていないことから、本考察ではR→STPについて議論することとする。

Researchにおいてまず、マクロ環境分析をPEST手法により分析する。（1）政治・法的要因(P)として、産業界への法的規制、政府助成、政府の介入度が挙げられる。再生医療製品一般にあっては、薬事規制を受けるという前提があり、例外的に医師法・医療法下にて医師が自ら実施する臨床研究として、ヒト幹細胞を用いる臨床研究に関する指針への適合性も課題である。本研究にて開発しているライソゾーム病を適応症とするヒト脂肪組織由来多系統前駆細胞は、ヒト幹細胞を用いる臨床研究に関する指針にのっとるため、機関内倫理審査委員会にて審議ののち、厚生労働省厚生科学審議会科学技術部会ヒト幹細胞臨床研究の審査に関する委員会にて審議されるべく、ヒト幹細胞を用いる臨床研究に関する指針（平成20年厚生労働省告示第380号）に適合すべく研究開発を進めている。本研究グループには、平成24年5指針の策定を主導した早川堯夫がアドバイザーとして参画しており、密接な研究打ち合わせと相まって、対応可能である。（2）経済的要因(E)として、GDP、為替、金利水準、所得水準について議論するのが一般的であるが、本再生医療製品に展開にあっては、我が国固有の国民皆保険制度（特に保険点数の上限）について評価すべきである。再生医療製品は先端的医薬品であるため、「革新的医薬品医療機器創出に係る5カ年計画」にあっても、高薬価にてインセンティヴを付与することとなっており、将来的に治験が実施されれば、本再生細胞製剤にあっても、十分な薬価が公示されると想定される。また、対象疾患がオーファンであることからも、インセンティヴを与えられる蓋然性が高い。（3）社会的要因(S)としては、宗教、道徳観、文化的価値観について分析を加えるが、当該再生細胞製剤ににあってはゼロリスクを求める国民性と、疾病構造としてライソゾーム病患者がオーファンであることを念頭に入れる必要がある。患者数が少ないため展開性が低いのではないかとの議論に関しては、患者数が少ないため競合研究者・機関が少ないという利点でもある。再生細胞治療は

個別化医療に近く、供給側が律速となることを考えると、共同他者が少ないことは、当該マーケットを確実に得られるということであり、むしろ強みとなる。（4）技術的要因(T)に関しては、最新技術、技術特許について議論することとなる。当該再生細胞製剤にあっては、特許・ノウハウ等知的財産および、競合他者製品の研究開発状況について分析を進める必要がある。間葉系幹細胞の基本特許のうち有効とみなされているのが1990年にUCLAより出願された骨髄由来間葉系幹細胞である。当該知財に追加する形で実施例なくして脂肪組織由来幹細胞の存在が示されたのが1994年である。これら知財は、米国では成立しているが我が国では放棄されている。脂肪組織由来間葉系幹細胞として実施例を伴って出願されたのが、UCLAとPittsburg大学の共同出願である出願2000-603416があるが、我が国では2回の拒絶査定を受けている。脂肪組織由来幹細胞として、我が国では「脂肪組織から幹細胞を調製するための方法およびシステム」（登録番号4217262）が成立しているものの、コラゲナーゼ処理を工程に含んでいない。

ついで、ミクロ環境分析として外部分析を試みる。ミクロ環境の外部分析要因として5 Forcesを考慮する。Forcesとは、競争者（競合者）、新規参入者、代替品、供給者（原材料供給業者）、買い手（保険者・病院・医師・患者）である。（1）競争者に関しては、本再生細胞製剤にあっては研究者・開発者間の敵対関係はどの程度か、という分析である。脂肪組織由来細胞を用いる臨床研究を実施している研究グループも多数あるが、美容外科等が主体で遺伝性疾患を対象としているグループはない。（2）新規参入者については、新規参入の脅威はどの程度かを議論することとなる。本再生細胞製剤では、希少難病を対象としているため、他研究グループが被験者（患者）リクルート可能であるか、という議論となろう。患者団体への働きかけなどが、今後の課題となろう。ついで、（3）代替治療法の脅威はどの程度か分析する。ライソゾーム病に対する酵素補充療法が試みられ、すでに3薬剤の承認が得られている。これら薬剤はム多糖症の病型の一つ一つについてBiologicsの開発が必須であるが、我々が開発中の細胞製剤は、本薬剤によって広範は病型を網羅可能であり、著しい優位性を持つ。（4）供給業者（原材料供給業者）については、試薬メーカー等の交渉力はどの程度か、という議論となる。臨床展開後に、試薬メーカーから原材料の値上げを通告されても薬価は変わらず、利益率は低下することとなる。我々は、研究開発当初から、「essential facilitiesは与えない」という戦略のもと、すべての培地、試薬は併売者がいる試薬のみを原材料として選定、かつ必ず併売者間で見積もりを取って競合させるとの戦略をとってきた。実際、基礎培地（DMEM-Low glucose: MCDB201, 6:4）は、当初500mL3500円で納入されていたが、平成24年度末にて700円にて納品され、fibronectinコート培養皿にあっては1枚1200円であったものが540円になり、かつfibronectinのヒト由来ウイルス検査まで実施した後に納品されている。これらにより、製造（品質管理・人件費とCPC賃借料を除く）に1ロットで350万円程度かかっていたものが、90万円程度にまで低コスト化できている。ついで、5

Forcesの最終要素である（５）買い手の交渉力は、将来的に十分な保険点数が公示されうるか、どの程度利用していただけるかを左右する。前者に関しては、再生医療製品は先端的医薬品であるため、「革新的医薬品医療機器創出に係る5カ年計画」にあっても、高薬価にてインセンティヴを付与することとなっており、将来的に治験が実施されれば、治験中に厚生労働省医政局経済課と折衝を持つこととしている。 買い手（保険者・病院・医師・患者）に着目した解析手法が、VALSである。VALSとはValues & Life-styles調査手法で、顧客の価値観でsegmentationをする手法である。開発しているヒト脂肪組織由来多系統前駆細胞にあっては、その投与により疾患の増悪を予防できる可能性があり（機能的価値）、保険診療費用の低減（経済的価値）も意思決定に寄与しうる。臨床利用の決定に大きな影響力を有する医師（主治医）を想定すれば、先端医療を用いて治療しているという充足感（心理的価値）もモティベーションになると考えられる。一方で、innovatorであるヒト幹細胞臨床研究の実施施設となる研究開発病院（特定機能病院等）と、差別化を希求するearly adaptorたる大学病院・専門病院と、early majorityとなる一般病院では臨床利用の意思決定に差があるのは明白である。まず、innovatorたる特定機能病院にてヒト幹細胞臨床研究を実施し、研究期間終了時には治験としてearly adaptorたる大学病院・専門病院への導入を目指すこととなる。

ミクロ分析として、内部分析を試みる。ミクロ環境の内部分析要因はValue chainの分析であり、 「アウトプットする価値はさまざまな活動の連鎖からなる」という考えに基づく。購買物流、製造、出荷物流、販売、サービスからなる主活動と、インフラストラクチャー、人事/労務管理・技術開発・調達活動を行う支援活動とに分けられる。確固とした知財がValue Chainの根幹であることを考えると、「肝臓関連疾患治療薬」（特許第4965000号）として特許が成立しており、EU特許も平成24年度末に成立していることから優位な位置にある。今後、スタッフの増員と教育によりValue chain体制の構築が急務であり、自らの企業による社会好悪権も含め、今後の課題である。

これまで、マクロ環境分析とミクロ環境分析を実施してきた。これらをもとに、SWOT/SWOTクロス分析を行い、もってResearchとする。S:はStrength（強み）、WはWeakness(弱み)でともに内部環境因子である。OはOpportunity（機会）、TはThreat（脅威）でともに外部環境因子である。本研究にて開発しているヒト脂肪組織由来多系統前駆細胞たる「肝臓関連疾患治療薬」は、特許が成立していることに加え、請求項にて広範囲に肝臓関連疾患を対象とすることを認めていることから、将来的に本研究対象疾患であるライソゾーム病など遺伝性肝疾患のみならず肝炎・肝硬変への展開も可能であり、十二分な強みがある。外部要因としては本研究事業にて開発している再生細胞製剤は、PEST分析・5 Forces分析の結果にみるように市場展開の機会を十分に有している。従って、SWOTクロス分析をみるにportfolio上事業機会を有しているとの結論に至る。

ついで、マーケティングの第2段階としてのSTP (Segmentation, Targeting and Positioning)について考察したい。再生医療製品は

限りなく個別化医療に類似している。マーケティング的にいえば、マス・マーケティングからターゲット・マーケティングへの転換である。再生医療はこれまで治せなかった疾患を治療する等アンメット・メディカル・ニーズへの応答であって、従来のマス・マーケティングでは展開不可なのは自明であることからも、ターゲット・マーケティングの発想が重要であることが分かる。共通ニーズを持つ集団（セグメント）を発見し、その集団をターゲットに価値（ポジショニング）を提供して市場占有を確保することがSTPの本質であることから、ヒト脂肪組織由来多系統前駆細胞の展開可能な疾患Segmentは知財の範囲から広範な肝臓関連疾とできる。ライソゾーム病はセグメントとして小さすぎるとの議論もあろう。市場をセグメント以上に細分化したこれら集団はサブセグメント、あるいはニッチとも呼ばれる。本研究開発シーズにおいては、positioningをしっかり押さえれば、グローバル・ニッチ・トップ、ついで適応拡大として遺伝性肝疾患、最終的には肝硬変治療剤としての展開を射程に入れうる。

Positioningとは、ジャック・トラウトによれば「あなたが狙う顧客の心の中であなたの製品をどう独自化するか、それがポジショニングの意味」である。本研究事業に当てはめれば、ライソゾーム病患者あるいはその主治医の治療法選択肢のなかで、ヒト脂肪組織由来多系統前駆細胞製剤を独自化することといえる。まず、梯子の法則にのっとり当該細胞製剤の存在を想起させしめ、Positioning mapポートフォリオ分割にて高機能かつ低価格（国民皆保険下では低負担と定義した方が適切と思われる）であることから選択に導くことが肝要である。

STPが終了すると、Marketing Mixにいたる。代表的手法として製品：product、価格：price、流通チャンネル：placeおよびプロモーション：promotionによりmarketingを進める「4P」が用いられる。再生細胞治療製剤に当てはめれば、Product：どのような再生医療製品を、Price：どの程度の価格で、Place：どのような流通経路で、Promotion：どのように宣伝するか、である。しかし、4Pはあくまで造り手の視点であるとの批判もあり、「4C」によるmarketingが行われるようになっている。4Cとは、Customer Solution：ニーズにこたえるサービスであるか？、Customer Cost：サービスに見合った価格であるか？Convenience：利便性をもったサービスであるか？、そしてCommunication：どのように顧客と意思疎通するのか？である。顧客視点への転換であると言われる所以である。再生細胞治療製剤に焦点を絞れば、Customer Solution：患者・医師のニーズにこたえる治療法であるか？、Customer Cost：医療保険財政・制度からみて、治療による費用と治療により得られる社会的利益が見合っているか？Convenience：患者・医師にとって選択しやすい治療法であるか？、そしてCommunication：どのように患者・医師と意思疎通するのか？である。ライソゾーム病を適応症とするヒト脂肪組織由来多系統前駆細胞の細胞製剤としての開発にあっては、1）Customer Solution：根治的治療法がなかったライソゾーム病患者とその主治医にとって、ヒト脂肪組織由来多系統前駆細胞投与が求められる治療法となりうるのか？　2）Customer Cost：医療保険財政・

制度からみて、ヒト脂肪組織由来多系統前駆細胞投与にかかる費用と本治療により得られる社会的利益が、経済的・QOL・社会正義の観点から見合っている（trade-off可能）か？３）Convenience：患者・医師にとって選択しやすい治療法として提供が可能か？、そして４）Communication：患者・主治医と意思疎通が現実的に可能なのか？である。本研究グループは、これらのマーケティング上の課題に一つ一つ答えを見出すべく、研究開発をすすめなければならない。

E. 結論

本研究では、生体内で分化生着した再生肝細胞がライソゾーム加水分解酵素を持続的に分泌、全身の細胞組織に供給補充し続けることを機序とした新規概念の細胞医薬品と位置付けられるとの特色を有する。当該概念による製剤はこれまで皆無であり独創的である。

ADMPCの投与により、ライソゾーム病の代表例としてのGM1-gangliosidosisモデルマウス（βガラクトシダーゼKOマウス）の血中βガラクトシダーゼ活性が改善した。ADMPCがライソゾーム病に対する再生医療等製品（細胞製剤）として研究開発を進めることに合理的根拠を示しえた。

なお、本研究事業では、費用のキャッピングから慢性試験を十分には実施できなかった。今後は、企業等へのスムーズなライセンスアウトにむけ、薬事戦略相談・治験・製造販売承認取得を念頭に置いた研究開発を進めることとしている。

F. 研究発表

1. 論文発表

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2. 「自主開発の経験から」・大倉 華雪・第 5 回ミニマム・コンセンサス・パッケージ（MCP）策定会議、第 5 回再生医療薬事講習会「非臨床安全性試験データパッケージの提案」—何のために非臨床試験？何のための非臨床試験？ 2013 年 10 月 4 日（金）
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G. 知的所有権の取得状況

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Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅲ. 研究成果の刊行物・別刷

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Intracoronary artery transplantation of cardiomyoblast-like cells from human adipose tissue-derived multi-lineage progenitor cells improve left ventricular dysfunction and survival in a swine model of chronic myocardial infarction

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ABSTRACT

Transplantation of human cardiomyoblast-like cells (hCLCs) from human adipose tissue-derived multi-lineage progenitor cells improved left ventricular function and survival of rats with myocardial infarction. Here we examined the effect of intracoronary artery transplantation of human CLCs in a swine model of chronic heart failure. Twenty-four pigs underwent balloon-occlusion of the first diagonal branch followed by reperfusion, with a second balloon-occlusion of the left ascending coronary artery 1 week later followed by reperfusion. Four weeks after the second occlusion/reperfusion, 17 of the 18 surviving animals with severe chronic MI (ejection fraction <35% by echocardiography) were immunosuppressed then randomly assigned to receive either intracoronary artery transplantation of hCLCs hADMPCs or placebo lactic Ringer's solution with heparin. Intracoronary artery transplantation was followed by the distribution of DiI-stained hCLCs into the scarred myocardial milieu. Echocardiography at post-transplant days 4 and 8 weeks showed rescue and maintenance of cardiac function in the hCLCs transplanted group, but not in the control animals, indicating myocardial functional recovery by hCLCs intracoronary transplantation. At 8 week post-transplantation, 7 of 8 hCLCs transplanted animals were still alive compared with only 1 of the 5 control ($p = 0.0147$). Histological studies at week 12 post-transplantation demonstrated engraftment of the pre DiI-stained hCLCs into the scarred myocardium and their expression of human specific alpha-cardiac actin. Human alpha cardiac actin-positive cells also expressed cardiac nuclear factors; nkx2.5 and GATA-4. Our results suggest that intracoronary artery transplantation of hCLCs is a potentially effective therapeutic strategy for future cardiac tissue regeneration.

1. Introduction

End-stage heart failure remains a major cause of death worldwide, mainly due to myocardial ischemia. Cardiac transplantation and mechanical support using implantation of the left ventricular assist system (LVAS) were established as the ultimate means of support for these patients [1,2]. However, these treatment entities have certain limitations including donor shortage, rejection, and LVAS durability, and alternative strategies are needed in such circumstances.

Cellular cardiomyoplasty was developed as a new approach to restore normal heart function, [3,4] using a variety of cell types [3–5]. Mesenchymal stem cells (MSC) seem particularly advantageous for cellular therapy in general because they are multipotent, potentially immune privileged [6]. MSC also proliferate rapidly and differentiate into cardiomyogenic cells [7–10]. MSC can be isolated from human adipose tissue, which can be resected easily and safely in most patients [11,12]. In fact, we have reported that adipose tissue-derived multilineage progenitor cells (ADMPCs), which met the criteria as mesenchymal stem cells [13], can differentiate into hepatocytes both *in vitro* and *in vivo* [14,15]. Recently, we demonstrated that human cardiomyoblast-like cells (hCLCs) from human adipose tissue-derived multi-lineage progenitor cells transplanted into rats with chronic myocardial infarction reversed wall thinning

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in the scarred area with the engrafted cells forming a thick stratum, and that the hCLCs reversed left ventricular dysfunction in the long term and survival of rats with experimentally-induced myocardial infarction [16].

The present study is an extension to the above study and was designed to accelerate the clinical application of hCLCs. Specifically, we examined in pre-/non-clinical studies the effects of hCLCs transplantation on cardiac dysfunction and on long-term survival with swine chronic myocardial infarction model. We also documented the histological regeneration of damaged myocardium after transplantation of hCLCs *in vivo*.

2. Materials and methods

2.1. Adipose tissue

Adipose tissue samples were resected from five human subjects during plastic surgery (all females, age, 20–60 years) as excess discards. Ten to 50 g of subcutaneous adipose tissue were collected from each subject after obtaining of 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.

2.2. Isolation of hADMPCs and preparation of hCLCs

Human adipose tissue-derived multi-lineage progenitor cells (hADMPCs) were prepared as described previously [13–17]. After passaging 5 to 6 times, the hADMPCs were replated and treated with 0.1% dimethyl sulfoxide (DMSO) (Cryoserve, GE Healthcare Biosciences, Uppsala, Sweden) for 48 h.

2.3. Reverse transcriptase–polymerase chain reaction

Total RNA was isolated from hADMPCs and cardiomyoblasts using an RNAeasy kit (Qiagen, Hilden, Germany). After treatment with DNase, cDNA was synthesized from 500 ng total RNA using Superscript III reverse transcriptase RNase H minus (Invitrogen, Carlsbad, CA). Real-time PCR was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). 20X Assays-on-Demand™ Gene Expression Assay Mix for *nkx2.5* (Hs00231763_m1), *islet-1* (Hs00158126_m1), *GATA-4* (Hs00171403_m1), *alpha-cardiac actin* (Hs01109515_m), *cardiac troponin I* (Hs00165957_m1), *myosin light chain* (*MLC*) (Hs00166405_m1), *myosin heavy chain* (*MHC*) (Hs00411908_m1) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) (Hs99999905_m1) were obtained from Applied Biosystems. TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2X), was also purchased from Applied Biosystems. Reactions were performed in quadruplicate and the mRNA levels were normalized relative to human GAPDH expression. Then the fold-inductions of hCLCs were compared to hADMPCs.

2.4. Animal model of myocardial infarction and cell transplantation

Five weeks before transplantation, the first diagonal branch (D1; #9) of the coronary arteries of 24 pigs (8-week-old female, 30.5 ± 0.7 kg, mean ± standard error of the mean) was balloon-occluded for 60 min followed by reperfusion, (Fig. 1A). One week later, the left ascending coronary artery of the same animals was balloon-occluded just proximal of the first septal branch divergence (#6), followed by reperfusion (Fig. 1A). To rescue the better baseline survivals and to obtain severe old myocardial infarction swine model, two separate reperfused infarcts one week apart were performed. From 5 days before cell transplantation to the end of the experiment, the swine received tacrolimus as an immunosuppressant (0.1 mg/kg/day intramuscularly) (Fig. 1B) as previously reported [18] with modification. Four weeks after the second occlusion/reperfusion (day 0), we examined 17 animals with chronic severe MI (ejection fraction <35% by echocardiography) of only 18 survivors. The tacrolimus-immunosuppressed chronic MI swine were randomly assigned to receive intracoronary transplantation of hCLCs (3×10^5 cells/mL concentration of cell

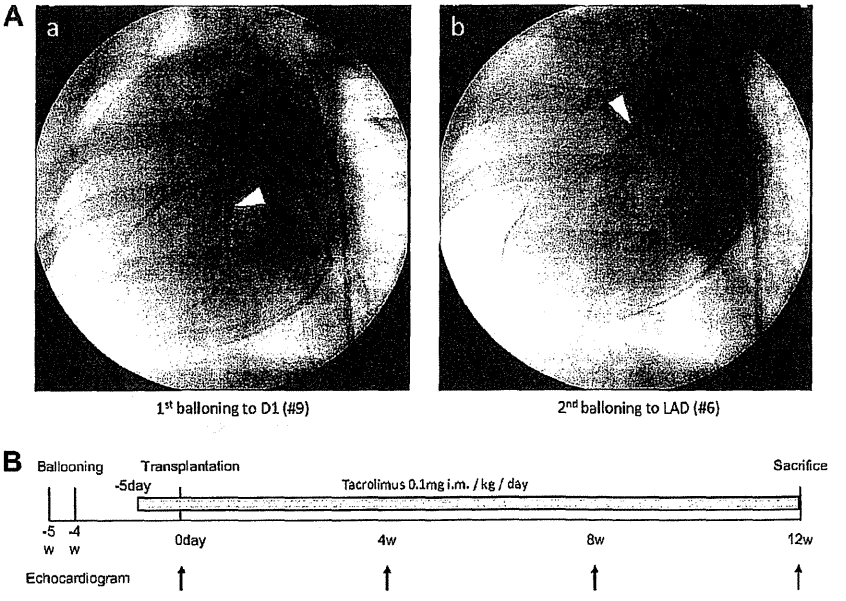

Fig. 1. Study protocol and angiographic demonstration of transient coronary artery occlusion. (A) Five weeks before transplantation, the first diagonal branch (D1; #9) of the coronary arteries was balloon-occluded followed by reperfusion (a, arrowhead). One week later, the left ascending coronary artery of the same animals was balloon-occluded just proximal of the first septal branch divergence (#6), followed by reperfusion (b, arrowhead). (B) From 5 days before cell transplantation to the end of the experiment, the swine received tacrolimus as an immunosuppressant. At day 0, 17 animals with chronic severe MI were applied for the experiment.

Table 1
Cardiocytic induction of hCLCs.

	Fold induction	
	Mean	SE
nkx2.5	2.49	1.02
islet-1	1.32	0.36
GATA-4	6.84	1.47
Alpha-Cardiac actin	1.46	0.22
Cardiac troponin I	2.36	0.47
Myosin light chain	1.89	0.49
Myosin heavy chain	109.89	6.13

suspension, 1 mL/kg cell suspension was transplanted.) ($n = 8$), hADMPCs (3×10^5 cells/mL concentration of cell suspension, 1 mL/kg cell suspension was transplanted.) ($n = 4$), or placebo lactic Ringer's solution with heparin ($n = 5$), at 4 weeks after the second occlusion/reperfusion. Transplantation procedure was performed as following, the transarterial catheter was placed in the left coronary artery, and then the cell-suspensions or placebo control solutions were transplanted into LAD (#6). The Osaka University Graduate School of Medicine Standing Committee on Animals approved all experimental protocols.

2.5. Assessment of swine cardiac function and histological analysis

Cardiac ultrasound studies were performed before cell-transplantation and at 4, 8 and 12 weeks after transplantation using a VIVID 7 system (GE Healthcare Biosciences, Uppsala, Sweden) and the data at the day transplantation, 4- and 8-week-after transplantation were applied for the statistical analysis. The studies were shown as M-mode with short axis view observed from left fifth intracostal space.

For histological analysis, the swine hearts were dissected out at the end of the experiment and immediately fixed overnight in 4% paraformaldehyde and processed for embedding in paraffin wax. Sections were cut at 3-μm thickness, deparaffinized and then rehydrated through a graded ethanol series into distilled water. The sections were then immersed in Target Retrieval Solution (Dako, Glostrup, Denmark) and boiled, followed by cooling at room temperature for 20 min. Sections were incubated overnight with 10% blocking solution (Nacalai tesque) in TBS-T, and then in a humidity chamber for 16 h at 4 °C with mouse monoclonal antibodies to human alpha-cardiac actin (American Research Products., Belmont, MA), human myosin heavy chain (MHC) (mouse monoclonal anti-human myosin heavy chain cardiac antibody, Cat: 05-833., Upstate, NY) and CD34 (ab81289 [EP373Y], Abcom) diluted in blocking solution, followed by Alexa Fluor 488-labeled anti- IgG (Molecular Probes, Eugene, OR) with counter DAPI-staining. Hematoxylin and eosin stain, Masson trichrome stain and Sirius red stain were also performed. The stained all slides were viewed on a BioZero laser scanning microscope (Keyence, Osaka, Japan). The scarred area percentages of the middle portion and apex side of LV were calculated by area stained blue with Masson's trichrome staining/total of 10 each independent sections using software Dynamic Cell Count (Keyence, Osaka, Japan).

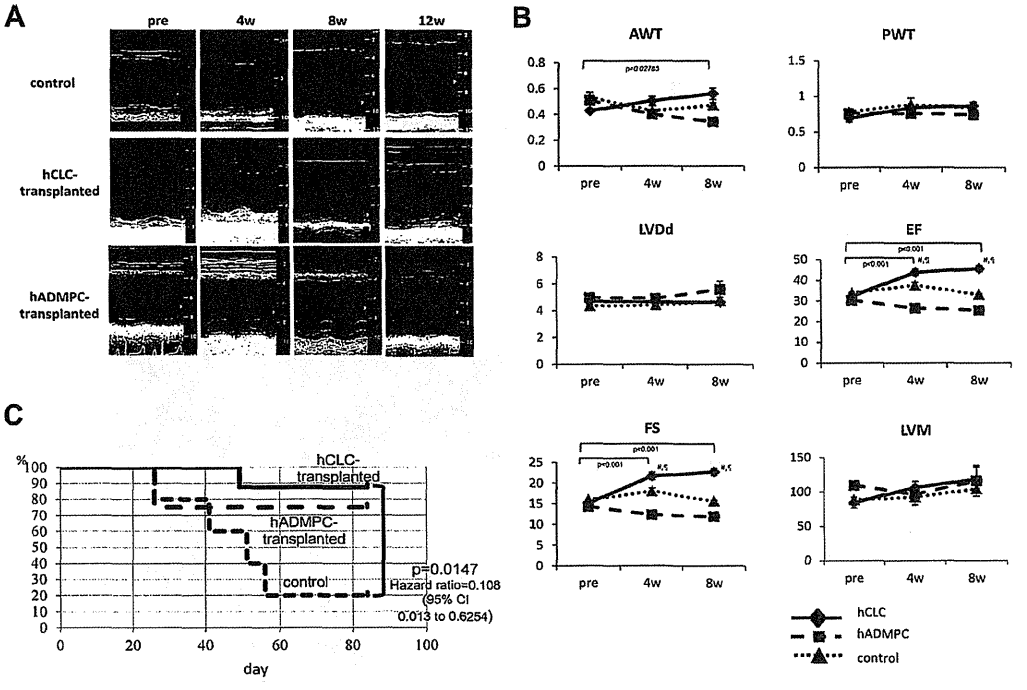

Fig. 2. Effects of hCLCs transplantation on cardiac function and survival rate. (A) In the hCLCs transplanted group, M-mode echocardiography showed improved wall motion within 4 weeks of transplantation. In contrast, worsening of the wall motion was noted in the mock-transplanted control swine. (B) Anterior wall thickness (AWT), ventricular ejection fraction (EF) and fractional shortening (FS) improved significantly in the hCLCs transplanted group, as confirmed by echocardiography. In the hCLCs transplanted swine, cardiac functions were recovered from transplantation to the end of the study. In the hADMPCs transplanted swine, cardiac functions were maintained from transplantation to the end of the study. In contrast, worsening of these cardiac function parameters was noted after mock-transplantation. The left ventricular diastolic dimension (LVDd) was maintained during the course of the experiment in hCLCs transplanted swine, but increased in the control groups. Posterior wall thickness (PWT) and left ventricular mass (LVM) showed no significant difference in the groups. Solid lines and squares indicated the transplanted group and the dashed lines and open squares indicated the control group. The symbol # indicated $p < 0.01$ hCLCs -transplanted vs control and indicated $p < 0.01$ hADMPC-transplanted vs control, respectively. Bars indicated mean ± standard error of the mean (SEM). (C) Effect of hCLCs transplantation ($n = 8$), hADMPCs transplantation ($n = 4$) and lactic Ringer's solution injection ($n = 5$) on long-term survival rates of swine. Kaplan–Meier survival curve analysis demonstrated significant difference in the survival rates between the hCLCs group and the lactic Ringer's solution group.

2.6. Statistical analysis

Longitudinal changes between groups were tested with the use of mixed-model repeated-measures analysis of variance, with adjustment for baseline values. When the overall P value for the main effect of group or time, or interaction between group and time was less than 0.05, the post hoc multiple comparisons with the use of the single-step adjustment method as implemented by Hothorn et al. were performed [19]. Survival curves were constructed by the Kaplan–Meier method and survival among groups was compared using the Log-Rank test (StatMate III for Windows, Atoms, Tokyo).

3. Results

3.1. Cardiocytic commitment of hADMPCs into hCLCs

The potential for hADMPCs to commit into CLCs was evaluated from the mRNA expression of several cardiocytic markers by quantitative reverse transcriptase-PCR before and after DMSO induction, as follows: *islet-1* is a cardiac stem cell marker; *nkx2.5* and *GATA-4* are transcription factors required for subsequent cardiac differentiation; and *alpha-cardiac actin*, *myosin light chain* (*MLC*), and *myosin heavy chain* (*MHC*) are markers of cardiocytic commitment (Table 1). After induction, hADMPCs expressed all markers with increment, indicating that hADMPCs could be successfully committed into cells of the cardiac lineage, hCLCs.

3.2. Effects of hCLCs transplantation on cardiac function and survival rate

Cardiac function was assessed by echocardiography. Four weeks after intracoronay transplantation of hCLCs, wall motion was improved but not in the placebo group (Fig. 2A). The wall motion of control swine worsened at 12 weeks after transplantation, while the improved motion was maintained after the hCLCs transplant (Fig. 2A). In the early post-transplantation period, there was no significant difference in left ventricular diastolic dimension (LVDd) between hCLCs -transplanted swine and the control. During the course of the study, LVDd exacerbated gradually in the control swine while it did not change significantly in the transplant swine (Fig. 2B). Likewise, the left ventricular ejection fraction (EF) and fractional shortening (FS) improved in the implanted group, but not in control swine (Fig. 2B). After hCLCs transplantation via left anterior descending (#6), the anterior wall thickness improved in the implanted group, but not in control swine. These results indicate that intracoronary transplantation of hCLCs resulted in recovery of cardiac function.

The Kaplan–Meier survival curves showed higher long-term survival rates for the hCLCs transplanted group than the control (Fig. 2C). Notably, only 1 of 8 swine died after transplantation of hCLCs. Survival at 12 weeks after transplantation was significantly higher in the hCLCs group (87.5%) than the control group (20%, 1 of 5) (Log-rank test: $p = 0.0147$. Hazard ratio = 0.108: 95% CI 0.013 to 0.625). These results suggest that transplantation of hCLCs

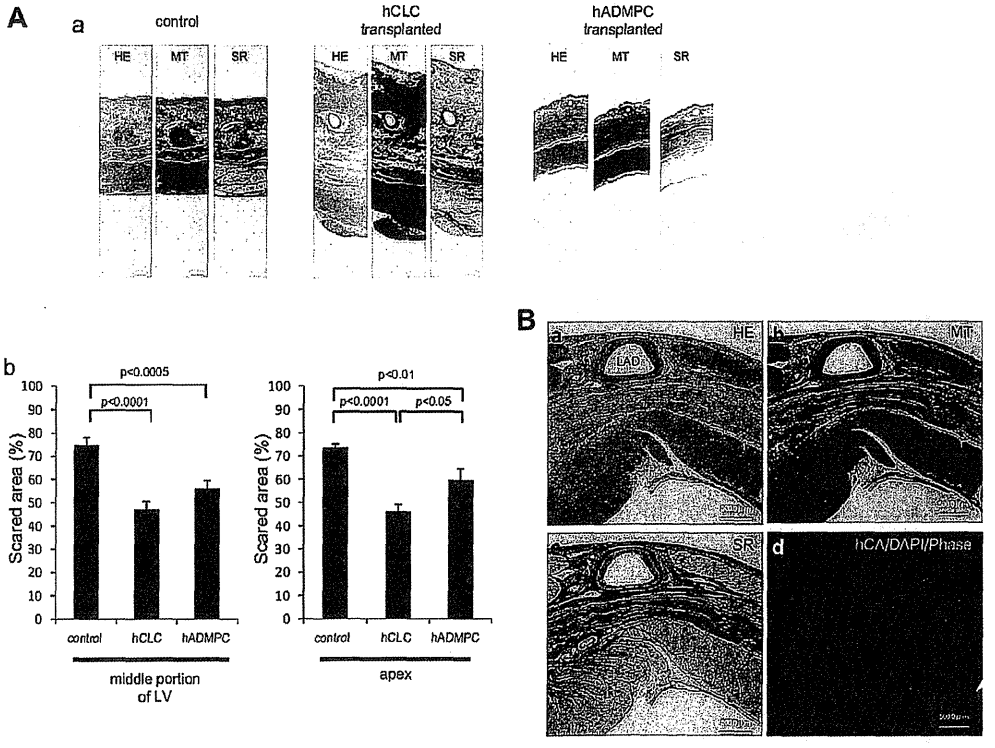

Fig. 3. Effects of hCLCs transplanted via coronary artery on cardiac structure. (A) (a) Photomicrographs of representative myocardial sections of the scarred area stained with hematoxylin/eosin (HE), Masson trichrome (MT) and Sirius red (SR) in the hCLCs-, hADMPC-transplantation and mock-transplanted control groups. Transplantation of hCLCs improved myocardial wall thickness in the infarcted myocardium and resulted in the development of new cardiac muscles on the surface. Bars = 500 μm. HE; hematoxylin and eosin staining, MT; Masson trichrome staining, and SR; Sirius red staining. (b) The scarred area percentages of the middle portion and apex side of LV. The scarred area percentages of hCLCs-, hADMPC-transplantation and control groups were calculated by area stained blue with Masson's trichrome staining /total of 10 independent sections. The error bar indicated SEM. (B) Photomicrographs of representative myocardial sections of apical side of the anterior wall stained with HE (a), MT (b), SR (c) or phase contrast merge image of neighboring sections stained with anti-human alpha-cardiac actin (hCA; green), and DAPI as counter staining (d). In the HE-, MT-, and SR-stained sections, cardiac muscles were distributed on the scarred areas, and some parts of these muscles expressed human alpha-cardiac actin (green). Bars = 500 μm. LAD; left anterior descending.

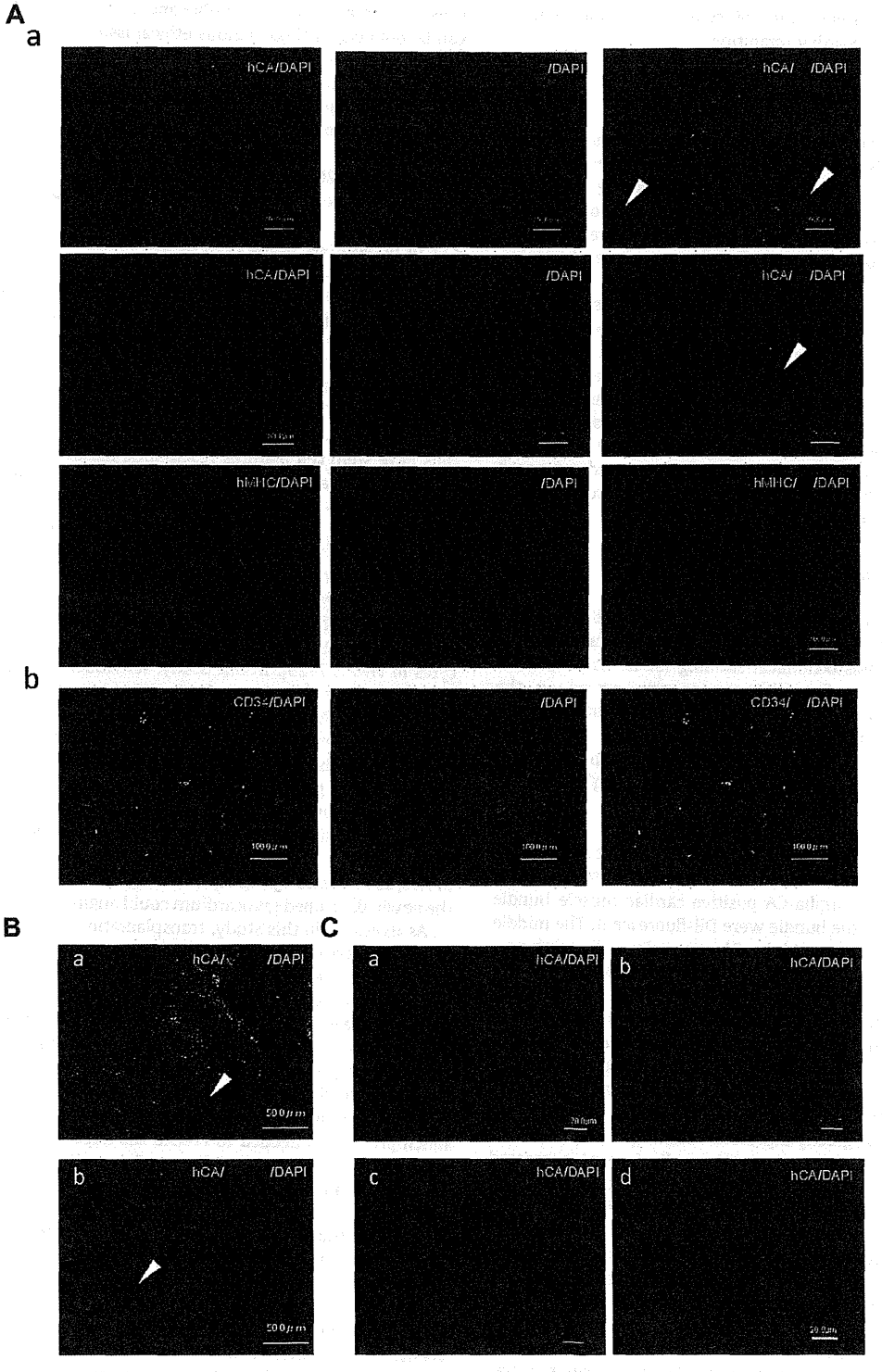

Fig. 4. hCLCs survive *in situ*. (A) (a) *In situ* survivals of the fluorescent DiI-prestained hCLCs into cardiomyocytes at 12-week- after transplantation. Note the presence of human alpha-CA positive cardiac muscle bundles or cells and that almost all cells exhibit DiI-fluorescence. Only minor part of DiI-positive cells did not express human alpha-CA (arrowheads). DiI-prestained cells were also positive for human myosin heavy chain (lower panel). (b) Survivals of hCLCs outside of vessel capillaries. The vessel capillaries were stained with anti-CD34 antibody and localization of DiI-positive cells were examined using fluoromicroscpy. DiI-positive cells exist outside of vessel capillaries which were stained with anti-CD34 antibody (B) Co-expression of human alpha-CA (green) and Nkx2.5 (purple) (a) or GATA-4 (purple) (b) in the nuclei of human alpha-CA positive cells. (C) Typical expression patterns of human alpha-CA on the cells. Human alpha-CA exhibited a brush pattern in oval cells (a), a spot pattern in cell-to-cell contact areas (b), as a sarcomeric structure beneath around the cell surface (c), and in a pattern resembling cardiomyocytes (d). Bars = 20 μm.

improves long-term survival rate of swine with heart failure induced by chronic myocardial infarction.

3.3. Effects of hCLCs transplantation on cardiac structure

Twelve weeks after transplantation, the treated swine were sacrificed and cardiac tissues prepared for histological examination for further analysis of cardiac structure and delineate the difference between hCLCs transplanted animals and controls (Fig. 3). Hematoxylin/eosin, Masson's trichrome and Sirius red staining showed the presence of a thin layer of cardiac muscles and massive fibrosis in the scarred anterior left ventricular wall of the control and hADMPCs transplanted swine (Fig. 3Aa). In contrast, the same staining techniques in hCLCs -transplanted swine showed significant thickening of the infarcted myocardium and layers of cardiomyocytes on the anterior ventricular wall (Fig. 3Aa). Next, to confirm the hCLCs could rescue from the fibrosis on cardiac structure, the scarred area percentages of the middle portion and apex side of LV were calculated. As shown in Fig. 3Ab, the percentage of scarred area of hCLCs -transplantation heart reduced compared to the control swine heart and hADMPC-transplanted one in both middle portion of LV and apex side.

3.4. hCLCs integrated in situ with the cardiac milieu

The *in situ* differentiation capacity of the implanted hCLCs into cardiomyocytes after grafting onto the scarred myocardium was assessed by immunohistochemical staining for human alpha-CA (Fig. 3B). Thin layers of cardiomyocytes were noted on the scarred myocardium by hematoxylin and eosin staining and Masson trichrome staining. Furthermore, clusters of human alpha-CA-positive cells were identified on the scarred myocardium (Fig. 3B; Green, arrowhead), indicating that hCLCs might integrate *in situ* with the cardiac milieu.

To confirm that the transplanted hCLCs survived *in situ*, we chased the fluorescent DiI-prestained hCLCs *in situ* 12 weeks after transplantation using histochemical technique. The top panel of Fig. 4Aa shows human alpha-CA positive cardiac muscle bundle and almost all cells of the bundle were DiI-fluorescent. The middle panel shows that all human alpha-CA-expressing cells were prestained DiI-fluorescent. DiI-prestained cells were also positive for human myosin heavy chain (Fig. 4Aa lower panel). On the other hand, DiI-positive cells exist outside of vessel capillaries which were stained with anti-CD34 antibody (Fig. 4Ab). Since cardiomyocytes are known to express the nuclear transcriptional factors; Nkx2.5 and GATA-4, we examined the expression of these molecules on human alpha-CA positive cells. The nuclei of human alpha-CA positive cells (green) expressed Nkx2.5 (purple) (Fig. 4Ba) and those of human alpha-CA positive cells (green) expressed GATA-4 (purple) (Fig. 4Bb), adding further confirmation that hCLCs might differentiate into cardiac marker positive cells.

The expression patterns of human alpha-CA on the cells were presented in Fig. 4C. The first pattern of human alpha-CA expression was the brushed pattern in oval-shaped cells (Fig. 4Ca). Alpha-CA also showed a spot pattern in the cell-to-cell contact areas (Fig. 4Cb). Resident alpha-CA-like immunoreactivity also appeared as sarcomeric structure beneath and around the cell surface (Fig. 4Cc). The fourth pattern of alpha-CA was cardiomyocyte structure-like pattern (Fig. 4Cd). These results indicate that hCLCs survive *in situ* and integrate into the cardiac milieu.

4. Discussion

There are several advantages to intracoronay transplantation of hCLCs for regeneration therapy. First, the source of adipose-derived cells is easily and safely accessible and large quantities of the cells can be obtained without serious ethical issues. Second, hCLCs can survive *in vivo* within the myocardial milieu. Finally, the reconstruction of a thick myocardial wall rescued cardiac dysfunction after chronic myocardial infarction and improved long-term survival in our swine model.

The choice of cell source is critical for realizing success in cellular therapy [19,20]. The adipose tissue is easily and safely accessible without serious ethical issues, and the cells can be obtained in large quantities since liposuction surgeries yield from 100 ml to >3 L of lipoaspirate tissue [21]. In the literature, isolation of cells from adipose tissue was first described by Bjorntorp et al. [22]. This procedure was then modified for the isolation of cells from human adipose tissue specimens [23–25]. In this context, Zuk et al. [11] reported the presence of cells with properties resembling those of mesenchymal stem cells resident in adipose tissue and they renamed the cell populations as adipose tissue-derived stromal/stem cells (ADSC). Recently, we have reported hADMPC as a novel cell population in human adipose tissue and indicated that these cells have stem cell features resembling mesenchymal stem cells including their ability to differentiate into cardiomyocytes in rat infracted cardiac milieu, into hepatocytes in rabbit hepatic milieu *in situ*, and into clusters of islet-like cells and hepatocytes *in vitro* [13–16]. Based on the above advantages, hADMPCs represent a potentially promising source of cells for cellular therapy, including patients with severe heart failure.

While the differentiation of ADSCs *in vitro* has been reported [26], only a few studies reported their differentiation into cardiomyocytes *in vivo* [27–29]. In one study, rat ADSCs were isolated and grown in intact monolayer sheets using temperature-responsive culture dishes. Placement of the rat ADSC sheets onto scarred myocardium in rats reduced the scarring and enhanced cardiac structure and function. Histological analysis demonstrated that the engrafted rat ADSC sheets grew to form a thickened layer that included newly formed vessels and few cardiomyocytes. In this context, Gimble et al. [20] suggested that hADSCs might secrete angiogenic factors. In our previous study, hCLCs survived within the rat myocardial milieu *in vivo*, as indicated by immunohistological results, suggesting that the newly developed myocardium could augment cardiac function.

As indicated in this study, transplantation of the hCLCs via the coronary artery resulted in the development of a new thick myocardial tissue, rescued cardiac dysfunction after MI in the swine model, and improved long-term survival rate compared to the control. Our findings suggest that hCLCs can be engrafted and survive within the myocardial infarct milieu, acquire phenotypic markers consistent with cardiomyocytic lineages, and have a positive impact on structural and functional endpoints. These are desirable outcomes for cardiac function and survival. Despite these encouraging results, much progress is needed to realize the hope of cell therapies for myocardial damage. First, delivery of the cell number to patients should be optimized for each given disease. Second, the risk–benefit based approach should be considered in the infracted or affected tissues after transplantation. Finally, the value and impact of hCLCs -transplantation should be confirmed in Investigational New Drug approval before embarking on clinical trials and applications.

In conclusion, we showed that the hCLCs were successfully engrafted into the scarred myocardium. The hCLCs -transplantation via the coronary artery also resulted in recovery of cardiac function and improved survival rate. Thus, transplantation of hCLCs in heart patients is a potentially effective therapeutic strategy for cardiac tissue regeneration within a few years.

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