Spherical Bullet Formation *via* E-cadherin Promotes Therapeutic Potency of Mesenchymal Stem Cells Derived From Human Umbilical Cord Blood for Myocardial Infarction

Eun Ju Lee[1], Sung Jung Park[1,2], Soo Kyoung Kang[1], Gi-Hwan Kim[1], Hyun-Jae Kang[1,2], Sae-Won Lee[1], Hong Bae Jeon[3] and Hyo-Soo Kim[1,24]

[1]National Research Laboratory for Stem Cell Niche and IRICT, Seoul National University Hospital, Seoul, Korea; [2]Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea; [3]Biomedical Research Institute, MEDIPOST Co., Ltd., Seoul, Korea; [4]Molecular Medicine and Biopharmaceutical Sciences, Seoul National University, Seoul, Korea

The beneficial effects of stem cells in clinical applications to date have been modest, and studies have reported that poor engraftment might be an important reason. As a strategy to overcome such a hurdle, we developed the spheroid three dimensional (3D) bullet as a delivery method for human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) through the maintenance of cell–cell interactions without additional xenofactors, cytokines, or matrix. We made spheroid 3D-bullets from hUCB-MSCs at 24 hours' anchorage-deprived suspension culture. To investigate the *in vivo* therapeutic efficacy of 3D-bullets, we used rat myocardial infarction (MI) model. Transplantation of 3D-bullet was better than that of single cells from monolayer culture or from 3D-bullet in improving left ventricular (LV) contractility [LV ejection fraction (LVEF) or LV fractional shortening (LVFS)] and preventing pathologic LV dilatation [LV end-systolic diameter (LVESD) or LV end-diastolic diameter (LVEDD)] at 8 weeks. In the mechanism study of 3D-bullet formation, we found that calcium-dependent cell–cell interaction was essential and that E-cadherin is a key inducer mediating hUCB-MSC 3D-bullet formation among several calcium-dependent adhesion molecules which were nominated as candidates after cDNA array analysis. In more specific experiments with E-cadherin overexpression using adenoviral vector or with E-cadherin neutralization using blocking antibody, we found that E-cadherin regulates vascular endothelial growth factor (VEGF) secretion *via* extracellular signal-regulated kinase (ERK)/v-akt murine thymoma viral oncogene homolog1 (AKT) pathways. During formation of spheroid 3D-bullets, activation of E-cadherin in association with cell–cell interaction turns on ERK/AKT signaling pathway that are essential to proliferative and paracrine activity of MSCs leading to the enhanced therapeutic efficacy.

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INTRODUCTION

Stem cell transplantation for the treatment of cardiac dysfunction has received considerable interest given the increasing prevalence of patients with ischemic cardiomyopathy.[1,2] Several studies have shown that cell therapy administered after myocardial infarction (MI) can reduce the infarct size and improve cardiac function.[3–5]

Recently, transplantation of umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) was proposed as an alternative source of bone marrow-derived MSCs (BM-MSCs) because obtaining cord blood is less invasive than acquiring BM.[3] In addition, human umbilical cord blood-derived MSCs (hUCB-MSCs) have a capacity similar to that of BM-MSCs for multilineage differentiation and immune modulation.[6–9] Although hUCB-MSCs may be a good cell type for use in stem cell transplantation, enhancement of therapeutic efficacy requires an advanced method to augment engraftment like other cell sources.[10,11] A lack of initial engraftment and retention due to cell death is a major roadblock to achieving clinical significance.[12] To improve engraftment, the best cell type, timing, and delivery method need to be optimized. Even after finding the optimized cell type for transplantation, low engraftment after cell delivery still remains a major obstacle to effective myocardial regeneration.[12] Some researchers transplanted genetically modified MSCs,[13,14] whereas others pretreated MSCs with cytokines or hypoxia.[15,16] However, these techniques cannot avoid the disruption of the cell–cell interaction, which induce the cell death in preparation step prior to transplantation.[17,18] We hypothesized that a new method which does not disrupt the cell–cell interaction would lead to more augmented engraftment and efficacy of transplanted cells. To augment engraftment efficiency of intramyocardial injection, we developed a new method to restore the disrupted cell–cell interaction during cell preparation process and thus to allow the cells to invade the interstitial spaces of myocardium more effectively.

Recently, a series of reports have indicated that formation of MSCs sphere enhances therapeutic potency.[19–21] However, limitation is that they require exogenous matrix and cytokines.

Correspondence: *Hyo-Soo Kim, Molecular Medicine and Biopharmaceutical Sciences, World Class University Program, Seoul National University/ Innovative Research Institute for Cell Therapy (IRICT), Seoul National University Hospital, 101 DaeHak-ro, JongRo-gu, Seoul 110-744, Republic of Korea. E-mail: hyosoo@snu.ac.kr*

Furthermore, the basic mechanism of the MSC sphere was not well described. Understanding the molecular mechanisms contributing to the formation of the potentiated MSC spheres may be very important, because it would be helpful to extend MSC applications and to develop the more economic methods for the potentiated MSC in regenerative medicine.

In this study, we developed spheroid three dimensional (3D) bullets as a new method to enhance engraftment of hUCB-MSCs by restoring cell–cell interactions without addition of xenofactors, cytokines, or matrix. Next, we proved that E-cadherin is a key mediator in the formation of hUCB-MSC 3D-bullets. Finally, we showed that E-cadherin-mediated extracellular signal-regulated kinase (ERK)/v-akt murine thymoma viral oncogene homolog1 (AKT) pathways stimulate not only proliferative but also paracrine activity of the hUCB-MSC 3D-bullet.

RESULTS

The restoration of cell–cell interaction *via* 3D-bullets formation improved proliferative, paracrine activity of hUCB-MSCs

To restore cell–cell interaction *via* 3D-bullets formation, we used anchorage-deprived suspension culture condition with bullet medium containing serum replacement. After single cells dissociated from the monolayer were exposed to bullet medium, we observed 3D-bullet formation, which was initiated through cell–cell adhesion and small bullet–bullet adhesion processes (**Figure 1a**). Because the 3D structure may have less access to nutrients, it was of importance to evaluate whether the cells in the 3D structure remained viable or not. After 24 hours in the 3D-bullet, almost 90% of the dissociated cells were viable and 35.64% were in S phase as assayed by propidium iodide uptake (**Figure 1b**).

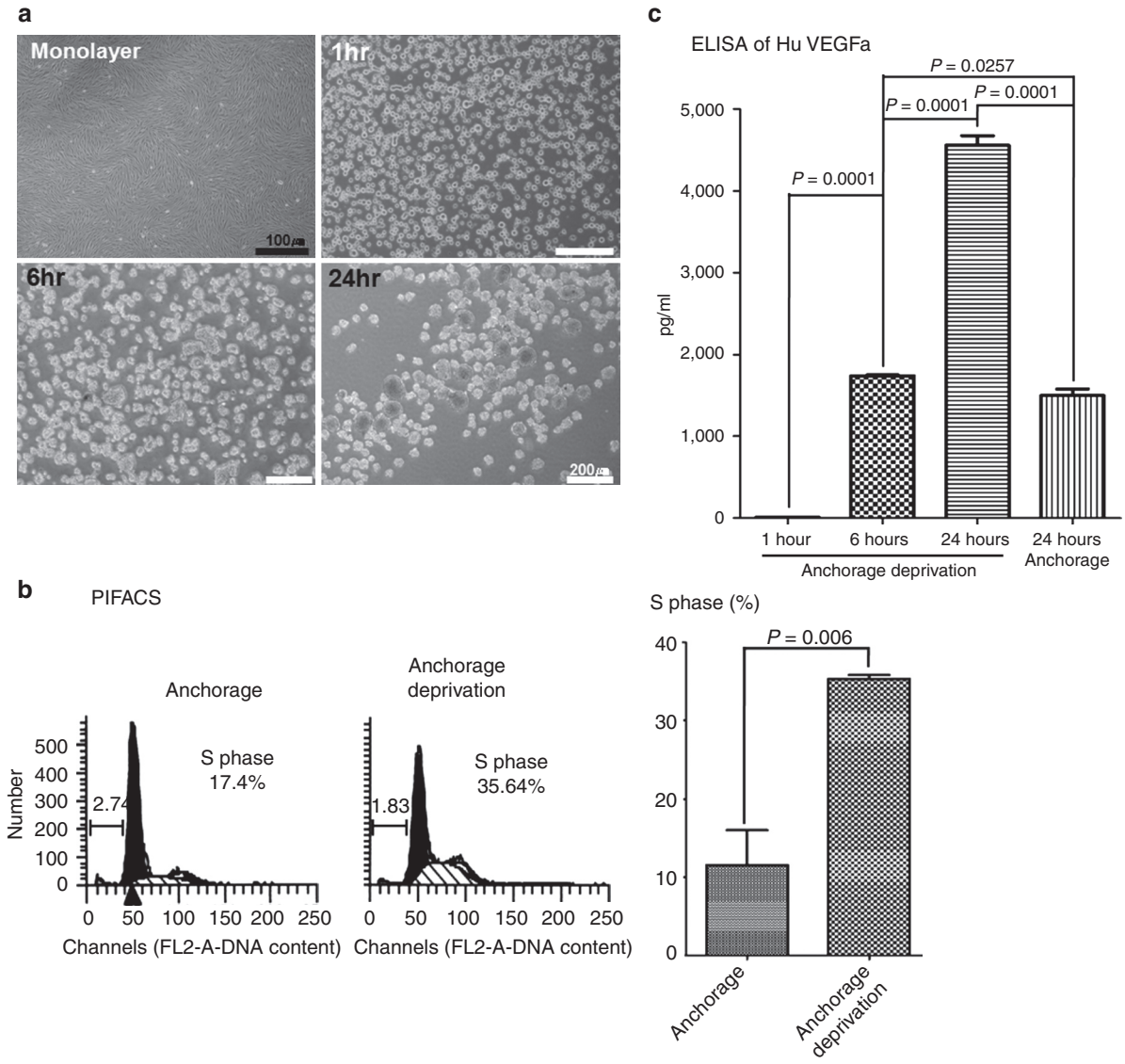

Figure 1 Three dimensional (3D)-bullet formation increases vascular endothelial growth factor (VEGF) amount and S-phase fraction of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs). (**a**) Phase contrast microscopy showing the time course of 3D-bullet formation of hUCB-MSCs over 24 hours. (**b**) Propidium iodide (PI) fluorescence-activated cell sorter (FACS) of 3D-bullet hUCB-MSCs at 24 hours from anchorage deprivation. Increased S phase in 3D-bullets was observed. (**c**) Enzyme-linked immunosorbent assay (ELISA) measurement of human VEGF secretion at 1, 6, and 24 hours from anchorage deprivation in hUCB-MSCs.

Paracrine secretions, such as vascular endothelial growth factor (VEGF), and immune suppressive properties are suggested as the most important benefits of MSCs in regenerative medicine. The VEGF amount gradually increased over time (**Figure 1c**) and the average size of the 3D-bullets also increased. To evaluate whether the MSC characteristics remained unchanged after 3D-bullet formation, the 3D-bullets were dissociated, attached, and cultured in MSC medium. After 3–5 days, we performed a fluorescence-activated cell sorter (FACS) analysis that revealed those cells were still MSCs-expressing specific markers (**Supplementary Figure S1**).

3D-bullets of hUCB-MSCs is superior to single cell suspension from conventional culture in the therapeutic efficacy for MI

Using rat MI model (**Figure 2a**), we compared the therapeutic efficacy of the four differently conditioned hUCB-MSCs: (i) single cell

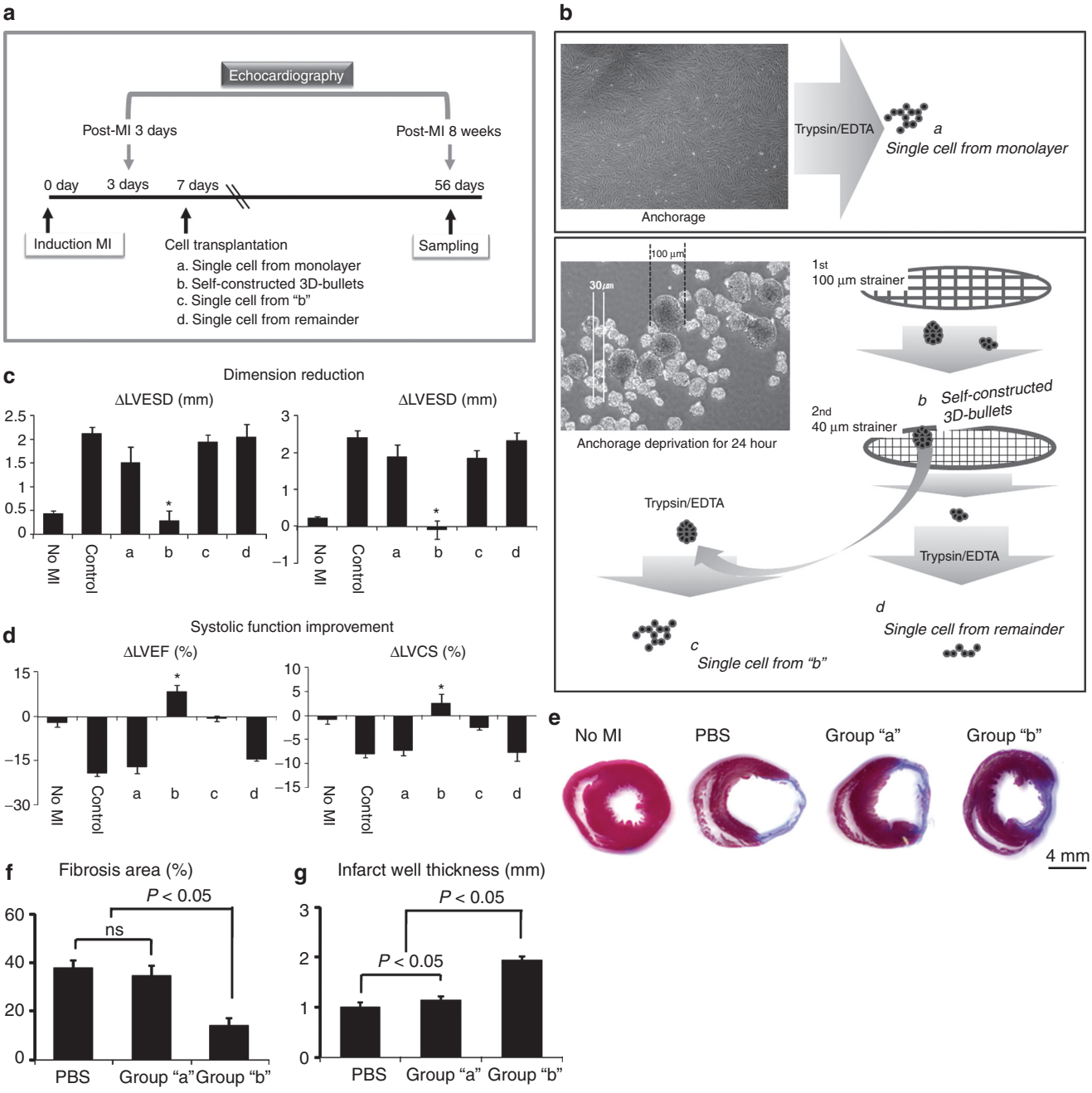

Figure 2 Therapeutic efficacy of human umbilical cord blood-derived mesenchymal stem cell (hUCB-MSC) is enhanced by transplantation as three dimensional (3D)-bullet rather than as single cells in postinfarction left ventricular (LV) remodeling. (**a**) Time table for the transplantation of hUCB-MSCs in the rat myocardial infarction (MI) model. (**b**) Experimental scheme. (**c**) Left ventricular end-systolic diameter (LVESD) and delta LVESD, and left ventricular end-diastolic diameter (LVEDD) and delta LVEDD showed significant repression of remodeling in the 3D-bullet group (group "b"). (**d**) Left ventricular ejection fraction (LVEF) and delta LVEF, and left ventricular fractional shortening (LVFS) and delta LVFS showed significant improvement in systolic function in group "b" compared to the other groups. $P < 0.05$ versus control group. (**e**) Masson's trichome staining. (**f**) Efficacy in reducing infarct size. (**g**) Efficacy in preserving infarct wall thickness. Group "b" showed the highest efficacy in reducing infarct size and preserving infarct wall thickness compared to single cells from monolayer group "a." $n = 7$ per group.

suspension from standard adherent monolayer culture, (ii) self-constructed 3D-bullets sized 40–100 µm, (iii) single cell suspension of the 3D-bullets from "b" group, (iv) single cell suspension of the residual 3D-bullets in culture smaller than 40 µm (**Figure 2b**). At 3 days post-MI, no significant differences were observed among the five groups with respect to LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), LV ejection fraction (LVEF), or LV fractional shortening (LVFS) (data not shown).

At 7 weeks after cell injection, transplantation of 3D-bullet was better than any other group in preventing pathologic LV dilatation (**Figure 2c**, a, c, d, control versus b; $P < 0.05$). Simultaneously, transplantation of 3D-bullet (group b) significantly improved LV contractility compared to the other treatment groups (delta LVEF in **Figure 2d**, a, c, d, control versus b; $P < 0.01$) (delta LVFS in **Figure 2d** a, c, d, control versus b; $P = 0.04$). The regrettable effects in group c suggested that the maintenance of the 3D-bullet structure is an important factor to achieve therapeutic efficacy.

In the further analysis of histopathologic changes of myocardium after cell transplantation, we compared the efficacy of only two groups: hUCB-MSCs in conventional standard monolayer culture condition (group a) versus our new 3D-bullet form of hUCB-MSCs (group b). Using Masson's trichome staining, we measured the area of fibrosis and wall thickness of infarcted hearts harvested 8 weeks after MI (**Figure 2e**). Transplantation of 3D-bullet hUCB-MSCs(group b) resulted in a significantly smaller fibrosis area (**Figure 2f**) and a thicker infarct wall than did the transplantation of single cells of hUCB-MSCs from the monolayer culture (group a) (**Figure 2g**). This pathologic data were corroborated by the *in vivo* echocardiographic one. In other words, the akinetic segments of the LV anterior wall at eight weeks after MI were smaller after transplantation of the 3D-bullet (group b) than after single cell from the monolayer (group a) (**Supplementary Figure S2**).

Enhanced engraftment after transplantation as 3D-bullet

To evaluate engrafting cells after transplantation as 3D-bullets or as single cells, we used DiI dye for *in vivo* tracking. Using fluorescence confocal microscopy of the peri-infarct area in the heart at 4 weeks, we detected a larger number of DiI-labeled MSCs in the 3D-bullet transplantation group (**Figure 3a**). Through an in vitro study, we observed that VEGF secretion was higher from 3D-bullet hUCB-MSCs and thus expected that greater engraftment of cells may lead to more angiogenesis. Using isolectin B4 endothelial cell-specific staining, we confirmed that capillary density was significantly higher in the 3D-bullet transplantation group (group b) than in the monolayer transplantation group (group a) (**Figure 3b**), which is correlated with the number of engrafting cells. In addition, we observed the evidence that DiI-labeled MSCs differentiated into endothelial cells expressing isolectin B4 (**Supplementary Figure 3a**) and cardiomyocyte expressing α-sarcomeric actin (**Supplementary Figure S3b**).

Formation of 3D-bullet is dependent on calcium-dependent adhesion molecule, E-cadherin

To reveal the mechanism of 3D-bullet formation, we first treated it with the calcium chelator EDTA. A previous report suggested that many cell types use one of two main aggregation mechanisms: either a calcium-dependent or a calcium-independent

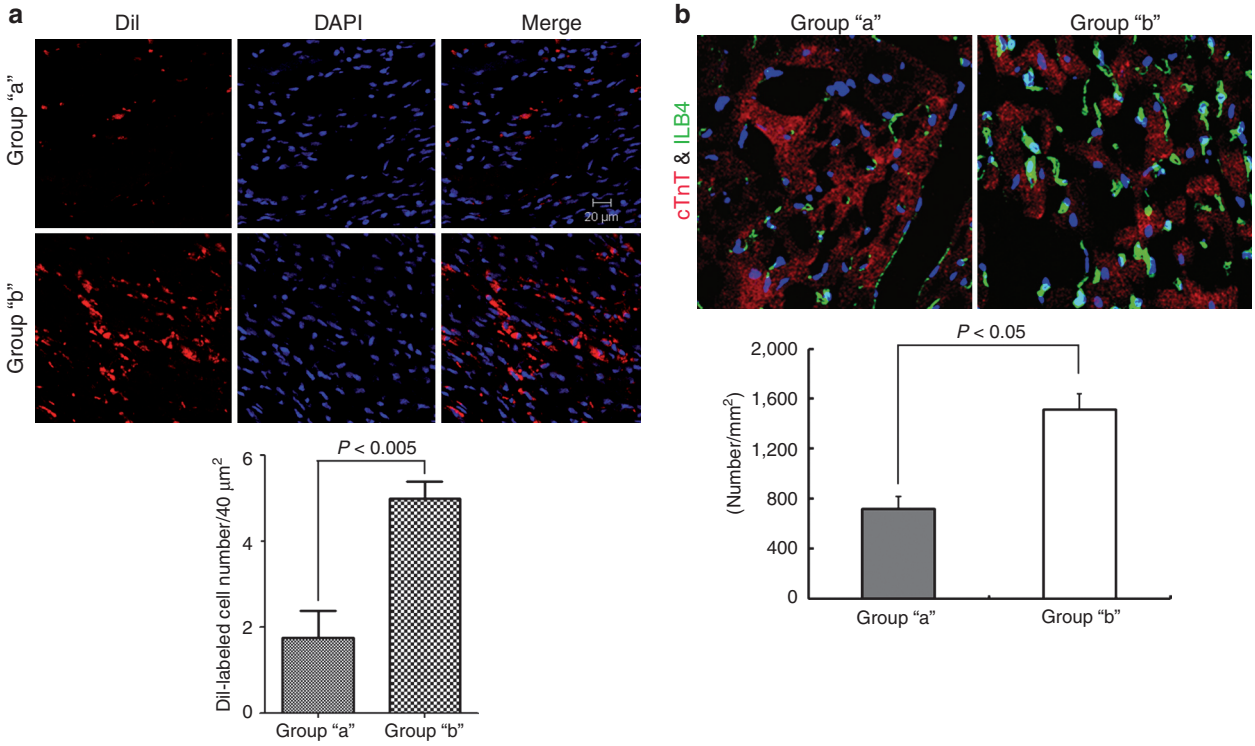

Figure 3 Engraftment of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) is improved after transplanted as three dimensional (3D)-bullets rather than as single cells. (**a**) The infracted heart at 3 weeks after cell transplantation was observed under fluorescence confocal microscopy. We used DiI-labeled hUCB-MSCs for tracking *in vivo*. DiI-positive cells were more frequently observed in group "b." (**b**) Lectin-positive capillary density was higher in group "b" than "a" in peri-infarct tissue. Four weeks post- myocardial infarction (MI); $n = 7$ per group.

one. Formation of the 3D-bullet was inhibited by EDTA treatment (**Figure 4a**). Next, to screen candidate genes for 3D-bullet formation, we compared the hUCB-MSCs in monolayer versus in 3D-bullet in terms of the RNA expression level of calcium-dependent adhesion molecules using a cDNA array (**Figure 4b**). Expression levels of four genes, *CDH1*, *CDH9*, *CDH16*, and *SELE* in the 3D-bullet were significantly higher than those in the monolayer. The RNA levels of the four genes were validated by real-time PCR. Among these four genes, CDH1 (E-cadherin) was most prominent in the preferential expression in the 3D-bullet than in the monolayer (**Figure 4c**).

To assess the role of E-cadherin in intercellular adhesion and 3D-bullet formation, we performed E-cadherin blocking or knockdown experiments. Neutralization of the extracellular domain of E-cadherin using antibody inhibited 3D-bullet formation (**Figure 5a**). For a more delicate observation of the role of E-cadherin, we induced aggregate formation within a limited volume and traced 3D-bullet formation.

During 3D-bullet formation, we observed that paracrine activity, such as VEGF secretion, was augmented (**Figure 1c**). When E-cadherin was blocked by neutralizing antibody, VEGF mRNA, and VEGF protein secretion were significantly decreased (**Figure 5b,c**). We further examined the mechanism underlying VEGF regulation by E-cadherin. Among cell survival molecules, ERK and AKT are known to be downstream signaling molecules of E-cadherin, but the regulatory mechanism by E-cadherin is controversial. Blocking of E-cadherin significantly inhibited ERK and AKT phosphorylation compared with the naive (nontreated) or immunoglobulin G-treatment (**Figure 5d,e**).

Conversely when we overexpressed E-cadherin using adenoviral vector (**Figure 6a**), ERK and AKT phosphorylation significantly increased compared with the control adeno-LacZ(**Figure 6b**). The activation of ERK and AKT by E-cadherin overexpression led to the increase in VEGF RNA (**Figure 6c**) and protein secretion (**Figure 6d**). Such an induction of VEGF by E-cadherin overexpression was once again blocked by inhibitor of ERK or AKT (**Figure 7a,b**).

DISCUSSION

Advantages of hUCB-MSCs in stem cell therapy

MSC transplantation has recently emerged as an attractive choice in the cardiovascular field for its therapeutic effect on the infarcted myocardium.[3,15,22] Among MSCs, hUCB-MSCs were tested extensively in many laboratories because of their biological and practical advantages.[3] Multiple cardiovascular risk factors in patients are known to suppress the function of their own stem cells.[23,24] In contrast, hUCB-MSCs from younger donors are fresher and have better cell vitality. In addition, they are relatively easy to acquire from numerous donors and could be cost-effectively stored for future therapeutic use. Moreover, academic and clinical applications are not limited by the ethical problems raised in the case of embryonic stem cells.

Reasons of the limited efficacy and strategies to overcome

Low therapeutic efficacy is frequently pointed out as a major obstacle before full-scale clinical application.[13,14] Uncertain efficacies are related to the poor survival rate of transplanted cells.[25]

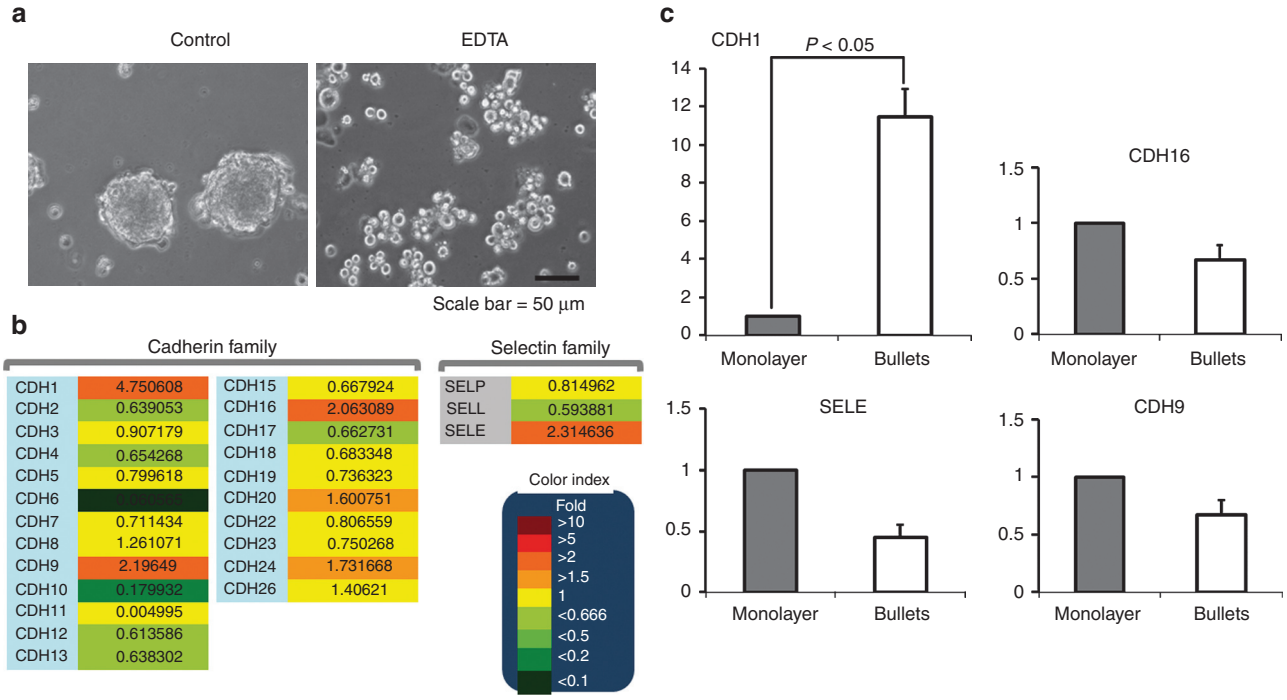

Figure 4 Calcium-dependent adhesion molecule is involved in three dimensional (3D)-bullet formation. (**a**) Calcium-dependent cell–cell interaction was required for 3D-bullet formation in human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs). The calcium chelator EDTA inhibited 3D-bullet formation in hUCB-MSCs. (**b**) Screening of calcium-dependent cell adhesion molecules in a cDNA array between the 3D-bullet and monolayer. (**c**) Four genes (*CDH1*, *CDH9*, *CDH16*, *SELE*) showing a twofold or greater expression in the 3D-bullet were validated by real-time PCR. E-cadherin CDH1 was significantly more highly expressed in the 3D-bullet.

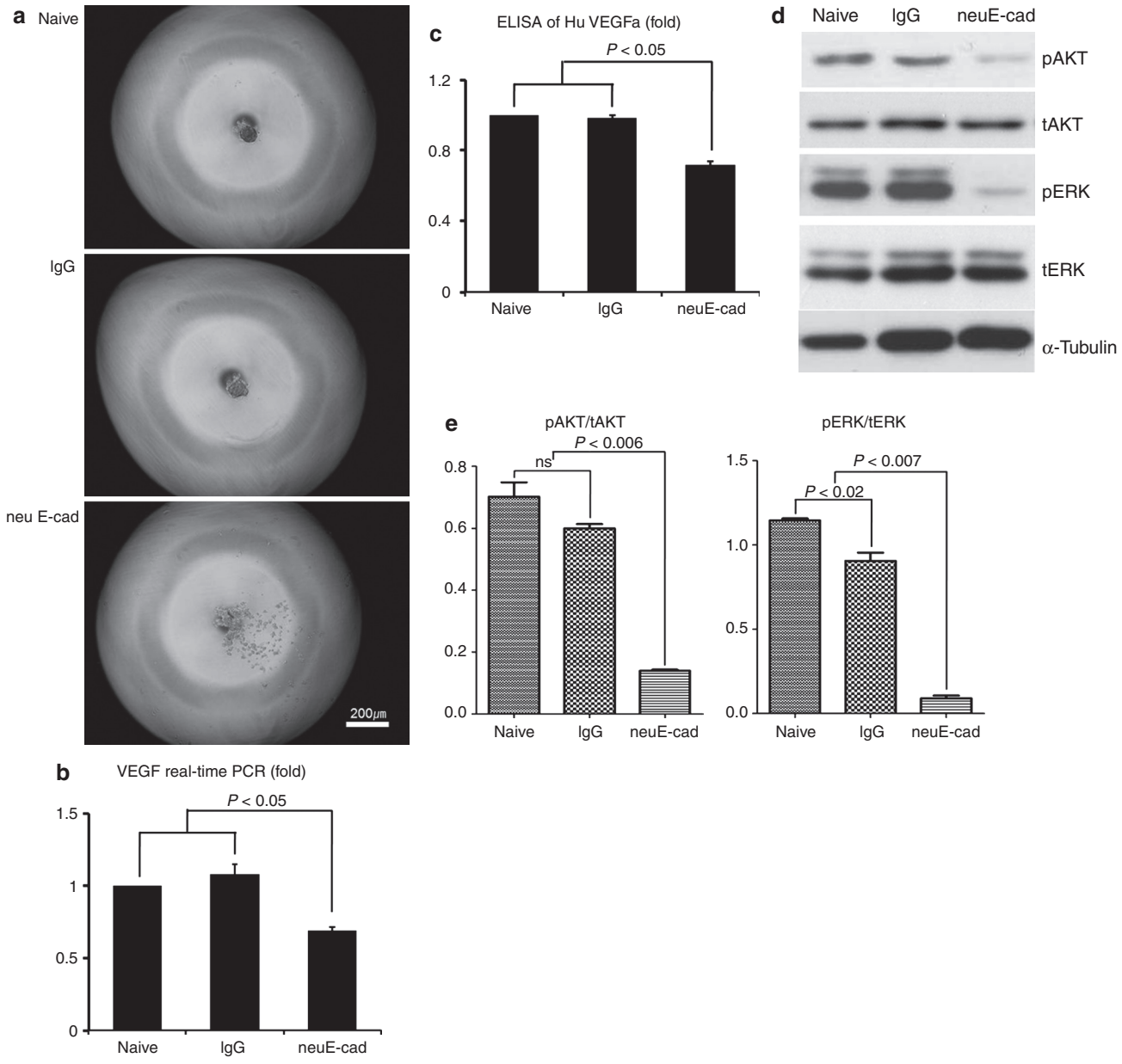

Figure 5 E-cadherin is necessary for three dimensional (3D)-bullet formation in human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs). (**a**) Phase contrast microscopy image of 3D-bullets in hanging drops. E-cadherin neutralization inhibited 3D-bullet formation. Monolayer cells were dissociated and induced to form 3D-bullet under E-cadherin blocking antibody (40 μg/ml; Sigma, U3254) or immunoglobulin G (IgG) for 6 hours. (**b**) mRNA of vascular endothelial growth factor (VEGF) was downregulated by E-cadherin neutralization. (**c**) Amount of VEGF secretion was reduced by E-cadherin neutralization, which was correlated with transcriptional regulation. (**d**) E-cadherin neutralization led to the inhibition of extracellular signal-regulated kinase (ERK) and AKT activation. (**e**) Quantification of ERK and AKT expression. The western blot was quantified using the software TINA 2.0 (Raytest, Straubenhardt, Germany). The hanging drop approach was used for this experiment.

Most transplanted stem cells are reported to die within 1 week after implantation because of the harsh conditions of the diseased target tissues, such as hypoxia, poor nutrient supply, inflammation, and immunological attacks from host cells.

In the immediate post-MI period, the first week is full of excessive oxidative stresses. Some researchers have investigated the optimal time point to deliver stem cells to improve grafted cell survival. Meanwhile, genetic modifications are tried targeting some antiapoptotic genes. MSCs treated with the *Bcl-2* gene showed less apoptosis and secreted more VEGF compared to control MSCs, especially under hypoxic conditions.[13] Incorporation of the *Akt* gene increased MSC survival until 7 days after MI, which led to greater restoration of LV systolic function.[14] In addition to genetic modifications, pretreatment of MSCs with various cytokines or growth factors were attempted with favorable results. Our previous experience[15] demonstrated that priming of MSCs with a combination of fibroblast growth factor 2, insulin-like growth factor-1, and bone morphogenetic protein-2 potentiated their viability under hypoxic conditions. These pretreated MSCs had enhanced gap junction formation between them and cardiomyocytes, which

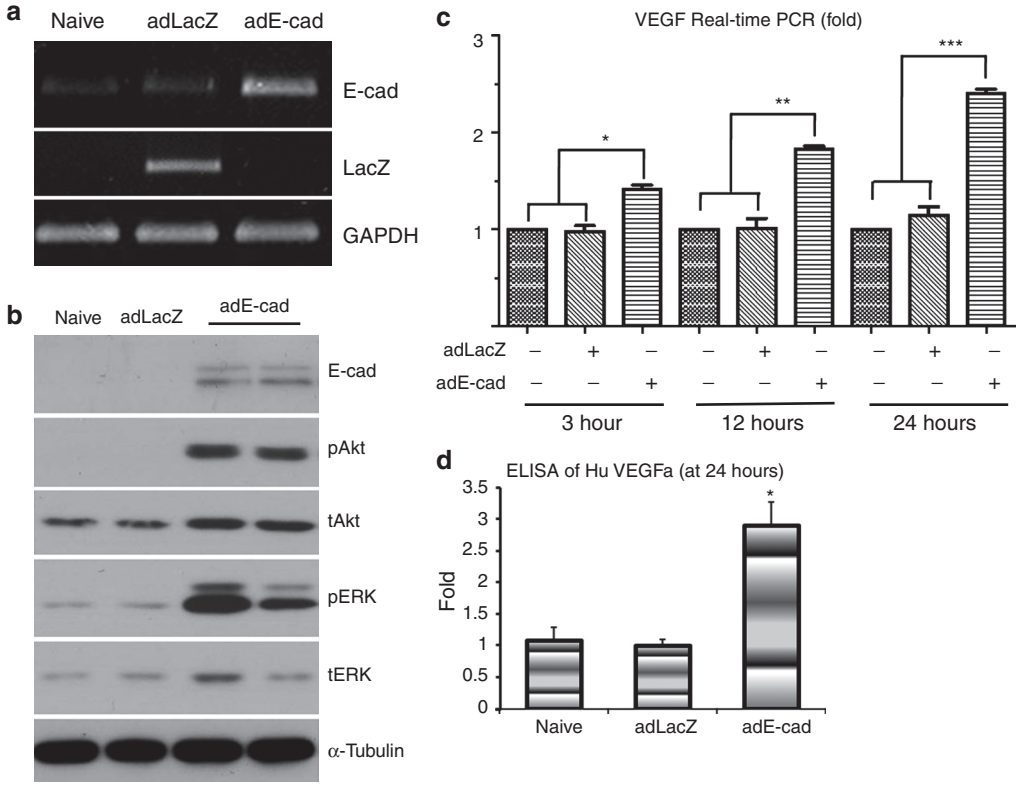

Figure 6 Overexpression of E-cadherin activates extracellular signal-regulated kinase (ERK) and AKT leading to vascular endothelial growth factor (VEGF) increase during three dimensional (3D)-bullet formation. (**a**) mRNA validation of E-cadherin overexpression of the adenoviral vector using RNA PCR. The *LacZ* gene was used as an adenoviral vector control. (**b**) Overexpression of E-cadherin induced ERK and AKT activation. (**c**) Expression of VEGF mRNA was increased by the overexpression of E-cadherin. *$P \leq 0.5$, **$P \leq 0.005$, ***M*f* 0.0005&(l) The amount of VEGF secretion was increased after E-cadherin overexpression at 24 hours, which was correlated with transcriptional regulation.

led to a greater cytoprotective effect on cardiomyocytes compared to naive MSCs. Therefore, LVs injected with pretreated MSCs showed smaller infarct sizes and better cardiac function versus other hearts implanted with control MSCs.[15]

Why a self-assembled 3D-bullet?

Although alternative approaches have reported an augmentation in therapeutic efficacy, they still have many limitations. They also require expensive cytokine or growth factors, or modification of innate genetic information. Moreover, typical cell transplantation techniques require the disruption of the extracellular matrix that was constructed by cell–cell interactions during *in vitro* cultivation. We wanted to develop more economical and safer method with high efficacy than preexisting methods. To achieve our purpose, we hypothesized that the delivery of cells without the disruption of the extracellular matrix could lead to higher engraftment efficacy and greater therapeutic benefit. We induced bullet formation under anchorage-deprivation conditions, which produced extracellular matrix *via* cell–cell interactions until they reached sufficient physical size (between 40 and 100 μm). For intramuscular injection, needles with a diameter of 60–250 μm were useful (Engel J, Hilgard P, inventors; AstaMedicaAktiengellschaft, assignee. Long-acting injection suspensions and a process for their preparation. US patent 5 773 032. 30 June 1998).

Self-assembled 3D-bullets do not require exogenous matrix or cytokines, and are also useful for developing biomimetic 3D organoids that could be used as in vitro model for studying differentiation, organogenesis, migration, and tumor biology.

E-cadherin is a key molecule in formation of 3D-bullet of hUCB-MSCs

Self-assembly and anchorage-independent survival are not general traits of MSCs. But, MSC spheroids were reported by several researchers[19–21] whose limitations include that they only dealt with augmentation of therapeutic potency or differentiation but did not reveal the mechanism of spheroid formation.

After we confirmed that 3D-bullet formation enhanced proliferative, paracrine, and immune modulatory activity of hUCB-MSCs and that 3D-bullet was superior to usual single cell suspension in repairing the infarcted myocardium, we analyzed the underlying mechanism for 3D-bullet formation. In the cDNA microarray comparative analysis of 3D-bullet–MSCs versus single cells MSCs from usual adherent monolayer culture, we found that E-cadherin was the adhesion molecule to significantly increase during the process of 3D-bullet formation.

No previous reports have described MSCs-expressing E-cadherin, which is a member of the cadherin family that calcium-dependently mediates cell–cell contact in epithelial cells.[26,27] Based on our results, the E-cadherin homophilic interaction between MSCs could activate the representative survival signals such as serine/threonine protein kinase AKT and extracellular signal-regulated protein kinase (ERK), as suggested in epithelial cells.[28–30]

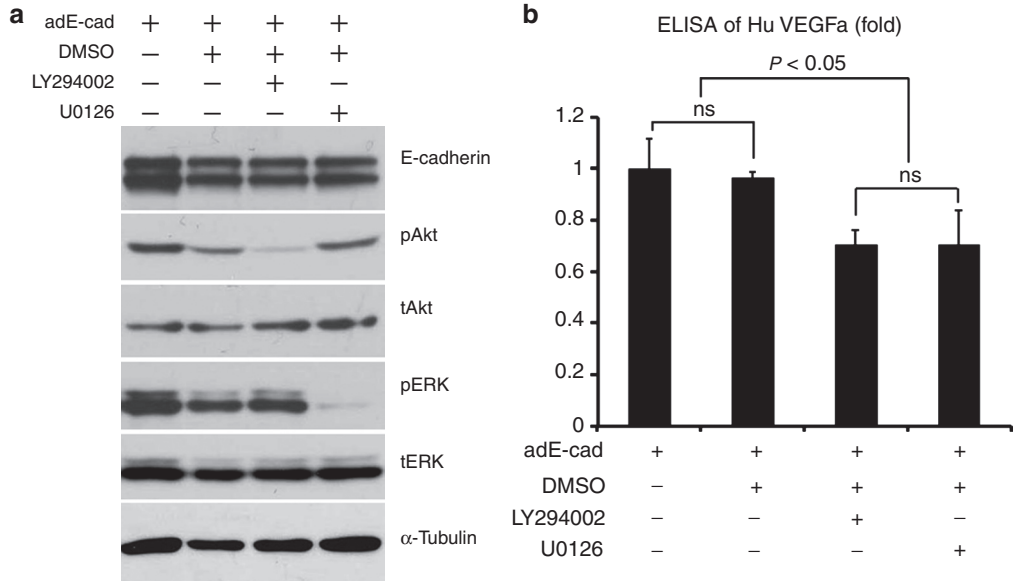

Figure 7 E-cadherin-induced vascular endothelial growth factor (VEGF) secretion is dependent on extracellular signal-regulated kinase (ERK)/AKT activation in three dimensional (3D)-bullet human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs). (**a**) Specific inhibition of ERK and AKT activation under E-cadherin overexpression. DMSO, a solvent for inhibitors, was used as a control. LY294002 was used to inhibit AKT activation. U0126 was used to inhibit ERK activation. (**b**) Inhibition of ERK and AKT activation suppressed VEGF secretion. ns, not significant.

In order to confirm the E-cadherin/ERK-AKT/VEGF axis to augment the cellular potency of hUCB-MSCs during the process of 3D-bullet formation, we performed several experiments using an overexpressing E-cadherin adenoviral vector and an E-cadherin neutralizing antibody, as well as inhibitors of ERK or AKT. In a series of experiments, we demonstrated that activation of E-cadherin in association with cell–cell interaction during formation of 3D-bullet turns on ERK/AKT signaling pathway, leading to activation of proliferative, paracrine property of hUCB-MSCs. To our knowledge, this is the first report to describe the role of E-cadherin in hUCB-MSCs.

MATERIALS AND METHODS

Isolation and culture of hUCB-MSCs. Medipost (Seoul, Korea) provided MSCs isolated from hUCB (UCB-MSCs) as previously described,[7] all these processes were carried out with informed consents. In brief, mononuclear cells were isolated by centrifugation (400G, 50 minutes) in a Ficoll hypaque gradient (density 1.077 g/cm^3; Sigma-Aldrich, St Louis, MO). These cells were cultured in α-minimum essential medium (Gibco BRL, Carlsbad, CA), supplemented with 10% fetal bovine serum (Gibco BRL), penicillin G sodium (10,000 U/ml), amphotericin B (25 μg/ml; Invitrogen, Madison, WI) and streptomycin sulfate (10,000 μg/ml), the concentration of which was 5.0×10^6 cells/cm^2. Cultures were maintained in a humidified 37 °C cell incubator with 5% CO_2 with a change of culture medium twice a week. Three weeks later, monolayer of fibroblast-like adherent cells were trypsinized (0.25% trypsin; HyClone, Logan, UT), washed, resuspended in culture medium and subcultured at a concentration of 1.0×10^6 cells/cm^2.

UCB-MSCs of 5–8th passage from three different donors were used for this experiment and purified in good manufacturing practice systems. These UCB-MSCs were collected at March 2005 and stocked in liquid nitrogen (–196 °C). All UCB-MSCs donor were characterized by a normal karyotype. These MSCs were tested to identify whether they have capacities of self-renewal and differentiation into osteogenic, chondrogenic, and adipogenic tissues. FACSCalibur system (Becton Dickinson, BD Bioscience, Sparks, MD) was used to detect surface antigens of MSCs isolated from hUCB.

Formation of 3D-bullet from monolayer hUCB-MSCs. After trypsinization (0.025% trypsin; HyClone) of the MSCs in monolayer culture state (**Figure 2b**, group "a"), culture them in suspension method using low attachment Petri dish containing bullet medium; bovine serum-free Dulbecco's modified Eagle's medium/F12 (Invitrogen) supplemented with 20% serum replacement (Gibco BRL), in a humidified 37 °C cell incubator with 5% CO_2. Then, 3D-bullet of hUCB-MSCs was formed about 24 hour later. After this suspension culture over 24 hours, 3D-bullet (**Figure 2b**, group "b") are sorted out using a 100 and 40 mesh strainer. Following this separation process, self-constructed 3D-bullets are once again trypsinized into single cells (**Figure 2b**, group "c") to detect 3D structural effect. The remnant MSCs are very smaller aggregates (<40 μm) passing out of the strainer, which dissociated into single cells to remove the interference of aggregate effect (**Figure 2b**, group "d"). We used gage 31 Hamilton syringe (nominal inner diameter 133 μm) for less scar and complete ejection.

E-cadherin blocking. After trypsinization (0.025% trypsin; HyClone) of the monolayer MSCs as described above, culture them in hanging drops in 20 μl of bullet medium containing 1,000–3,000 cells/drop with E-cadherin neutralizing antibody (Sigma; 40 μg/ml) or immunoglobulin G (40 μg/ml).

Rat MI models. The investigators conformed to the National Research Council's "Guide for the Care and Use of Laboratory Animals" (revised 1996) and all experimental protocols were approved from Institutional Animal Care and Use Committee of Seoul National University. For this experiment, Sprague-Dawley rats weighing 240–290 g and aged 8–10 weeks from OrientBio (GyeongGi-Do, South Korea), were maintained on Rat & Mouse Cubes and fed with standard pellet feed and water *ad libitum*.

They were anesthetized with zoletil (15 mg/kg; Virbac Lab, Carros Cedex, France) by intraperitoneal injection. After intubation, rats were positive pressure ventilated with room air supplemented with oxygen using a small animal volume controlled ventilator (model 683; Harvard Apparatus, Holliston, MT). A left thoracotomy was performed in the fourth intercostal space and the pericardium was opened. Induction of MI was performed by ligation of the left anterior descending coronary artery with 6-0 silk suture as described previously.[31] After ligation of proximal left anterior descending coronary artery, an irreversible pale area was demarcated on the surface of the middle and apical portion of left ventricle, and then the chest was closed.

hUCB-MSCs transplantation. Before the transplantation of hUCB-MSCs, development of MI was confirmed by echocardiography 3 days after MI. Then Sprague-Dawley rats randomly assigned into four groups as defined above (group a, b, c, d, $n = 7$ for each group, respectively) with phosphate-buffered saline injected control group, were anesthetized, ventilated, and the chest was opened in the same way of the initial surgery. We implanted the minimal effective dose (1×10^5, 100 µl) of cells from each hUCB-MSCs group, as already determined in the previous study.[3] hUCB-MSCs were injected using 31 Hamilton syringe into three different peri-infarct zones with same distance apart each other.

In vivo *echocardiographic evaluation.* Transthoracic echocardiography using 11.5 MHz transducer (Vivid i; GE Healthcare, Piscataway, NJ) was performed 3 days before and 7 weeks after hUCB-MSC transplantation. For clear images acquisition, hairs on the chest walls of the Sprague-Dawley rats were removed. LV end-diastolic and end-systolic dimensions (LVEDD and LVESD) were measured according to the leading-edge method of the American Society of Echocardiography. The LV percent fractional shortening was calculated as 100× (LVEDD-LVESD)/LVEDD. LVEF was automatically calculated and given by the built-in software.

Histological preparations and staining. After echocardiographic evaluation, rats were euthanized and the hearts were removed. The excised heart was retrograde perfused with phosphate-buffered saline for coronary vasculature and LV washing, and fixed with 4% paraformaldehyde overnight at 4 °C and then 15% sucrose for overnight at 4 °C. Each tissue was embedded in paraffin or frozen in optimal cutting temperature compound (Tissue-Teck; SAKURA, Torrance, CA). Sections (7 µm) stained with Masson's trichome were used to calculate infarct size and wall thickness using Image analysis system (Image Pro version 4.5; MediaCybernetics, Bethesda, MD). To detect hUCB-MSCs injected into peri-infarct area of rat heat, hUCB-MSCs were prestained with DiI before transplantation. The number of capillary vessels was counted in the peri-infarct area of all groups, using isolectin B4 (Vector Laboratories, Burlingame, CA) and staining. Five high-power fields in each area were randomly selected, and the number of capillaries in nonconsecutive sections were averaged and expressed as the number of capillary vessels per square millimeter.

All examination was observed under confocal laser scanning microscopy (LSM 510; Carl Zeizz, Oberkochen, Germany).

Flow cytometric analysis

Cell cycle analysis: hUCB-MSCs (3D-bullet versus monolayer) were fixed with *P* -formaldehyde, permeabilized with Triton X-100, and nuclear DNA was labeled with propidium iodide. Cell cycle phase distribution of nuclear DNA was determined on FACS having fluorescence detector equipped with 488-nm argon laser light source and 623 nm band pass filter (linear scale) using Cell Questpro software (Becton Dickinson). Total 10,000 events were acquired and flowcytrometric data were analyzed using Cell Questpro software (BD CellQuest Pro Software).

Cell surface marker analysis; the cells were first dissociated by incubation at 37 °C for 1 minute in 0.25% trypsin/EDTA (Invitrogen), washed with phosphate-buffered saline containing 2.5% fetal bovine serum, and incubated for 30 minutes with various combinations of saturating amounts of antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): CD45-PE, CD34-FITC, CD44-FITC, CD73-PE, CD166-PE, and HLA-ABC-PE (Becton Dickinson), isotype-matched control (Pharmingen; BD Bioscience, Sparks, MD), and CD90-FITC (Becton Dickinson).

At least 10^4 events were analyzed by FACSCalibur system (Becton Dickinson) with the Cellquest software (BD CellQuest Pro Software).

cDNA microarray. Total RNAs from hUCB-MSCs (3D-bullet or monolayer) were isolated using TRIzol (Invitrogen) and the RNeasy Mini kit (Qiagen, Valencia, CA). The purity and integrity of the total RNA were checked by Bioanalyzer (Agilent, Santa Clara, CA). The microarray experiment procedures including probe synthesis from total RNA, hybridization, detection, and scanning were taken from manufacturer's protocols (Affymetrix, Santa Clara CA). Briefly, cDNA was synthesized from total RNA using the One-Cycle cDNA Synthesis Kit. After clean up with a sample cleanup module, double strand cDNA was used for *in vitro* transcription (IVT). cDNA was transcribed using the GeneChip IVT Labeling Kit, in the presence of biotin-labeled CTP and UTP. After clean up with a sample cleanup module, labeled cRNA was fragmented by fragmentation buffer. Fragmented cRNA was hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array. After hybridization, the arrays were washed in a GeneChip Fluidics Station 450 with wash buffer. After washing, the arrays were stained with a streptavidin–phycoerythrin complex. After staining, intensities were determined with a GeneChip scanner 3000, controlled by GCOS Affymetrix software. As a normalization process, Robust Multi-Array Average normalization was applied to remove systematic variance. By applying Robust Multi-Array Average, the raw intensity values are background corrected, log2 transformed and then quantile normalized. We identified genes that were differentially expressed using *t*-test ($P < 0.05$) among triplicated experiments.

Transduction of human E-cadherin gene using adenoviral vector. The Ad5 vector used in this study was replication deficient (E1-E3 deleted), expressing human E-cadherin or lacZ under cytomegalovirus promoters, as previously described (generous gifts from Dr Meenhard Herlyn, Wistar Institute, Philadelphia, PA)

The hUCB-MSC was transduced with adenoviral supernatants for 24 hours. Efficacy of transduction was evaluated by reverse transcription-PCR and western blot western blot.

Real-time PCR analysis. Total RNA from the cultured hUCB-MSCs was extracted using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The cDNA was synthesized from ~1 µg of total RNA using the Reverse Transcription System (Promega, Madison, SI) and subjected to PCR amplification with specific primers; CDH1 F- TGCCGCCATCGCTTACACCA, CDH1 R- AGGCACCTGACCCTTGTACGTG; CDH9 F- ACCCTCCTC GATTTCCCCAGAGTAC, CDH9 R- CTGCACCATCTCCTTCAGCAA TGCT; CDH16 F- CTGCCTGGCTGTGGCTGCTT, CDH16 R- TGCT CCTCTCGGTCCAGGGC; SELE F- TGCCCTATGCTACACAGCTGCCT, SELE R- TGCTGCTTGGCAGGTAACCCC. The reactions were performed using the ABI Prism 7000 sequence detection system (Applied Biosystems) with SYBR Green I dye (Sigma). GAPDH was simultaneously run as a control and used for normalization. Nontemplate control wells without cDNA were included as negative controls. Each test sample was run in triplicate. The results are reported as the relative expression after normalization of the transcript amount to the endogenous control using the $2^{-\Delta\Delta CT}$ method.[32] Threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold.

Enzyme-linked immunosorbent assay. Cell-free supernatant were collected at 1, 6, 24 hours from anchorage deprivation or at 24 hours from anchorage condition of hUCB-MSCs. They were used to detect the level of Hu VEGFa quantitatively determined by Bio-Plex 200 system (Bio-Rad, Hercules, CA) according to the manufacturers' protocol.

Western blot analysis. Cell lysates were obtained by incubation in lysis buffer containing protease inhibitors (Roche, Hague RD, IN) for 20 minutes. Total protein (10–30 µg) was immunoblotted with specific primary antibodies overnight at 4 °C. The α-tubulin was used as an internal control. ECL or ECL-PLUS (Amersham; GE Healthcare) was used for detection.

Statistical analysis. Continuous variables are expressed as mean ± SE. Differences between continuous variables were analyzed by the unpaired Student *t*-test. Group differences of continuous variables were tested by analysis of variance and followed by a Scheffe's *post-hoc* test. A probability

value of <0.05 was considered significant. Statistical analysis was performed with SPSS 13.0 statistical package (SPSS, New York, NY).

SUPPLEMENTARY MATERIAL

Figure S1. Cell surface marker analysis.
Figure S2. 2D-Echocardiography.
Figure S3. Immunofluorescence assay.

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