Amniotic mesenchymal stem cells have robust angiogenic properties and are effective in treating hindlimb ischaemia

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Aims
In this study, we aimed to evaluate whether human amniotic mesenchymal stem cells (AMMs) have angi-vasculo-genic properties and to determine their therapeutic effects on experimental ischaemia. Although AMMs are a promising source of stem cells, their angi-vasculogenic properties are not fully understood.

Methods and results
We have characterized AMMs by quantitative real-time polymerase chain reaction, Matrigel tube formation assays, and various in vitro endothelial differentiation assays. AMMs expressed significantly higher levels of representative proangiogenic genes, vascular endothelial growth factor-A, angiopoietin-1, hepatocyte growth factor, and fibroblast growth factor-2 (FGF-2) than adipose-derived mesenchymal stem cells. In addition, the anti-apoptotic factor Akt-1 was highly expressed in the AMMs. Cells were directly transplanted into the ischaemic hindlimbs of mice to evaluate their angi-vasculogenic and therapeutic effects. They spontaneously differentiate into vascular-like structures and exhibit endothelial-specific genes and proteins. In an in vivo study on hindlimb ischaemia, implantation of AMMs augmented blood perfusion and capillary density, indicating AMM-augmented neovascularization. The engraftment rate of AMMs was high, and the transplanted AMMs showed vasulogenic potential.

Conclusion
AMMs are not only markedly angiogenic but also vasculogenic, thus ameliorating hindlimb ischaemia. Our data suggest that AMMs have considerable therapeutic effects on ischaemic hindlimb through high angiogenic and engraftment abilities.

Keywords
Amnion • Angiogenesis • Cell-based therapy • Ischaemic hindlimb • Mesenchymal stem cells

1. Introduction
Peripheral arterial disease (PAD) is a life-threatening problem in health care in aged societies.¹ In the later stages of PAD, progression of tissue hypoperfusion leads to ischaemic ulceration and gangrene. Thus, PAD often results in infections, limb amputation, and increased mortality. Although surgical or percutaneous revascularization could be the main treatment for increasing blood perfusion in patients with PAD, it has rarely been successful in restoring flow capacity. Moreover, there are limited medications or procedures to improve the blood perfusion rate of the affected tissues. Rapid revascularization or regeneration of ischaemic tissues is necessary to restore their physiological function.

Mesenchymal stem cells (MSCs) derived from various tissues have been shown to contribute to tissue regeneration. Human cord blood (CB) or placenta-derived amniotic membrane has been evaluated as a good cell source for allogeneic cell transplantation.² ³ In fact, we previously reported the potential of CB-derived MSCs for improving peripheral circulation and rest pain in clinical studies.³ However, CB-derived MSCs are generally less abundant, and it is more difficult to isolate them than any other tissue-derived MSC. Therefore, as an alternative stem cell source, amniotic mesenchymal stem cells

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(AMMs) have been widely studied. Since AMMs are abundant in the wasted placenta, they can be easily isolated and are not associated with prominent ethical concerns. They have a low expression of major histocompatibility complex (MHC) class I that is associated with immunological tolerance. In addition, AMMs were demonstrated to have a high capacity to transdifferentiate into various tissue lineages. However, their angiogenic property and therapeutic contributions to ischaemic blood perfusion has not been fully investigated.

Although stem cells have emerged as a promising therapeutic model, there have been controversies regarding therapeutic mechanisms for ischaemic tissues. In particular, the plasticity of bone marrow (BM)-derived stem cells, which transdifferentiate into cardiomyocytes, smooth muscle cells, and endothelial cells (ECs), has been widely debated. Since the extent of vasculogenesis or myogenesis was low, it was hard to elucidate the therapeutic effects after cell transplantation. Transdifferentiation is mostly driven by the fusion of stem cells. On the other hand, paracrine mechanisms mediated by the factors secreted from stem cells have been widely accepted as convincing mechanisms for the therapeutic outcomes observed in various ischaemic diseases.

In this study, we evaluated the angio-vasculogenic property of AMMs and investigated their therapeutic mechanisms by comparing them with other stem cells for allogeneic cell therapy.

2. Methods

2.1 Cell culture

Normal human dermal fibroblasts (HDFs) and human umbilical vein ECs (HUVECs) were purchased from ATCC (Manassas, VA, USA). Human adipose-derived MSCs (ADMs) and AMMs from different donors (n = 4 per group) were purchased from Thermo Scientific Inc. (Rockford, IL, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer. HDFs, ADMs, and AMMs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA). Multilineage differentiation potential and cell characterization data for these MSCs were provided by the manufacturer.

2.2 Flow cytometry

Passage 6 ADMs and AMMs were suspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. Cells were incubated for 20 min with FITC- or phycoerythrin-conjugated monoclonal antibodies specific for CD14, CD29, CD44, CD73, CD90, CD45, CD105 (endoglin), and HLA-DR. All antibodies were purchased from BD Pharmingen (San Diego, CA, USA). Proper isotype-identical IgGs were used as controls. Cells were first stained and then analysed with a flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.3 Real-time polymerase chain reaction and reverse transcriptase–polymerase chain reaction analyses

Quantitative real-time (qRT)-polymerase chain reaction (PCR) assays were conducted as described previously. In brief, total RNA was isolated from cultured ADMs, AMMs, and HDFs at Passage 4 by using RNA-stat (Iso-Tex Diagnostics, Friendswood, TX, USA), according to the manufacturer’s instructions. Extracted RNA was subsequently reverse transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. The synthesized cDNA was subjected to qRT-PCR or reverse transcriptase–PCR (RT)–PCR using human-specific primers and probes. RNA levels were quantitatively assessed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Relative mRNA expression normalized to GAPDH expression was calculated as described previously. We purchased primer/probe sets from Applied Biosystems.

2.4 qRT-PCR and RT–PCR primers

The primers used for qRT-PCR were human vascular endothelial growth factor (VEGF)-A (Hs00999907_m1), angiopeitin (Ang)-1 (Hs00181613_m1), hepatocyte growth factor (HGF) (Hs00300159_m1), fibroblast growth factor-2 (FGF-2) (Hs00266645_m1), Akt-1 (Hs00178289_m1), stromal cell-derived factor (SDF)-1a (Hs00171022_m1), and GAPDH (Hs99999905_m1). The primers used for qRT-PCR were Mouse VEGF-A (Mm01204733_m1), Ang-1 (Mm00456503_m1), and GAPDH (Mm9999915_g1). The following paired RT–PCR primers were used: 5′-aagacattttccggctcac/ggcactttagtagttctcc-3′ for e-NOS (548 bp), 5′-ctggt ttgcagcatatgacaagacgtgctggaggg-3′ for KDR (266 bp), 5′-ccaaaggtct cacgccttatacgctgacacaccgc-3′ for vWF (189 bp), 5′-aagacattttccggctcac/-aggccccggacatgc-3′ for flt-1 (617 bp), 5′-ttacttggcaggcagctctttattcaaggg accttacagctggagggagtt-3′ for Tie-2 (223 bp), and 5′-gggacccggccggatc/ gcgccggtgcctccttgag-3′ for GAPDH (198 bp). All primer/probe sets were purchased from Applied Biosystems.

2.5 Matrigel tube formation assay

Culture media (CM) were collected as described previously. ADMs and AMMs (1 × 10⁶ cells each) were seeded into T-75 cell culture flasks and grown in normal medium or low-glucose DMEM (Gibco) containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco) for 48 h until the cells reached approximately a confluency of 80%. CM from each sample were then centrifuged at 1000 g for 10 min, and the supernatants were collected and used as CM for the study. To evaluate the tube formation potential, HUVECs were seeded with EGM-2 complete medium and each CM was derived from the ADMs and AMMs at a concentration of 1 × 10⁶ cells/well in basement membrane matrix gel (Matrigel, BD)-coated two-well glass slides (NUNC). To investigate the direct vasculogenic capacity of AMMs, Matrigel tube formation assays were also conducted. The ADMs and AMMs, at a concentration of 1 × 10⁶ cells/well in basement membrane matrix gel (Matrigel, BD)-coated two-well glass slides, were seeded with EGM-2 complete medium. After 6 and 12 h of incubation, representative fields were randomly photographed using fluorescence microscopy, and the tube length and branching point from each sample were examined.

2.6 Immunoblot analysis

Western blot assays were conducted using a previously described method. Briefly, protein extracts (each 100 µg) were separated on 8% SDS–PAGE (Bio-Rad Laboratories) and electrotransferred onto PVDF membranes (GE Healthcare). Specimens were probed with the following antibodies: Akt-1, phospho-Akt (Thr 308) (308) and β-actin (Santa Cruz Biotechnology, Inc.). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody and signal was detected using an LAS-3000 chemidoc system (Fuji Photo Film, Japan).

2.7 Transplantation of cells in the ischaemic hindlimb animal model

Experimental protocols were approved by the Dong-A University Institutional Animal Care and Use Committee, and all procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).
Male NOD/severe combined immunodeficiency (scid) mice (NOD.CB17-Prkdcscid/J strain, The Jackson Laboratory, Bar Harbor, ME, USA) that were 8–10 weeks old and weighed 18–22 g were used.

Mice were anaesthetized with isoflurane (induction: 450 mL air, 4.5% isoflurane, maintenance: 200 mL air, 2.0% isoflurane, Baxter International, Inc., Deerfield, IL, USA) and the depth of anaesthesia was monitored by the respiratory rate and the lack of withdrawal reflex upon toe pinching.

To induce hindlimb ischaemia, the right femoral artery was surgically ligated as described in our earlier report. A solution of $1 \times 10^6$ 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (Dil)-labelled cells prepared in PBS was then intramuscularly injected into the ischaemic hindlimb area after surgery (n = 7 for each transplantation). Euthanasia was conducted by intravenous injection of thiopental sodium (40 mg/kg).

We used a laser Doppler perfusion image (LDPI) analyzer (Moor Instrument, Axminster, UK) to measure the serial blood flow in the hindlimb after the operation.

**2.8 Histological analysis**

The adductor muscles were harvested, fixed in 4% paraformaldehyde for 4 h, and incubated overnight in a 15% sucrose solution. The tissues were embedded in OCT compound (Sakura Finetek USA, Torrance, CA, USA), snap frozen in liquid nitrogen, and sectioned in thickness increments of 10–20 mm. For capillary density measurement, five frozen sections from each group of ischaemic tissue from the adductor muscles were stained with biotinylated isolectin B4 (ILB4, 1:250; Vector Laboratory Inc., Burlingame, CA, USA) primary antibody followed by streptavidin Alexa Fluor 488 (1:100; Invitrogen) secondary antibody. Five fields from five tissue sections were randomly selected, and the number of capillaries was counted in each field. To detect human cells, tissue sections were stained with human nuclear antigen (HNA, Chemicon, Temecula, CA, USA) and fluorescence in situ hybridization (FISH) was performed using a Cy3-conjugated (Cambio, Cambridge, UK) human X chromosome probe. Photographs were taken using fluorescent inverted microscopy or confocal microscopy.

**2.9 Quantification of cell engraftment in ischaemic hindlimbs**

Cell engraftment in the ischaemic hindlimbs was quantified by histological analyses. Briefly, Dil-labelled cells were injected into ischaemic hindlimbs of mice. After 4 weeks, the ischaemic hindlimbs were harvested, and tissue sections were embedded and sectioned. Five fields from four tissue sections were randomly selected, and the number of Dil-labelled cells was counted in each field.

**2.10 Statistical analysis**

All data are presented as mean ± SEM. Statistical analyses were conducted using Student’s t-test for comparisons of the two groups, and ANOVA with Bonferroni’s multiple comparison test using SPSS v11.0. Data with $P < 0.05$ were considered statistically significant.
3. Results

3.1 Characteristics of AMMs
Cultured AMMs showed spindle fibroblast-like shapes and exhibited a high nucleus-to-cytoplasm ratio (see Supplementary material online, Figure S1A). The growth kinetics of ADMs and AMMs were measured, revealing that ADMs had a higher cell proliferation rate than AMMs (see Supplementary material online, Figure S1B). AMMs underwent more than 120 population doublings (PDs), and AMMs at 6–8 PDs were used in the present study. To identify cell characteristics, we performed FACS analysis by multiple surface epitopes. Results revealed that ADMs and AMMs minimally expressed (<1%) the haematopoietic cell markers (CD14 and CD45) and MHC class II molecules (HLA-DR) (see Supplementary material online, Figure S1C). In contrast, ADMs and AMMs expressed high levels of the MSC-specific markers CD29, CD44, CD73, CD90, and CD105. AMMs maintained their phenotypes and did not spontaneously differentiate during the cell expansion period.

3.2 AMMs show high angiogenic gene expression
To evaluate the angiogenic and anti-apoptotic potential of AMMs, we analysed them with qRT-PCR. Surprisingly, the AMMs expressed significantly higher levels of representative proangiogenic genes, VEGF-A, Ang-1, HGF, and FGF-2 when compared with the ADMs, HDFs, and HUVECs (Figure 1A). In addition, the anti-

Figure 2 Matrigel tube formation analysis. (A) Representative images of Matrigel tube formation using CM. The tube length and branching point numbers of HUVECs were significantly higher in the AMM-CM-treated group compared with the ADM-CM group. **p < 0.01 AMM-CM vs. ADM-CM; †p < 0.01 AMMs vs. control. Control-CM in the absence of cytokine cocktail was used as a negative control group. (B) Representative images of direct Matrigel tube formation. Matrigel tube formation assay was performed to measure the in vitro vasculogenic potential of AMMs and ADMs. Tube length and branching point numbers were markedly higher in the AMMs than in ADMs. **p < 0.01. Bars: A and B, 200 μm.
apoptotic factor Akt-1 was highly expressed in the AMMs when compared with the ADMs and HDFs. However, a well-known chemokine, SDF-1α, which plays a pivotal role in repopulating the circulating progenitor cells, was highly expressed in the ADMs when compared with the AMMs, indicating the presence of angiogenic capacity in the ADMs. Next, we performed immunoblotting to confirm the phosphorylation of Akt-1 (Figure 1B). As expected, the phosphor protein levels were also found to be significantly elevated in the AMMs when compared with HDFs and ADMs, supporting the prior Akt-1 mRNA expression data. Taken together, these data demonstrated that AMMs are a promising source of stem cells that are enriched with multiple angiogenic and anti-apoptotic factors.

3.3 Secreted factors derived from AMMs improve endothelial network formation

To test the angiogenic effects of secreted factors derived from AMMs, we performed Matrigel tube formation assays. EGM-2 complete medium and each CM derived from ADMs and AMMs were seeded at a concentration of 1 × 10⁵ HUVECs/well in Matrigel-coated two-well glass slides. After 6 h of incubation, 10 fields from each sample were randomly photographed by inverted microscopy, and tube lengths and branching points were calculated by Image analysis system (Image J, Windows version; National Institutes of Health, USA). The results revealed that AMM-CM induced significantly higher tube lengths (1.44-fold) and branching points (1.55-fold) when compared with ADM-CM (Figure 2A). These data indicate that the secreted factors derived from AMMs have strong angiogenic potential. Next, in order to investigate the direct vasculogenic capacity of AMMs, Matrigel tube formation assays were also conducted. We measured the tube lengths and branching points of each type of cells after a 12 h-long incubation. Markedly higher numbers of branching points and longer tube lengths were observed in the AMMs compared with the ADMs (Figure 2B).

3.4 In vitro EC differentiation and EC-specific gene expression

To compare the EC differentiation potential of AMMs and ADMs, each cell type was cultured in EGM-2, which is endothelial cell basal media-2 with 2% FBS and cytokine cocktail (SingleQuots; Lonza), for
Figure 4 Implantation of AMMs is effective for the recovery of ischaemia and induced neovascularization in vivo. (A) Laser Doppler perfusion images showed recovery of blood perfusion in ischaemic hindlimbs, while quantitative analysis (B) showed improved blood perfusion in the AMM when compared with the ADM and PBS groups over 4 weeks after cell injection. The blue represents low perfusion and the red represents high perfusion. n = 7 per group. *P < 0.01 AMMs vs. PBS; †P < 0.05 AMMs vs. ADMs. (C) Capillary density in hindlimb muscles and quantitative analysis of capillary density (D) revealed significantly increased capillary growth in the AMM group when compared with the ADM and PBS groups. n = 8 per group. *P < 0.01 AMMs vs. PBS; †P < 0.05 AMMs vs. ADMs. Bar: C, 100 μm. (E) Increased expression of angiogenic factor in tissues transplanted with AMMs. n = 5 per group. *P < 0.01 AMMs vs. PBS; †P < 0.05 AMMs vs. ADMs.

Figure 5 Engraftment of AMMs in vivo. (A) Representative photographs of localized Dil-labelled ADMs and AMMs at 4 weeks after cell injection. (B) Quantification of engrafted ADMs and AMMs. Dil-labelled cells (red) in the ischaemic hindlimb area were quantified by histological analysis. *P < 0.05 AMMs vs. ADMs. (C) Representative images of HNA-stained ADMs and AMMs (arrows) at 4 weeks after cell transplantation. (D) Quantification of engrafted human cells. HNA (red)-positive cells were quantified. *P < 0.05 AMMs vs. ADMs.
10–20 days. After the ADMs and AMMs differentiated into ECs on day 10, we observed an intriguing phenomenon; some cells aggregated and spontaneously formed linear tubular structures, thus mimicking vasculogenesis (see Supplementary material online, Figure S2). These tubular cells were stained with CD146, which is an EC-specific marker. However, ADMs tubular structures were consistently negative for CD146. This suggests that these ADMs may have partially differentiated into ECs. In addition, immunocytochemistry results demonstrated that the high number of AMMs simultaneously exhibited the EC marker kinase insert domain receptor (KDR) and lectin more than ADMs (see Supplementary material online, Figure S2), indicating the relatively low EC differentiation capacity of the ADMs when compared with the AMMs.

Next, to examine the change of EC-specific gene expression during EC differentiation, we performed RT–PCR. Undifferentiated ADMs and AMMs expressed endothelial nitric oxide synthase (eNOS), Flt-1, Tie-2, and Von Willebrand factor (vWF). Specifically, undifferentiated AMMs exhibited a high level of vWF (Figure 3A). Five days after EC differentiation of AMMs, the levels of Flt-1, KDR, and Tie-2 expression were weakly elevated. Quantification of gene expression results demonstrated that the levels of Flt-1 (1.8-fold), KDR (14.1-fold), and Tie-2 (1.6-fold) expression were significantly higher in AMMs than ADMs at 15 days after EC differentiation (Figure 3B). In addition, EC-differentiated AMMs showed strong levels of expression of endothelial genes such as eNOS, Tie-2, and vWF at levels similar to those of HUVECs.

### 3.5 Favorable therapeutic effects of AMMs in hindlimb ischaemia

After investigating the angiogenic and vasculogenic capacities of AMMs in vitro, we examined the in vivo therapeutic ability of AMMs to restore experimentally induced ischaemia. LDPI analysis revealed that blood perfusion was significantly higher in the AMM-injected limbs than those injected with either ADMs or PBS at days 14, 21, and 28 (Figure 4A and B). The capillary density in the ischaemic hindlimb adductor muscles after cell transplantation was significantly higher when using AMMs than that observed when using ADMs or PBS (Figure 4C and D).

To determine the therapeutic mechanism of transplantation of AMMs on cytokine expression pattern in vivo, the mice were sacrificed and the tissues from the hindlimbs were collected for analysis. The expression levels of Ang-1 and VEGF-A were significantly up-regulated in the limbs transplanted with AMMs than those receiving transplanted ADMs or PBS (Figure 4E), suggesting that AMMs up-regulate the expression of multiple angiogenic factors involved in neovascularization.

### 3.6 Higher engraftment/survival potential and in vivo EC transdifferentiation of AMMs

The engraftment and survival potentials of AMMs were studied in an induced ischaemic hindlimb model. A total of 1×10^6 Dil-labeled cells of each type were directly transplanted into the ischaemic region of the hindlimb. Four weeks after cell transplantation, hindlimb tissues were collected and analysed by immunohistochemistry. Immunohistochemistry results demonstrated that AMMs exhibited a significantly higher engraftment potential (21.3±4.1) compared with ADMs (5.6±2.3) at 28 days after cell transplantation (Figure 5A and B). To detect injected human cells in hindlimb tissues, sections were stained with HNA. Immunohistochemistry data also revealed that a significantly high number (5.3±1.6) of AMMs were detected in hindlimb tissues compared with ADMs (1.6±1.5) at 28 days after cell transplantation (Figure 5C and D).

The majority of engrafted AMMs were localized in the perivascular or pericytic areas, and a low number of AMMs displayed the EC-specific marker ILB4 and vascular-like structures (Figure 6A). To demonstrate whether these ILB4 expressing Dil-labelled cells were truly derived from the injected AMMs, FISH analysis using a human chromosome was performed. The FISH results revealed that these double-positive cells present in the hindlimb tissue originated from a human donor, confirming the transdifferentiation of AMMs into ECs (Figure 6B).

### 4. Discussion

In the present study, we demonstrated that AMMs have potent angiogenic and vasculogenic potentials and that these cells are therapeutically useful for recovering tissue from ischaemia. First, we demonstrated that AMMs are enriched with proangiogenic genes. Secondly, AMMs differentiated into ECs in culture. Thirdly, transplantation of AMMs into ischaemic hindlimb promoted recovery of tissue ischaemia. Fourthly, implanted AMMs exhibited high cell survival/engraftment capacity, and thereby provided a favourable environment for neovascularization.

Using AMMs as an allogeneic stem cell source can be highly beneficial, since there are no ethical problems involved, they are easy to collect, have a high transdifferentiation capacity, and express only low levels of the MHc antigen. In fact, amniotic membrane is of foetal origin and may possess superior differentiation, proliferation potential, and a lower immunological reaction than other adult-derived stem cells. In addition, using amniotic membrane for isolating MSCs removes the possibility of contamination with unwanted cells such as endothelial and hematopoietic cells, since these are unlikely to be present in placenta-derived cells or CB cells. Because the amniotic membranes are devoid of any blood, vascularization is the only source of MSCs. Despite the advantages offered by AMMs, they have not been more actively studied than other adult stem cells.

Recently, many studies regarding human MSCs derived from various tissues such as CB, BM, and adipose tissues have been conducted. MSCs express angiogenic and arteriogenic genes and induce angiogenesis in ischaemia. Recently, Alviano et al. reported investigations on the in vivo EC differentiation potential of AMMs. However, detailed angiogenic characteristics of AMMs such as angiogenic gene expression (associated with paracrine factors) and their therapeutic effects in vivo have yet to be fully evaluated. Therefore, we focused our studies on their specific angiogenic properties by comparison with ADMs (representative MSCs). Surprisingly, qRT-PCR results revealed that crucial angiogenic genes that are beneficial for vascular regeneration, such as VEGF-A, Ang-1, HGF, and FGF-2, are highly enriched in the AMMs compared with the ADMs (Figure 1). These angiogenic factors in combination have been proposed to induce synergistic effects during therapeutic neovascularization, indicating that the AMMs have strong angiogenic characteristics. However, HDF and ADMs exhibited significant levels of SDF-1α, a well-known chemotactic factor, when compared with AMMs, suggesting that these cells also have angiogenic capabilities. Taken together, these results strongly indicate that AMMs are potent angiogenic, suggesting that these cells could be one of the most suitable stem cells to use for the regeneration of injured ischaemic tissues.
Figure 6  EC differentiation of AMMs in vivo. (A) Engraftment and differentiation of transplanted AMMs in ischaemic hindlimbs. Three-dimensional z-stacked orthogonal and multipanel images clearly demonstrated that the injected AMMs incorporated into the vascular structure and exhibited EC markers. Tissue sections from ischaemic hindlimb 4 weeks after cell injection were stained with ILB4 antibody (green). Arrows indicate lectin and Dil double-positive cells. (B) FISH analysis of AMMs transplanted into hindlimb tissues. One cell (arrow), which localized to an ILB4-stained capillary, was also positive for the human X chromosome (red), indicating the in vivo transdifferentiation of transplanted AMMs into ECs. ILB4 (green); DAPI (blue).
To evaluate the angiogenic effects of secreted factors from AMMs, we conducted Matrigel tube formation assays using cultured medium. In concordance with the qRT-PCR data, AMM-derived CMs markedly affected the tube-like network formation of HUVECs when compared with ADM-derived CMs (Figure 2). These results could be explained by the mechanism that angiopoietin-1 stimulates a tube-like network formation by interacting with VEGF in a dose-dependent manner.24 In addition, we previously observed that AMM-derived CMs promoted cell migration from the scratch wound assay (unpublished data), demonstrating the high wound-healing properties of AMMs. In vivo cell transplantation studies further confirmed the in vitro angiogenic results. Transplantation of AMMs markedly augmented blood perfusion and capillary density in the ischaemic hindlimb adductor muscles. Local delivery of AMMs may have induced circulating stem or progenitor cells to home in the ischaemic tissues, and thereby contributed to the therapeutic outcomes, since AMMs expressed a high level of chemokines, including VEGF and HGF. In addition, it is likely that paracrine factors from local vascular cells by collateral effects of cell transplantation lead to the home circulation of angiogenic progenitor cells.

Cell survival factors prevent apoptosis and suppress the intrinsic cell death process. Several strategies to enhance stem cell engraftment or survival capacity in ischaemic tissues have been studied.25,26 Mostly, genetically engineered stem cells have been investigated to achieve the goal. In facts, MSCs overexpressing the anti-apoptotic factors Akt or Bcl-2 were able to significantly restore damaged heart function. However, there are still some hurdles regarding safety and efficacy issues before these can be clinically applied, and it is critical that these issues are addressed from a long-term view. Interestingly, we found that AMMs significantly express Akt (Figure 1A and B), which plays a pivotal role in cell survival27 and has been reported to repair the function of infarcted hearts and prevent heart remodeling.28 In line with the in vitro data, we also observed that an increased number of AMMs successfully engrafted and survived in vivo in the ischaemic hindlimb when compared with the ADMs (Figure 5A–D). These data suggest that AMMs have potent cell survival properties, which could be valuable for treating ischaemia. Because artificially modified stem cells are not necessary, there are no safety issues in treating ischaemic tissues or organs.

The current controversy arose regarding the (trans)differentiation potential of BM-derived cells or MSCs.10,28–30 To investigate the endothelial differentiation potential of AMMs, we differentiated them in vitro using EGM-2, i.e. EC culture medium. RT–PCR data demonstrated that AMMs already expressed a high level of VWF, indicating the possibility of endothelial commitment. During the EC differentiation period, AMMs revealed an increased expression of EC-specific genes, namely VEGF receptors 1 and 2 (Flt-1 and KDR) and Tie-2 (Figure 3A and B), confirming endothelial differentiation. Furthermore, immunocytochemistry results showed that differentiated AMMs clearly expressed the EC-specific proteins of lectin and KDR (see Supplementary material online, Figure S3). In addition, AMMs intriguingly formed vascular-like tubules (see Supplementary material online, Figure S2) without the aid of Matrigel, which is almost similar to the phenomenon observed in our previous study on the endothelial differentiation of human peripheral blood-derived CD31-positive cells.19 This in vitro EC differentiation capacity of AMMs was consistent with a previous report.20 To clarify this EC differentiation capacity of AMMs, we meticulously examined in vivo AMM-implanted hindlimb muscles using three-dimensional-reconstructed confocal microscopy images and FISH analysis. Although most transplanted AMMs localized to the interstitium or perivascular region, some AMMs were clearly incorporated into the vasculature and expressed EC markers (Figure 6A and B), suggesting the existence of robust vasculogenic potential. To quantify EC-transdifferentiated AMMs in the ischaemic limbs, it is necessary to develop more advanced cell tracing technologies and to perform long-term follow-ups.

In conclusion, AMMs are not only markedly angiogenic but also vasculogenic, thus ameliorating hindlimb ischaemia. Therefore, these cells offer a highly promising and easily applicable option as a novel therapy for treating ischaemic cardiovascular diseases. However, further investigation will be required to test whether AMMs are safe and capable of ameliorating ischaemia for allogenic cell therapy in clinical studies.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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