RESEARCH ARTICLE

Outgrowth endothelial cells form a functional cerebral barrier and restore its integrity after damage

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Abstract
Breakdown of blood-brain barrier, formed mainly by brain microvascular endothelial cells (BMECs), represents the major cause of mortality during early phases of ischemic strokes. Hence, discovery of novel agents that can effectively replace dead or dying endothelial cells to restore blood-brain barrier integrity is of paramount importance in stroke medicine. Although endothelial progenitor cells (EPCs) represent one such agents, their rarity in peripheral blood severely limits their adequate isolation and therapeutic use for acute ischemic stroke which necessitate their *ex vivo* expansion and generate early EPCs and outgrowth endothelial cells (OECs) as a result. Functional analyses of these cells, in the present study, demonstrated that only OECs endocytosed DiI-labelled acetylated low-density lipoprotein and formed tubules on matrigel, prominent endothelial cell and angiogenesis markers, respectively. Further analyses by flow cytometry demonstrated that OECs expressed specific markers for stemness (CD34), immaturity (CD133) and endothelial cells (CD31) but not for hematopoietic cells (CD45). Like BMECs, OECs established an equally tight *in vitro* model of human BBB with astrocytes and pericytes, suggesting their capacity to form tight junctions. Ischemic injury mimicked by concurrent deprivation of oxygen and glucose (4 hours) or deprivation of oxygen and glucose followed by reperfusion (20 hours) affected both barrier integrity and function in a similar fashion as evidenced by decreases in transendothelial electrical resistance and increases in paracellular flux, respectively. Wound scratch assays comparing the vasculoreparative capacity of cells revealed that, compared to BMECs, OECs possessed a greater proliferative and directional migratory capacity. In a triple culture model of BBB established with astrocytes, pericytes and BMEC, exogenous addition of OECs effectively repaired the damage induced on endothelial layer in serum-free conditions. Taken together, these data demonstrate that OECs may effectively home to the site of vascular injury and repair the damage to maintain (neuro)vascular homeostasis during or after a cerebral ischemic injury.

Key Words: *cell-based therapy; endothelial progenitor cells; endothelium; ischemic stroke; neurodegeneration; novel therapeutics; outgrowth endothelial cells; regenerative medicine; stem cells; translational medicine*

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Introduction
Ischemic stroke stemming from the occlusion of an artery leading to or within the brain continues to be one of the main causes of morbidity and mortality worldwide. Thrombolysis with recombinant tissue plasminogen activator (rt-PA) remains the only approved pharmacotherapy for this debilitating condition. Unfortunately, due to issues surrounding patients' eligibility, high cost, a short post-stroke therapeutic window to administer recombinant tissue plasminogen activator (the first 4.5 hours of stroke) and markedly elevated risk of intracranial bleeding beyond this point, globally < 1% of patients receive this therapy each year (Hacke et al., 2008; Hisham and Bayraktutan, 2013). Since, thousands of clinical trials assessing the therapeutic capacity of a wide range of so-called pharmaceuticals have failed to produce a safe and efficacious agent, the emphasis of stroke research has in recent years been tilting towards the cell-based approaches. In this regard, endothelial progenitor cells (EPCs) appear to attract much of the attention due their ability to differentiate into few cell lines including endothelial cells to help repair stroke-induced vascular and neuronal damage (Bayraktutan, 2019). However, the restricted availability of true EPCs in bone marrow or systemic circulation renders it impossible to isolate these cells in sufficient numbers to treat acute conditions such as ischemic stroke. This naturally necessitates growth of few isolated cells in culture to generate large quantities of clinically-applicable homogenous cells.

Accumulating evidence demonstrates that EPCs in culture produce two distinct types of cells, namely early EPCs (eEPCs) which appear early in culture and have limited proliferative and no tubulogenic capacity and late EPCs (or outgrowth endothelial cells; OECs), which appear late in culture (3–4 weeks), express progenitor cell markers and exhibit strong proliferative and tubulogenic capacity (Rehman et al., 2003; Hur et al., 2004). Taken together, these findings imply that OECs may represent the EPC subtype that can significantly contribute to vascular network formation and neurovascular repair. To elucidate this notion and considering the fact that structural and functional damage to blood-brain barrier (BBB) accounts for much of the ischemic stroke-related mortalities during (sub)acute phase of the disease, the current study for the first time explored the barrier-forming and barrier-restorative roles of OECs in *in vitro* settings using a well-established cell culture model of human BBB consisting of astrocytes, pericytes and brain microvascular endothelial cells (HBMECs). In doing so,

the study also assessed whether endothelial and progenitor characteristics of OECs are truly different than those of eEPCs and HBMECs.

Materials and Methods

Cell culture

Human astrocytes, pericytes and HBMECs were bought from TCS CellWorks Ltd. (Buckingham, UK) and cultured at 37°C in relevant specialised media (Sciencell, Caltag Systems, Buckingham, UK) in a humidified atmosphere consisting of 75% N_2, 20% O_2 and 5% CO_2. In related studies, cells cultured to about 90% confluence and subjected to 4 hours of oxygen-glucose deprivation alone (OGD, 94.95% N_2, 0.05% O_2, 5% CO_2) or followed by 20 hours of reperfusion (OGD + R). To conduct OGD experiments, D-glucose free medium (RPMI 1640, Sigma, Dorset, UK) and an MCO-18M multigas incubator (Sanyo Electric Co. Ltd, Japan) flushed with N_2 were used. Reperfusion was induced by switching the OGD media with the relevant specialized media and returning cells to normoxic conditions.

EPC culture

Isolated peripheral blood mononuclear cells from a 24-mL blood sample was seeded on 0.01 mg/mL fibronectin-coated 12-well plates and cultured in endothelial basal medium-2 containing 20% foetal calf serum and all the supplements provided with the media. Non-adherent cells were removed after 48 hours and culture media was changed every other day until colonies appeared. This protocol was very effective in identifying the true EPCs with actual endothelial properties as previously shown (Bayraktutan, 2019).

Immunocytochemistry

Both HBMECs and OECs (5×10^4) were grown on fibronectin-coated (1 mg/mL, Sigma) coverslips using endothelial cell growth medium 2 containing vascular endothelial growth factor (VEGF) (5 µL/mL, Fisher Scientific, Laughborough, UK). Once the cells were ~95% confluent, they were incubated with DiI-labelled acetylated-low density lipoprotein (Dil-AcLDL, 1 mg/mL, Invitrogen, Laughborough, UK) for 4 hours prior to staining with FITC-conjugated Ulex europaeus agglutinin (FITC-UEA-1, 1 mg/mL, Sigma) for 2 hours. The cells were then fixed with 4% formaldehyde and mounted on glass slides using mounting medium (Vector Laboratories, Peterborough, UK).

In additional experiments, the architecture of stress fibres was studied by staining of F-actin filaments in approximately 80% confluent cells cultured in normal and experimental conditions. The cells were then fixed for 20 minutes in 4% paraformaldehyde/PBS and permeabilised for 15 minutes in 0.1% Triton X-100/PBS prior to staining with 1× rhodamine phalloidin for 60 minutes (Abcam, Cambridge, UK). The coverslips were mounted on glass slides as above before viewing cells with fluorescence microscopy (Zeiss Axio Observer, Carl Zeiss Ltd, Cambridge, UK).

Flow cytometric analyses of HBMEC and OECs

Fully confluent OECs and HBMECs were trypsinized and resuspended in their specialised media before manually counting with a neubauer hemocytometer (Hawksley and Sons Ltd., Lancing, UK). The cells were then incubated with FcR blocker (Miltenyi Biotech, Bergisch Gladbach, Germany) to stop non-specific binding of antibodies and CD45, CD133, CD34 and CD31 antibodies tagged successively with fluorescein isothiocyanate (BD Biosciences, Franklin Lakes, NJ, USA), allophycocyanin (Miltenyi Biotech), phycoerythrin-cyanine7 (BD Biosciences) and phycoerythrin (BD Biosciences). Fluorescence minus one controls that contain all the fluorochromes except for the one that is being measured was used to properly interpret the positives from the negative population. For FACS analysis, 500,000 events were acquired using a FACS Canto II analyzer (BD Biosciences).

Matrigel tube formation assay

OECs and HBMECs were plated in 48-well plates (9×10^4 cells/well) pre-coated with growth factor-reduced Matrigel (BD Biosciences) and cultured for 8 hours to determine the *in vitro* angiogenic activity of cells. Tube formation was defined as the appearance of a circle-like structure and visualised by a light microscope. Images of tubes were obtained from five randomly chosen fields per sample.

Wound scratch assays

Same passage number HBMEC and OECs were seeded in 6-well plates (1.5×10^4 cells/well) and cultured in their specialised media until reaching full confluence. A scratch was then made manually down the centre of the well with a p1000 micropipette tip in one swift motion and the cells were maintained under normoxic conditions at 37°C. Pictures of wound closure were taken every 12 hours during 36 hours post-scratch period. Wound closure was quantified as the percentage of difference in scratch area between the initial image (0 hour image) and the images obtained at different time points using ImageJ software (version 1.52k, NIH, Maryland, USA).

Cell culture model of human BBB

Since HBMEC, human astrocyte and pericyte triple-culture model is regarded as one of the most clinically-relevant *in vitro* model of human cerebral barrier, this particular model has been employed throughout the present study (Allen and Bayraktutan, 2009a; Shao and Bayraktutan, 2013). To this end, about 7.5×10^4 human astrocytes were transferred onto the outer surface of Corning Costar transwell inserts (0.4 µm pore size, 12 mm diameter polyester membrane, High Wycombe, UK) placed upside down in a 6-well cell culture plate. After overnight adherence, the inserts were directed the right way and transferred into a 12-well plate holding fresh medium. Once, the astrocytes were about 90% confluent, HBMEC (~5×10^4 cells) were seeded onto the inner side of the membrane before growing both cell layers to full confluence. To set up the triple culture model, these inserts were transferred to 12-well dishes containing confluent pericytes.

In some experiments, a scratch was made manually down the centre of the endothelial layer with a p10 micropipette tip in one swift motion. Endothelial cell media was then replaced with serum-free media containing OECs (3.5×10^4). 48 hours after the induction of scratch, the characteristics of the BBB were assessed as follows.

Due to difficulties regarding clear photography of endothelial monolayer in the BBB model, in additional experiments HBMECs were seeded in 48-well plates (that provided

closer growth area to inserts used; 0.95 cm^2 *versus* 1.12 cm^2, respectively) and cultured to confluence in endothelial cell media. Wounds were created by scratching the monolayer with a p10 micropipette tip before replacing endothelial cell media with serum-free media containing OECs (3.5×10^4). Images were taken immediately after scratch and 8 hours after addition of OECs.

Measurement of BBB integrity and function

The structural and functional integrities of BBB were examined by assessments of transendothelial electrical resistance (TEER) and inter-endothelial cellular flux of sodium fluorescein, a low molecular weight permeability marker (NaF, 376 Da). An EVOM resistance meter connected to STX electrodes was used to study changes in TEER values (World Precision Instruments, Hertfordshire, UK). The inserts were then rinsed with Hank's Balanced Salt Solution (HBSS, Sigma) and placed into fresh 12-well dishes containing 2 mL of HBSS. NaF was then added to the luminal chambers at a concentration of 10 μg/mL before taking samples from both luminal and abluminal chambers every 20 minutes to measure NaF levels by fluorometry (excitation/emission: 440/525 nm) using a FLUOstar Omega plate reader (BMG Labtech Ltd., UK). The flux across the cell-free inserts was determined as before (Abdullah and Bayraktutan, 2014).

Statistical analyses

Data are illustrated as the mean ± SEM from at least three independent experiments. Statistical analyses were performed by one-way analysis of variance followed by Tukey's *post hoc* test or Student's *t*-test (where appropriate). All statistical analyses were performed with GraphPad Prism 7.0 statistical software package (GraphPad Software Inc., La Jolla, CA, USA). A value of $P < 0.05$ was considered as significant.

Results

OECs, but not eEPCs, display true endothelial characteristics

While both HBMEC and OECs possessed the classical endothelial phenotype i.e., cobblestone morphology, eEPCs displayed a spindle-shaped morphology. Again, while both OECs and HBMEC formed well-established and clearly identifiable tubules on matrigel, a marker of *in vivo* angiogenesis, eEPCs formed only few and less-organised tubes (**Figure 1**). Furthermore, both HBMEC and OECs, but not eEPCs, bound ulex europaeus agglutinin and endocytosed DiI-AcLDL (**Figure 2**).

OECs possess progenitor cell features

Flow cytometric analyses of OECs showed that these cells did not show any expression of hematopoietic cell marker CD45 (0.012% and 0.008% for OECs and HBMECs, respectively, $P = 0.1000$) but primarily expressed endothelial cell marker, CD31 (99.81%) which closely matched HBMEC expression of CD31 (99.71%, $P = 0.1000$), implying commitment of OECs to the endothelial lineage. Level of stemness marker CD34 was higher in OECs (91.18%) than in HBMECs (23.32%, $P < 0.0001$), exhibiting that OECs displayed a more immature phenotype which appeared to be the case for the progenitor cell marker CD133 as well (0.05% and 0.02% for OECs and HBMECs, respectively, $P = 0.0213$, **Figure 3**).

Effects of OGD and reperfusion on HBMEC and OEC cytoskeletal formation

Under normoxic conditions, both HBMEC and OEC displayed a cortical actin staining. Exposure of either cell line to OGD significantly increased the formation of actin stress fibres traversing the cells. Reperfusion led to more diffuse staining of actin filaments accompanied by the presence of stress fibres and some cortical staining (**Figure 4**).

Effects of experimental ischemic injury on BBB integrity and function

When cultured with astrocytes and pericytes, both HBMECs and OECs formed an equally tight and functional *in vitro* model of human cerebral barrier, indicating appropriate establishment of tight junctional complexes in both cases. Exposure of either model to 4 hours of OGD significantly perturbed BBB characteristics integrity and function as evidenced by similar changes in TEER and flux of NaF, respectively. Despite improving the barrier integrity and function, reperfusion (OGD + R) failed to return these levels to the levels observed in normoxic conditions (**Figure 5**).

OECs repair endothelial damage

Time-course assessment of cell migration and proliferation through wound scratch assays revealed that both HBMEC and OECs repaired endothelial damage in a time-dependent manner. However, the duration of wound closure was considerably shorter with OECs, proving the ability of these cells to proliferate and migrate at faster rates (**Figure 6**). Scrutiny of the endothelium-reparative feature of OECs in a triple culture model of human BBB, established with HBMEC, astrocytes and pericytes and maintained in serum-free conditions showed that OECs effectively homed into the scratch area, repaired endothelial damage and consequently restored barrier integrity and function (**Figure 7**). Although inserts with transparent polyester membrane, superior to polycarbonate and collagen-coated polytetrafluoroethylene membranes in allowing assessment of monolayer formation under phase contrast microscopy, had been used in these experiments, difficulties with effective visualisation of endothelial layer alone persisted. Additional wound scratch experiments performed with HBMEC monolayers, exposed to serum-free conditions, addressed this issue and further corroborated endothelial-reparative action of OECs (**Figure 8**).

Discussion

Loss of endothelial integrity and diminished potential for neovascularisation help promote ischemic events affecting the brain (Gibson et al., 2014). Hence, re-endothelialisation of cerebral vasculature by proliferation and lateral migration of existing mature endothelial cells and by intricate function of circulating EPCs may both substantially alleviate the risk of cerebral ischemic events and also attenuate the severity of post-ischemic damage (Liman and Endres, 2012). Indeed, through mediating the release of various growth factors and differentiating into mature endothelial cells, EPCs are thought to restore normal endothelial function and promote angiogenesis (Hur et al., 2004). Therefore

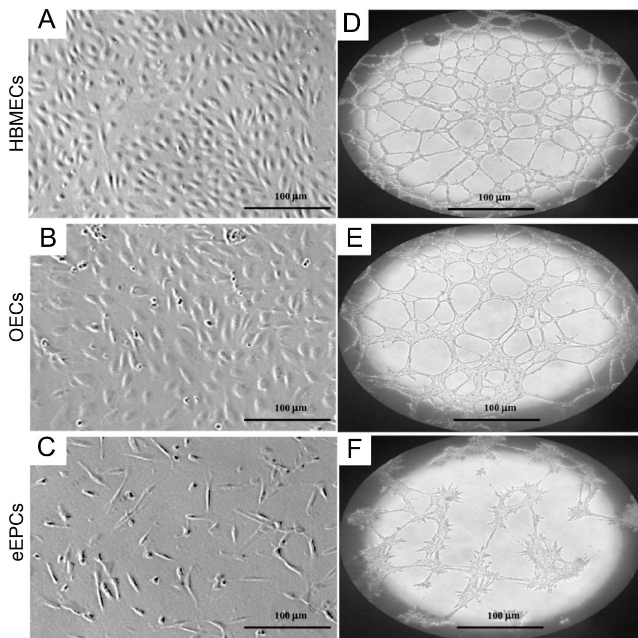

Figure 1 Morphological and tubulogenic features of HBMECs, OECs and eEPCs.
Both HBMECs (A and D) and OECs (B and E) display cobblestone morphology and form well-established tubules on matrigel. eEPCs, on the other hand, exhibit spindle-shape morphology and fail to form a coherent tubule network on matrigel (C and F). eEPCs: Early endothelial progenitor cells; HBMECs: human brain microvascular endothelial cells; OECs: outgrowth endothelial cells.

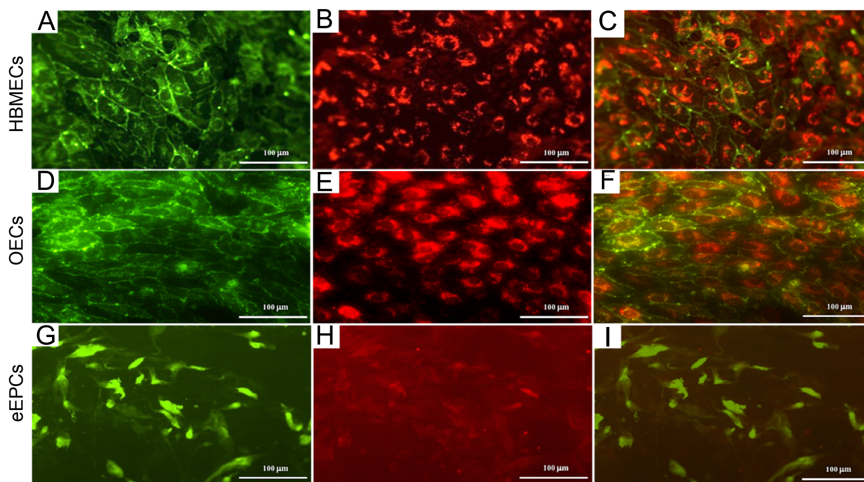

Figure 2 Characterisation of endothelial features of HBMECs, OECs and eEPCs.
Immunocytochemical analyses of HBMECs (A and B) and OECs (D and E) reveal that both cell types are stained positively with ulex europaeus agglutinin and can endocytose DiI-labelled acetylated-low density lipoprotein (DiI-AcLDL). eEPCs cannot be stained with ulex europaeus agglutinin and fail to take up DiI-AcLDL (G and H). The merged images showing the pattern of both staining in HBMECs, OECs and eEPCs (C, F and I). Scale bars: 100 μm. eEPCs: Early endothelial progenitor cells; HBMECs: human brain microvascular endothelial cells; OECs: outgrowth endothelial cells.

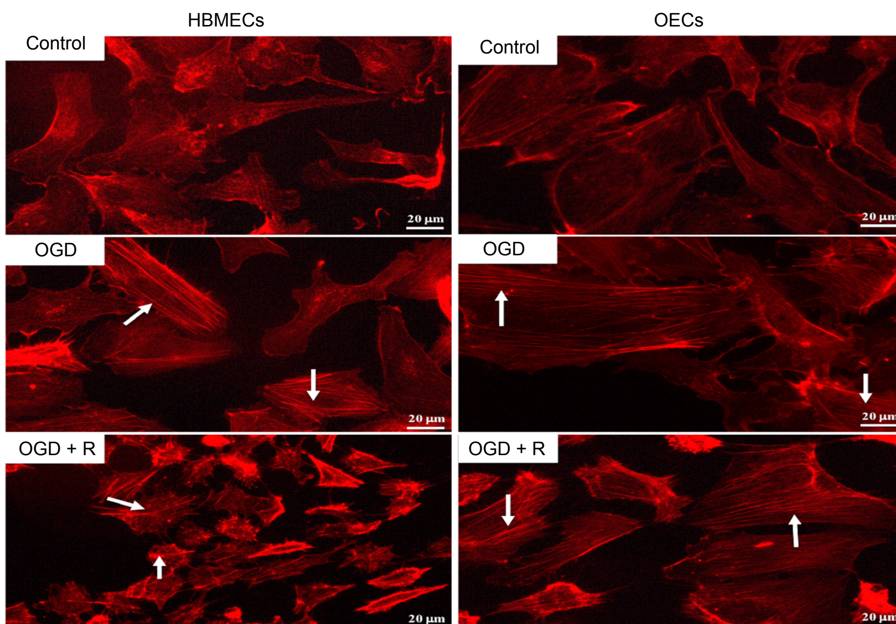

Figure 4 Actin microfilament staining in HBMECs and OECs.
Staining of actin filaments with rhodamine-labelled phalloidin lead to cortical and some cytosolic actin staining in HBMECs and OECs. Exposure of either cell line to oxygen-glucose deprivation alone (OGD, 4 hours) promote formation of thick actin stress fibres (indicated by white arrows). OGD followed by reperfusion (20 hours, OGD + R) diminish the intensity of actin stress fibres and return actin staining to plasma membrane. Scale bars: 20 μm. HBMECs: Human brain microvascular endothelial cells; OECs: outgrowth endothelial cells.

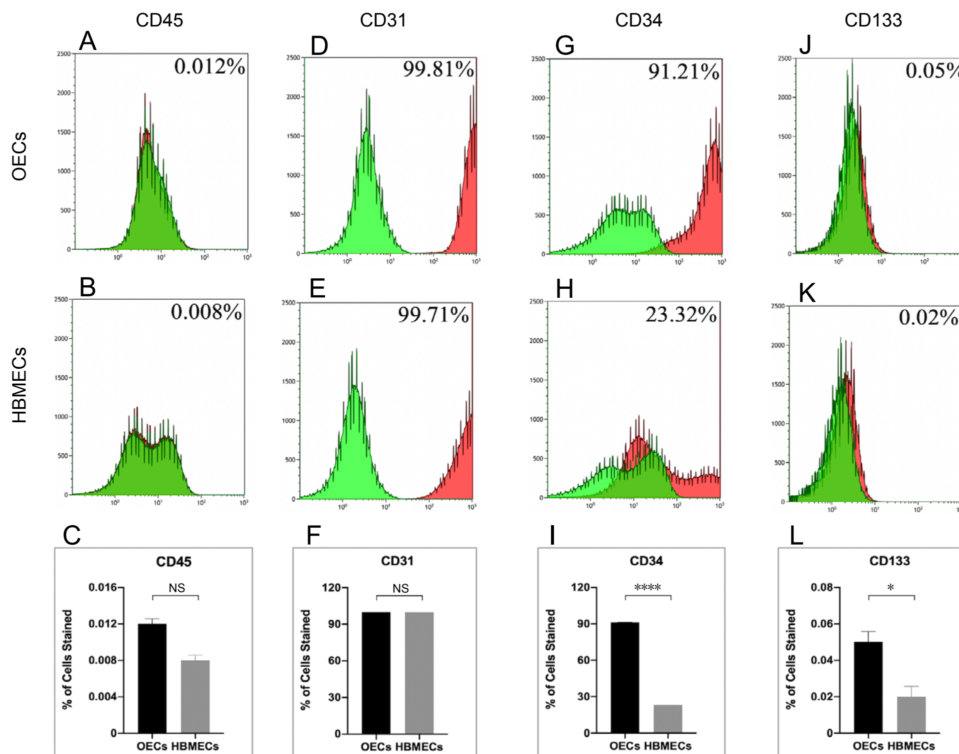

Figure 3 Cell surface immunophenotype of OECs and HBMECs by flow cytometry.
(A–C) Both OECs and HBMECs show no expression of haematopoietic cell marker, CD45 (0.012% and 0.008% respectively, P = 0.1000). (D–F) OECs (99.81%) and HBMECs (99.71%, P = 0.1000) express endothelial marker, CD31 in equal measures. (G–L) Level of the stemness marker CD34 is higher in OECs (91.18%) than in HBMECs (23.32%, P < 0.0001). This is also true for CD133 (0.05% and 0.02% for OECs and HBMECs, respectively, P = 0.0213). Data are expressed as percentage of the total number of cells and analyzed by Student's *t*-test. Not significant (NS) P > 0.05, *P < 0.05, ****P < 0.0001. All experiments were performed in triplicates. HBMECs: Human brain microvascular endothelial cells; OECs: outgrowth endothelial cells.

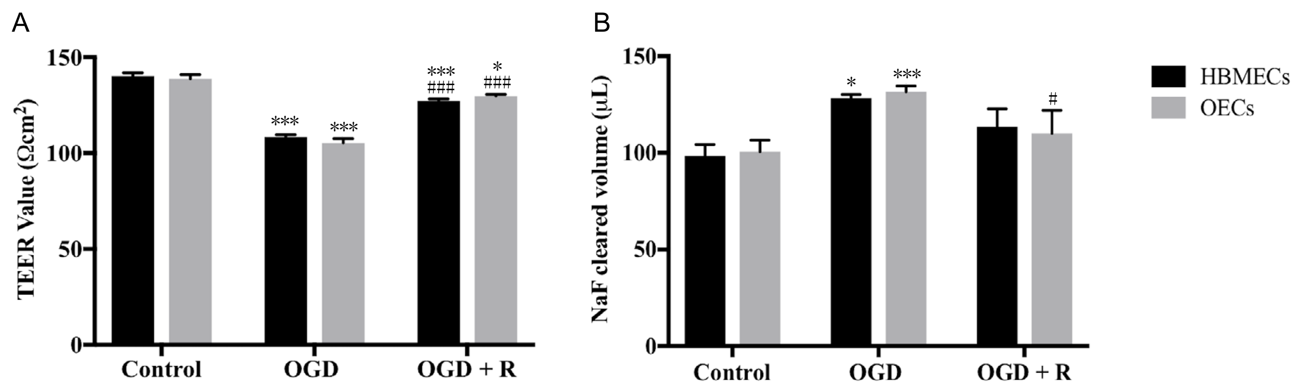

Figure 5 Assessment of the integrity and function of the BBB constructed with HBMECs or OECs.
Triple culture of HBMEC or OECs with human astrocytes and pericytes lead to generation of equally tight and functional BBB as evidenced by assessments of transendothelial electrical resistance (TEER, A) and paracellular flux of sodium fluorescein (NaF), a low molecular weight permeability marker (B). Exposure of both types of BBB models to oxygen-glucose deprivation alone (OGD) or followed by reperfusion (OGD + R) affect the integrity and function of both barriers in a similar fashion. *P < 0.05, ***P < 0.001, *vs.* control; #P < 0.05, ###P < 0.001, *vs.* OGD (one-way analysis of variance followed by Tukey's *post hoc* analysis). All experiments were performed in triplicate. BBB: Blood-brain barrier; HBMECs: human brain microvascular endothelial cells; OECs: outgrowth endothelial cells.

local and systemic interventions capable of enhancing EPC mobilisation and homing, e.g. by GM-CSF and SDF-1, may significantly elevate EPCs' regenerative capacity (Sprigg et al., 2006; Bogoslovsky et al., 2011; Cheng et al., 2017). Albeit somewhat effective, or at least harmless, in few preclinical and clinical stroke studies, these approaches require several days or weeks to increase circulating EPC numbers to the therapeutic levels that can positively affect disease outcome, implying that administration of EPCs themselves in sufficient numbers may be a better therapeutic strategy (Lee et al., 2005; Kawada et al., 2006; Sprigg et al., 2006). Studies investigating this very notion has generated inconsistent results, neurovasculo-protective versus neutral, and attributed this discrepancy largely to the heterogeneous nature of EPCs and the diversity of methods employed to isolate them. Indeed, flow cytometry constitutes one of the routinely used methods to detect EPCs in peripheral blood. It is based on the concomitant analysis and detection of several cell surface markers on individual cells in a mixture of cells. However, the marked similarities of markers expressed by EPCs and haematopoietic cells renders it challenging to detect EPCs by this method (Peichev et al., 2000; Fadini et al., 2006). Fluorescence-activated cell sorting represents another commonly employed methodology to isolate EPCs from bone marrow or blood to allow their application in clinical studies. However, mostly $CD34^+$ cells are sorted as EPCs by this technique (Sobrino et al., 2011).

Placement of blood mononuclear cells on specific substrates, e.g., fibrinogen, to promote their adhesion before culture in specific media supplemented with all the essen-

tial growth factors, hormones and foetal bovine serum is currently regarded as the best available method to generate copious amounts of homogenous EPCs (Reinisch et al., 2009). In accordance with previous findings, this approach produced two functionally and phenotypically different EPC subtypes, OECs and eEPCs in the current study (Hur et al., 2004). Comparison of functional characteristics that define endothelial cells in culture revealed that, similar to HBMECs, only OECs displayed cobblestone morphology, endocytosed DiI-AcLDL, bound to specific lectins and formed tubules on matrigel (Medina et al., 2010a). Collectively, these findings implied that OECs possessed most, if not all, the unique attributes required to effectively replace mature endothelial cells in vasculature to maintain overall vascular tone and functionality and also indicated that OECs could successfully induce angiogenesis when triggered by an appropriate stimulus. Determination of greater expression of specific markers for stemness (CD34) and immaturity (CD133) on OECs versus mature endothelial cells in the current study verified their inherent capacity to differentiate into few other cell types, possibly including those that make up neurovascular unit. Indeed, being a key element of neural stem cell niche, endothelial cells have previously been shown to promote endogenous neurogenesis (Shen et al., 2004; Ohab et al., 2006). Moreover, EPCs and various factors released by them are also implicated in post-stroke neurogenesis in that proliferation and migration of neuroblasts appear to play a pivotal role in recovery of nerve cell functions in the penumbra (Zhao et al., 2013). These alongside a stable phenotype and previously reported finite growth potential accompanied by a significantly reduced risk of tumourigenesis as well as amenability to genetic modification and ease of isolation from peripheral blood for therapy propose OECs as efficacious therapeutics (Medina et al., 2010b, 2013).

By employing a wound scratch assay, the present study has, for the first time, tested the therapeutic capacity of OECs on a well-established *in vitro* model of human BBB consisting of astrocytes, pericytes and HBMEC (Allen and Bayraktutan, 2009a; Shao and Bayraktutan, 2013). In this context, the initial wound scratch assays performed on HBMEC or OEC monolayers proved that both cell lines were capable of proliferation and directional migration where the rate of recovery was considerably faster with OECs. Subsequent experiments assessing the reparative function of OECs on the endothelial layer of the abovementioned BBB model revealed that treatments with OECs effectively repaired the integrity and function of BBB as evidenced by significant improvements in readings for TEER and paracellular flux of NaF, respectively. Omission of serum in culture media used for these experiments confirmed that the observed healing process could only be attributed to effective homing and differentiation of OECs rather than HBMEC growth. Similar experiments conducted in the presence of serum completely restored BBB integrity and function, indicating the additional contribution of OEC and possibly HBMEC proliferation to this process (data not shown). Again establishment of equally tight and functional *in vitro* models of human BBB with OECs and HBMECs, in the present study, further confirmed the capacity of OECs to differentiate into mature endothelial cells and form fully functional tight junctional complexes. Furthermore, mitigation of endothelial dysfunction by exogenous OECs through replacement of dead or dying endothelial cells may also be key in suppressing cerebral atherosclerotic disease and associated neurovascular structural or functional complications. In support of this hypothesis, attenuation of endothelial dysfunction by exogenously administered EPCs/OECs has been shown to reduce neointima formation, infarct volume and neurological deficits in translational models of acute ischemic stroke (Ohta et al, 2006; Liu et al, 2011). Contrary to these, other studies suggest that EPCs actually repair BBB damage in *in vivo* settings by activating resident endothelial cells through regulating secretion of various growth factors, notably VEGF and exosomes rather than turning into them (Rehman et al., 2003; Li et al., 2016). Regardless of the mechanism(s) involved, the data proving that OECs restore endothelial integrity and function in a timely and efficacious manner to prevent cerebral oedema remain to be of particular importance given that cerebral oedema continues to be the leading source of mortality within the first week of ischemic strokes (Qureshi et al., 2003; Dostovic et al, 2016).

Albeit remains to be confirmed by others, the presence of local hypoxia and apoptotic cells in ischemic zone are thought to enhance reparative and regenerative capacities of OECs through modulation of various growth factors and adhesion molecules, notably VEGF, vascular cell adhesion molecule-1, intercellular adhesion molecule-1 and E-selectin (Akita et al., 2003; Bhatwadekar et al., 2009; Di Santo et al., 2009). Although, the barrier-reparative function of OECs was not investigated in ischemic settings in the present study, exposure of BBB established with OECs or HBMECs to OGD similarly affected barrier characteristics. Despite significantly improving barrier integrity and function, reperfusion failed to normalise these parameters possibly due to exaggerated availability of reactive oxygen species during reoxygenation phase (Allen and Bayraktutan, 2009a, b).

After a certain number or replication in culture OECs become dysfunctional and start displaying the major signs of replicative senescence, notably telomere shortening, DNA damage, significant transcriptome changes and low telomerase activity (Medina et al., 2013). As senescence is the most reliable test for tumourigenicity in *in vitro* settings, it is plausible to assume that therapeutic application of OECs in clinical settings may not lead to any major undesirable side effects. Even so, determination of molecular mechanisms involved in OEC senescence is of pivotal importance to produce sufficient numbers of highly functional OECs *in vitro* and to regulate the length and level of activity *in vivo*. Amongst various components put forward, the inflammatory cytokines and chemokines, namely interleukin-8 (IL-8), IL-6, IL-1α, IL-1β, nitric oxide and homocysteine appear to be the most likely candidates involved in OEC senescence (Xu et al., 2000; Hayashi et al., 2008; Medina et al., 2013). However, their individual or collective relevance to OECs' therapeutic efficacy in stroke settings remain to be studied. Besides, issues pertaining to homing, survival, tracking, safety, time window and optimal dose of OECs also need to be addressed in stroke settings. It is likely that, as observed with mesenchymal stem cells, the quantity of OECs may determine their immunological status, immune-evading vs immune-privileged (Ankrum et al., 2014).

There are some limitations to this study. Firstly, scrutiny of OECs' barrier-forming and barrier-restorative roles in *in*

vitro settings mimicking ischemic injury by transient deprivation of oxygen and glucose would have perhaps provided clinically more relevant information about therapeutic potential of OECs. In this respect, further work with an animal model of ischemic stroke would better demonstrate whether OECs contribute significantly to vascular repair and attenuate neurological deficits.

In conclusion, this experimental study demonstrates that in *in vitro* settings, OECs can effectively repair BBB damage and restore its function thereby suggesting the idea that treatment with these oligopotent cells may be a viable therapeutic option to stem the loss of neurovascular cells after an ischemic stroke.

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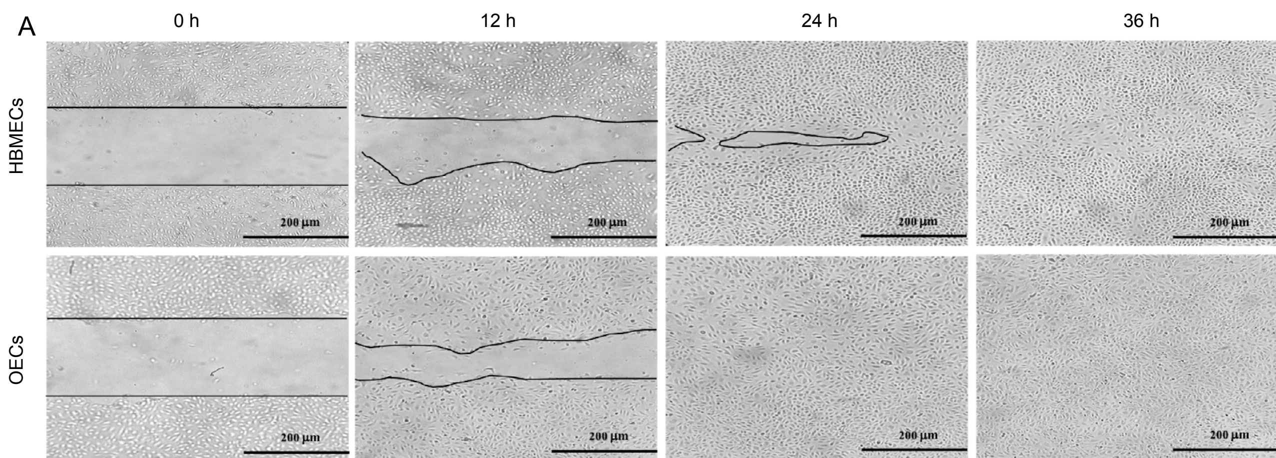

Figure 6 Wound scratch assays with HBMECs and OECs.
(A and B) Repair of wound induced on HBMEC and OEC monolayer shows that the speed of wound closure is significantly faster with OECs at different time points after scratch than these observed with HBMECs. Data are presented as mean ± SEM. *P < 0.05, ***P < 0.001 (one-way analysis of variance followed by Tukey's *post hoc* analysis). All experiments were performed in triplicate. HBMECs: Human brain microvascular endothelial cells; OECs: outgrowth endothelial cells.

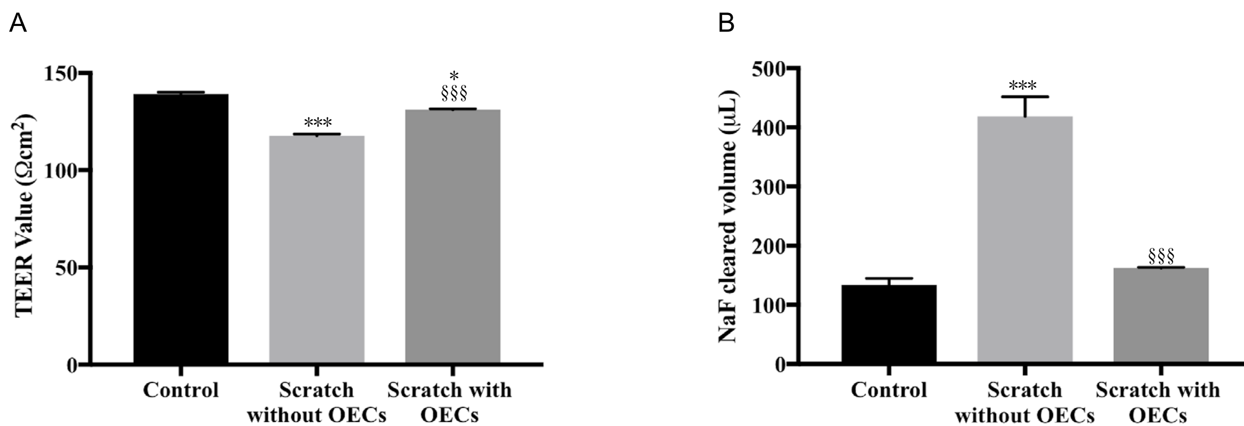

Figure 7 Analyses of the vasculoreparative function of OECs on an *in vitro* model of human BBB.
Exogenous OECs repair the wound scratch induced on endothelial layer of a triple culture model of human BBB established by astrocytes, pericytes and human brain microvascular endothelial cells and maintained in serum-free conditions as assessed by measurements of transendothelial electrical resistance (TEER, A) and paracellular flux of low molecular weight permeability marker sodium fluorescein (NaF, B). Data are presented as mean ± SEM. *P < 0.05, ***P < 0.001, *vs*. control; §§§P < 0.001, *vs*. scratch without OECs (one-way analysis of variance followed by Tukey's *post hoc* analysis). All experiments were performed in triplicate. BBB: Blood-brain barrier; HBMECs: human brain microvascular endothelial cells; OECs: outgrowth endothelial cells.

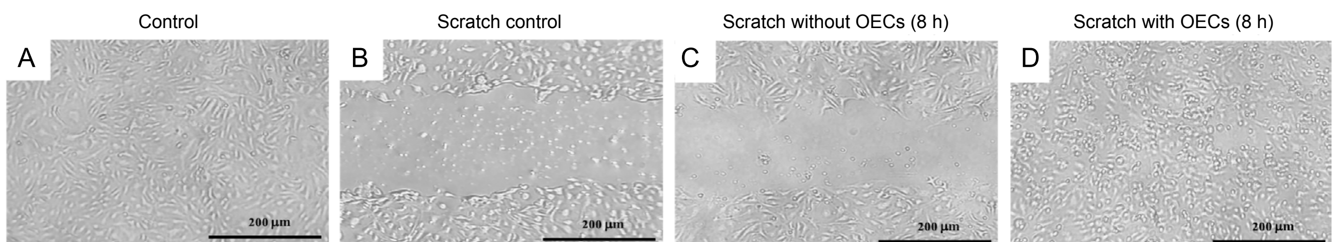

Figure 8 Analyses of endothelium restorative function of OECs with wound scratch assay.
(A–D) Exogenous OECs repair the wound scratch induced on human brain microvascular endothelial cells maintained in serum-free conditions. OECs: Outgrowth endothelial cells.

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