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EX VIVO EXPANSION OF HUMAN OUTGROWTH ENDOTHELIAL CELLS LEADS TO IL-8-MEDIATED REPLICATIVE SENESCENCE AND IMPAIRED VASOREPARATIVE FUNCTION

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ABSTRACT
Harnessing outgrowth endothelial cells (OECs) for vasoreparative therapy and tissue engineering requires efficient ex vivo expansion. How such expansion impacts on OEC function is largely unknown. In this study, we show that OECs become permanently cell-cycle arrested after ex vivo expansion, which is associated with enlarged cell size, β-galactosidase activity, DNA damage, tumor suppressor pathway activation, and significant transcriptome changes. These senescence hallmarks were coupled with low telomerase activity and telomere shortening, indicating replicative senescence. OEC senescence limited their regenerative potential by impairing vasoreparative properties in vitro and in vivo. Integrated transcriptome-proteome analysis identified inflammatory signaling pathways as major mechanistic components of the OEC senescence program. In particular, IL8 was an important facilitator of this senescence; depletion of IL8 in OECs significantly extended ex vivo lifespan, delayed replicative senescence, and enhanced function. While the ability to expand OEC numbers prior to autologous or allogeneic therapy remains a useful property, their replicative senescence and associated impairment of vasorepair needs to be considered. This study also suggests that modulation of the senescence-associated secretory phenotype could be used to optimize OEC therapy.

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INTRODUCTION
Harnessing the vasoreparative potential of endothelial progenitor cells (EPCs) has emerged as a viable therapeutic option for ischemic diseases such as myocardial infarction [1], peripheral arterial disease [2], and ischemic retinopathies [3]. In addition, generation of new blood vessels is a critical step in tissue engineering strategies, and EPCs are currently being considered for enhancing wound repair, osteogenesis, and development of 3D-vascularized liver organoids. The therapeutic potential of EPCs underpins the necessity to develop an efficient, clinically relevant EPC isolation and ex vivo expansion protocol for subsequent use in therapeutic angiogenesis and tissue engineering.

A frequently overlooked hurdle in adult stem cell therapies is the generation of sufficient cell numbers required for delivery into patients [4] and viability of an EPC-based cytotherapy relies on safe and efficient expansion of cell numbers ex vivo from tens to millions. Large-scale expansion of adult stem cells is often limited by the exhaustion of their replicative potential. Although many reports highlight the remarkable proliferative capacity of a distinct EPC subtype known as outgrowth endothelial cells (OECs) or endothelial colony forming cells (ECFCs) [5, 6], the implications for their long-term expansion remain largely unknown and detailed molecular and functional evaluation is required.

Cellular senescence has been reported to occur during ex vivo expansion of adult stem cells [7]. Senescence of mesenchymal stem cells and hematopoietic stem cells (HSCs) is associated with significant changes in their differentiation potential and gene expression profile [8, 9] which impact on their therapeutic efficacy. In this study, we have investigated the ex vivo growth dynamics and senescence program of this well-defined and characterized EPC subpopulation commonly known as OECs [10, 11] or ECFCs [5, 6]. We show that OECs can be successfully expanded ex vivo, although these cells ultimately undergo senescence upon exhaustion of their...
replicative potential. In addition, we demonstrate that in comparison to presenescent (early-passage) OECs, senescent OECs are dysfunctional, and by analysis of their transcriptome, proteome, and secretome, we have identified molecular players during OEC senescence. interleukin 8 (IL8) was recognized as a critical component and here we report that by knocking down expression of this cytokine in OECs, the establishment of cellular senescence was significantly delayed. Through better understanding of the molecular pathways that control OEC senescence, we may ultimately be able to overcome the major challenge of efficiently expanding cell numbers without diminishing functionality.

**MATERIALS AND METHODS**

**Cell Isolation and Culture**
This study was approved by the Office for Research Ethics Committees Northern Ireland. OECs were isolated from human peripheral (PB) and umbilical cord blood (CB) as previously described [11]. Human PB was obtained with full ethical approval from healthy, nonsmoking volunteers aged 22–35 years who were not receiving any medication. Umbilical CB was obtained with full ethical approval from full-term neonates. Operational definition for early-passage OECs in this study was cells that were in their exponential phase of the growth curve (between passage 4 and 6 for PB-derived OECs [PB-OECs] and between passage 8 and 12 for umbilical CB-derived OECs [CB-OECs]), while late-passage OECs were cells that have reached their Hayflick limit. The CASY cell counter-analyzer (Roche, Germany, http://www.roche-applied-science.com) was used for cell counts and cellular size assessment.

**Cytochemical Staining**
Senescence-associated β-galactosidase (SA-β-Gal), bromodeoxyuridine (BrdU) incorporation, and γH2AX immunochemistry assays were performed using standard protocols which are outlined in Supporting Information.

**Cell Cycle Analysis**
Detailed in Supporting Information.

**Telomere Length and Telomerase Activity Assays**
The telomere PNA kit/fluorescein isothiocyanate (FITC) for flow cytometry (DAKO, http://www.dako.com) was used to measure telomere lengths according to manufacturer’s protocol. For measurement of telomerase activity, the quantitative telomerase detection kit (US Biomax, http://www.biomax.us) was used in accordance with manufacturer’s guidelines.

**RNA Extraction and Microarray Analysis**
Total RNA was extracted using an RNAqueous kit (Ambion Life Technologies, http://www.ambion.com), and 1 µg of RNA from each cell sample was labeled and hybridized to an Illumina WG-6 v3.0 Expression Beadchip (Illumina, http://www.illumina.com). Samples analyzed in the array were three PB-OEC biological replicates of early and late passages. Gene expression data obtained from Illumina Beadstudio was normalized by using an “R” biocoductor with a “lumi” package. Data were processed and analyzed using various bioinformatics software packages detailed in Supporting Information. Microarray data are available at the ARRAYEXPRESS database with accession number E-MTAB-1388.

**Real-Time Reverse-Transcription Polymerase Chain Reaction**
Detailed in Supporting Information.

**Human Angiogenesis and Cytokine Protein Arrays**
OEC protein lysates and conditioned media were analyzed by using the Proteome Profiler human angiogenesis and cytokine arrays (R&D Systems, Minneapolis, MN, http://www.rndsystems.com) in accordance with manufacturer guidelines. Spots were detected by using chemiluminescence, and densitometry was quantified using ImageJ software.

**IL8 ELISA**
IL8 levels were assessed by ELISA using Human IL-8 ELISA MAX (Biolegend, http://www.biolegend.com) according to the manufacturer’s instructions.

**In Vitro Cell Functional Assays**
Tubulogenesis, migration, and clonogenic assays were performed following standard protocols detailed in Supporting Information.

**Murine Model of Retinal Ischemia**
C57BL/6 mice were purchased from Harlan Laboratories, UK. All experiments were performed in compliance with the Association for Research in Vision and Ophthalmology statement for the use of animals in Ophthalmic and Vision Research and UK Home Office regulations. Ischemic retinopathy was induced in C57BL/6 mice by using the previously described oxygen-induced retinopathy model (OIR) [11] and summarized in Supporting Information.

**IL8 Small Hairpin RNA (shRNA) Lentiviral Transduction**
IL8 gene expression knockdown was carried out using the Dharmacon SMARTVector 2.0 system (Thermo Fisher Scientific, http://www.thermofisher.com) targeting IL8, according to the manufacturer’s protocol. SMARTvector 2.0 Non-targeting Control Particles were used alongside the silencing constructs targeting IL8 for all downstream experiments.

**Protein Extraction and Western Blotting**
Detailed in Supporting Information.

**Statistical Analysis**
All data are expressed as mean ± SEM. Statistical significance of differences between groups was determined by using one-way ANOVA with the Student-Newman-Keuls post-test with Prism5 Graphpad software.

**RESULTS**

**OECs Have a Finite Replicative Lifespan In Vitro**
Human OECs were isolated following well-established protocols [5, 11]. OECs were characterized as highly proliferative cells that grow as a monolayer with cobblestone-like morphology (Fig. 1A), form adherens junctions through cadherin proteins (Fig. 1B), and express endothelial markers VEGR2 (Fig. 1C) and caveolin 1 (Fig. 1D). Furthermore, immunophenotypic analysis by flow cytometry (Fig. 1E) indicates this EPC subpopulation does not contain any hematopoietic cells (negative for CD45 and CD14), it is mainly composed of cells of endothelial lineage (≥99% positivity for CD31, CD105, and CD146), and also expresses the progenitor cell marker
CD34 (albeit with variation according to passage number). Human OECs have been previously characterized at the transcriptome, flow cytometric, and ultrastructural levels [10, 11]. To evaluate the growth kinetics and replicative potential of OECs, growth curves were generated following strict recording of cell numbers during serial passage of adherent cultures (Fig. 2A). As expected for progenitors, all OEC clones showed considerably enhanced replicative potential when compared to human aortic endothelial cells and human dermal microvascular endothelial cells (HDMECs), which were used as differentiated mature counterparts. CB-OECs exhibited greater proliferative capacity than PB-OECs with the former demonstrating, on average, 60 population doublings in 70 days, compared with PB-OECs which reached their Hayflick limit at 27 population doublings in 40 days. By contrast, mature endothelial cells displayed only 10 population doublings in 30 days.

Morphological assessment indicated a significant increase in cellular size during ex vivo expansion (Fig. 2B). Late-passage cells showed a fourfold enlargement of the cellular surface area (Supporting Information Fig. S1A) and an increase in cellular diameter occurred in unison with population doubling time (Fig. 2C). This physical enlargement of late-passage cells was even evident after monolayer trypsinization, with a significant increase in volume observed at the single cell level (p < .001) (Fig. 2D).

The percentage of cells retaining the ability to synthesize DNA and incorporate BrdU (Fig. 2E) decreased significantly from 13% to < 2% (p < .001) (Fig. 2F). In addition, DNA content analysis using flow cytometry indicated that most late-passage OECs were arrested in the G1 phase of the cell cycle (Fig. 2G). When compared to early-passage OECs, late-passage OECs showed a significant decrease in the percentage of cells at S and G2/M (p < .001) (Fig. 2H). Collectively, these data demonstrate that although OECs can be efficiently expanded in vitro, their replicative potential is limited. After long-term expansion of OECs, cell numbers reach a plateau exemplified by the Hayflick limit where most cells are growth-arrested in G1 and have acquired an enlarged flattened morphology.

**OEC Growth Arrest Induced by Continuous Culture Ex Vivo Is Replicative Senescence**

Since cellular growth arrest can be defined as quiescence or senescence, our next aim was to identify which process was contributing to OEC growth arrest ex vivo. Contrary to quiescence, senescence is an irreversible state and cells typically show a complete loss of proliferative potential. Despite appropriate growth stimuli, such as availability of nutrients and space, OEC proliferation eventually became completely halted (Fig. 1A), which was suggestive of a senescence process. To confirm this, we measured SA-β-Gal activity at pH6, which is currently the most commonly used biomarker for senescence. Late-passage OECs showed a marked increase in SA-β-Gal activity when compared to early-passage counterparts (Fig. 3A). Quantification indicated a significant increase in the percentage of cells that were positive for SA-β-Gal; 3% in early-passages increasing to 60% in late-passages (Fig. 3B). SA-β-Gal activity is associated with expansion of the lysosomal mass [12]; therefore a lysotracker probe was used. Consistent with SA-β-Gal data, there was a significant fourfold increase in the lysosomal mass per cell when comparing early- to late-passage OECs (Supporting Information Fig. S1B, S1C). DNA damage is a cardinal feature of cellular senescence, and the presence of the established DNA damage marker γ-H2AX was evaluated during the replicative lifespan of OECs (Fig. 3C). A significantly higher percentage of OECs positive for γ-H2AX was observed in the late-passage group when compared to early-passage cells (p < .001) (Fig. 3D).

Senescence is associated with major changes in gene expression therefore we performed transcriptome analysis. Total RNA was extracted from early- and late-passages for the same OEC clone, and three biological replicates were used for transcriptome analysis using the Illumina WG-6 v3.0 expression beadchips to assay more than 48,000 transcripts. Statistical assessment using National Institute on Aging array analysis distinguished a significant differential gene expression when comparing early- and late-passages (Fig. 3E). Overall, 782 transcripts were found to be significantly upregulated in early-passage OECs, while 654 transcripts were significantly upregulated in late-passage OECs. Differences in gene signatures were consistent among three different
biological replicates, as shown in heatmaps for the most altered genes (Fig. 3F). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to validate microarray results and showed comparable changes (Fig. 3G).

In order to gain an insight into the biological meaning behind this long list of genes, gene ontology analysis for biological processes was performed using the functional genomics tool FunNet. This highlighted that while gene ontologies for transcripts significantly upregulated in early-passage OECs were associated with cell cycle, mitosis, and DNA replication; gene...
Figure 3. OEC growth arrest induced by ex vivo expansion is a result of cellular senescence. (A): Representative images for early- and late-passage OECs stained for SA-β-Gal shown in blue. Scale bars = 100 μm. (B): Statistical analysis comparing percentages of SA-β-Gal positive cells in early-passage versus late-passage OECs (n = 4). (C): Representative immunofluorescence staining for DNA damage marker γ-H2AX in green. Nuclei are counterstained in red with PI. Scale bars = 100 μm. (D): Statistical analysis comparing percentages of γ-H2AX positive cells in early-passage versus late-passage OECs (n = 4). (E): Dot plot for genome-wide transcriptome analysis using National Institute on Aging array with false discovery rate (FDR) = 0.05 to compare early-passage with late-passage OECs. 782 transcripts were significantly upregulated in early-passage OECs (shown as red dots), while 654 transcripts were significantly upregulated in late-passage OECs (green dots). (F): Heat map for the top 30 upregulated and downregulated transcripts demonstrates a consistent gene expression change among paired biological replicates (n = 3). (G): Real time quantitative reverse transcriptase polymerase chain reaction for SOX18, HMGB2, CCNB2, IFI27, IL8, and CXCL6 serve to show consistent alterations between early and late-passage OECs and validate gene microarray results. (H): GO assessment using the functional genomics tool FunNET. (I): Molecular pathway network analysis using Ingenuity software for assessment of transcripts upregulated in late-passage OECs. (J): Western Blotting to evaluate expression of phospho-p53, p21, pRb, and cyclin A in early- and late-passage OECs. The error bars indicate SE; ***; p < .001. Abbreviations: EP, early passage; GO, gene ontology; IL, interleukin; LP, late passage; OEC, outgrowth endothelial cell; PI, propidium Iodide; SA-β-Gal, senescence-associated β galactosidase activity.
Statistical analysis to compare early-passage and senescent OECs between mean fluorescent intensities to arbitrary units (A.U.). (C): Quantification of telomere length by normalizing distances between telomere probes and control probes (gray filled histograms) with differences in mean fluorescence intensity histograms show differences in mean fluorescence intensities between telomere probes and control probes (gray filled histograms and white histograms, respectively). Note the larger distance between peaks in the early-passage OEC group compared to the late-passage group. Representative dot plots show two distinct cell populations which have been incubated with and without an fluorescein isothiocyanate (FITC)-conjugated peptide nucleic acid probe specific for telomere repeats. These are shown as gray and black dots, respectively. Note that overlap only exists in senescent cells. (B): Representative fluorescence intensity histograms show differences in mean fluorescence intensities between telomere probes and control probes (gray filled histograms and white histograms, respectively). Note the larger distance between peaks in the early-passage OEC group compared to the late-passage group. (D): Telomerase activity was assessed using the telomere repeat amplification protocol (TRAP) assay for pairs of OEC clones (n = 3). Note that there is a significant decrease in telomerase activity when comparing senescent OECs to their early-passage counterparts. The U937 cell line was used as a positive control for high telomerase activity, and HDMEC provided an appropriate control for low telomerase activity. The error bars indicate SEM.***, p < .001. Abbreviations: HDMEC, human dermal microvascular endothelial cells; OEC, outgrowth endothelial cell.

Thus, we examined whether replicative senescence impairs functional properties of OECs. First, we used a scratch wound assay to compare migratory capacities of early-passage and senescent OECs. Eight hours after creating the wound, early-passage OECs almost closed the gap while senescent OECs showed much reduced capacity (Fig. 5A). Quantification of wounded areas corroborated this qualitative data (p < .001) (Fig. 5B). Second, the Oris cell migration assay was used. In agreement with the scratch wound assay, we found impairment in the migratory capacity of senescent OECs (Fig. 5C), which was significantly lower than that of early-passage OECs (p < .001) (Fig. 5D). To test tubulogenic potential, a Matrigel-based 3D angiogenesis model was performed (Fig. 5E). After 72 hours, it was evident that in vitro tube forming capacity was significantly impaired in senescent OECs (p < .001) (Fig. 5F).

The impact of OEC senescence on their vasoreparative (therapeutic) potential was investigated in vivo using the OIR model. OIR provides a consistent and reliable preclinical system to test the therapeutic benefit of a cytotherapy by objectively assessing vascular repair in the ischemic retina. Mouse pups consistently developed vaso-obliteration of the central retina at P12 (Fig. 5G). At P13, mice were divided into three experimental groups (n = 6 animals per group) for the delivery of equal numbers of early-passage OECs, senescent OECs, or vehicle. Cells were prelabeled with fluorescent Qdots and delivered via intravitreal injection. Retinas were sampled at 72 hours and vascular areas were evaluated by Isolectin staining (Fig. 5G). Injection of both early- and late-passage OECs resulted in a significant reduction of the avascular area (p < .001). Although senescent OECs were still beneficial, they were less vasoreparative than their normal counterparts (Fig. 5H). Despite the observed reduction in retinal ischemia, it was apparent that most senescent OECs did not integrate into the resident vasculature as efficiently as the early-passage OECs. Further quantification of preretinal pathological neovascularization showed that only the early-passage OECs significantly decreased the amount of neovascular units (p < .001) (Fig. 5I). Quantification of neovascular areas confirmed that there was no difference among the groups treated with senescent OECs and vehicle (Fig. 5I). These findings indicate that replicative senescence not only affects the proliferative potential of OECs but also their migratory and tubulogenic capacities. Most importantly, when used as a cytotherapy for vascular repair, senescent OECs do not perform as well as their presenescent counterparts.

**Senescence Program of OECs Has a Major Inflammatory Component**

Key factors involved in OEC senescence were identified using transcriptomic analysis. Ingenuity Pathway software determined “Inflammatory Response” as the top BioFunction in senescent OECs. Alongside this, the resultant gene network established for transcripts upregulated in senescence included many inflammatory cytokines, such as IL8, IL1A, IL32, IL6ST, and CXCL1 (Supporting Information Fig. S4A). This was confirmed using REVIGO software which highlighted “Regulation of cytokine production” in the center of the semantic space for senescent OEC transcripts (Supporting Information Fig. S4B), suggesting an important role for inflammatory cytokines in modulation of senescence-upregulated genes. Ingenuity software also recognized STAT3, STAT2, and TP53 as transcription factors associated to senescent OECs upregulated genes. This was confirmed by Genomatix software, which assessed promoters of senescence-upregulated genes, indicating STAT1, STAT2, STAT3, TP53, and NFKB.

**Figure 4.** Telomere shortening as a hallmark of replicative senescence in OECs. (A): Telomere length was determined by flow cytometry using fluorescence in situ hybridization and a fluorescein-conjugated peptide nucleic acid probe. Representative dot plots show two distinct cell populations which have been incubated with and without an fluorescein isothiocyanate (FITC)-conjugated peptide nucleic acid probe specific for telomere repeats. These are shown as gray and black dots, respectively. Note that overlap only exists in senescent cells. (B): Representative fluorescence intensity histograms show differences in mean fluorescence intensities between telomere probes and control probes (gray filled histograms and white histograms, respectively). Note the larger distance between peaks in the early-passage OEC group compared to the late-passage group. (C): Quantification of telomere length by normalizing distances between telomere probes and control probes (gray filled histograms and white histograms, respectively). Note the larger distance between peaks in the early-passage OEC group compared to the late-passage group. (D): Telomerase activity was assessed using the telomere repeat amplification protocol (TRAP) assay for pairs of OEC clones (n = 3). Note that there is a significant decrease in telomerase activity when comparing senescent OECs to their early-passage counterparts. The U937 cell line was used as a positive control for high telomerase activity, and HDMEC provided an appropriate control for low telomerase activity. The error bars indicate SEM.***, p < .001. Abbreviations: HDMEC, human dermal microvascular endothelial cells; OEC, outgrowth endothelial cell.
as relevant transcription factors. All this in silico analysis of transcriptomic data, using a diverse range of bioinformatics software, indicated a strong link between OEC replicative senescence and inflammation-related pathways. Therefore, we next compared gene expression of inflammatory cytokines by qRT-PCR in early-passage versus senescent OECs. Although no difference was found in gene expression of the NFκB components rela and ikkb, there was a significant upregulation of IL8, IL1B, and IL1A (Supporting Information Fig. S5). To confirm these results at the protein level, an angiogenesisspecific proteome profiler was used. Proteins with increased expression in senescent OECs included IL8 and IL1B (Fig. 6A).

Recently, the senescence-associated secretory phenotype (SASP) [13] or senescence messaging secretome [14] has been included as a hallmark of cellular senescence. This information prompted the characterization of the secretome in senescent OECs. A cytokine proteome profiler was used to identify and quantify proteins present in the conditioned media of early-passage and senescent OECs. The most abundant protein in the senescent OEC secretome was IL8, which was expressed to a similar level as positive controls (Fig. 6B). Densitometric analysis confirmed a significant fivefold increase in IL8 when conditioned media from senescent OECs was compared to conditioned media of early-passage OECs (p < .001) (Fig. 6C). Other proteins shown to increase in the secretome during senescence were CXCL1, sICAM-1, and IL6; proteins that were present at similar levels were MIF and CCL2; while PAI-1 expression decreased in senescent secretomes (Supporting Information Fig. S6). Quantitative ELISA corroborated a significant increase in the IL8 levels secreted per cell when comparing early-passage to senescent OECs (p < .001) (Fig. 6D). Limulus amebocyte lysate (LAL) endotoxin testing confirmed that cell culture conditions at both early and late-passages were free of bacterial endotoxins or lipopolysaccharide (LPS) (Supporting Information Fig. S7).

These results at transcriptome, proteome, and secretome levels indicate that there is a clear association between inflammatory cytokines and OEC replicative senescence. This suggests a possible role for some of these cytokines in the induction, establishment, or reinforcement of OEC senescence.

**IL8 Depletion Delays Replicative Senescence in Cultured OECs**

A network of inflammatory cytokines was found to be significantly upregulated in senescent OECs and it was therefore important to establish if these might play a role in the senescence program of these cells. Our investigations focused on IL8 since it was consistently among the most differentially expressed mRNA and the most highly expressed cytokine in the secretome of senescent OECs. The SMARTvector 2.0 lentiviral shRNA technology was used to knockdown IL8 expression in OECs with approximately 93% transduction efficiency after Puromycin selection (Supporting Information Fig. S8A, S8B). qRT-PCR confirmed a significant decrease in IL8 mRNA when shRNA-IL8 transduced cells were compared to

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**Figure 5.** Replicative cellular senescence limits OEC regenerative potential by impairing vasoreparative properties. (A): Representative phase-contrast micrographs for the scratch wound assay on early-passage and senescent OECs. Wound edge delineated with yellow dots. (B): Quantification of migrated areas using NIS elements software (n = 10). Note significant impaired migration in senescent OECs. (C): Oris Cell Migration Assay with OECs prestained using the vital dye calcein red. The central area where cells migrated is outlined in yellow. (D): Quantitative analysis of migrated areas (n = 12). Note significantly smaller migrated areas in the senescent OEC group. (E): The tube-forming capacity of OECs was assessed using the 3D-angiogenesis model. Representative images of OECs embedded in Matrigel 7 days after starting the assay. Calcein staining in red. (F): Quantification of tube length per field of vision (n = 4) shows significantly decreased tube forming capacity in senescent OECs. (G): The oxygen induced retinopathy model was used to determine the therapeutic potential of OEC cytoterapy. Murine retinal flat mounts were stained using isoelectin (green) to identify retinal vasculature and multiple images per retina with magnification x10 were taken and stitched together using NIS elements software. The central avascular area is shown in black and delineated with a yellow line. Images at postnatal day 12 (P12) shows the ischemic state of retinas at the point of OEC delivery by intravitreal injection. All other images were taken 72 hours after cell or vehicle delivery. (H): Quantification of the avascular area of retinas 72 hours after intravitreal injection with either vehicle: EP-OECs, or LP-OECs. Note that OECs significantly decrease avascular areas but with less efficiency than early-passage counterparts. (I): Quantification of the pathological pre-retinal neovascularization 72 hours after intravitreal injection of OECs (n = 6). In contrast to early-passage OECs, note that senescent cells fail to significantly decrease pathological neovascularization. The error bars indicate SEM. *, p < .05; **, p < .01; ns, not significant. Scale bars (A, C, E): 250 μm. Abbreviations: EP, early passage; LP, late passage; OEC, outgrowth endothelial cell.
growth curve (Fig. 7C). Clonogenic assays corroborated results from growth curves, larger sized and greater numbers of single cell-derived colonies occurred in the shRNA-IL8 group (p < .001) (Fig. 7D). Analysis of SA-β-Gal revealed a significant decrease in the number of OECs positive for this senescence biomarker when IL8 was depleted (p < .001) (Fig. 7E). This is consistent with a marked decrease in DNA damage in IL8-depleted OECs as shown by immunocytochemistry for 53BP1 nuclear foci (Supporting Information Fig. S10).

To assess the impact of IL8 on senescence-related loss of functionality, Matrigel-based tubulogenesis assays were used. shRNA-mediated depletion of IL8 restored tube forming potential as indicated by the significant increase in the number of branching points when shRNA-IL8 OECs were compared to shRNA-control OECs at the same passage (p < .001) (Fig. 7F). In agreement with this, the migratory capacity of late-passage OECs was significantly enhanced (p < .001) when IL8 was depleted (Fig. 7G). These results provide evidence of a role for IL8 during OEC replicative senescence since attenuating expression of this cytokine not only delayed senescence but also increased proliferation rate, extended in vitro lifespan, and enhanced functionality.

We also investigated the functional role of shRNA-IL8 OECs in an in vivo model of ischemic retinopathy. Interestingly, these transplanted, virally modified OECs induced a marked inflammatory cell infiltration response when compared to vehicle-injected controls in the contra-lateral retina (Supporting Information Fig. S11A). Myeloid cells accumulated preferentially around endothelial tubes formed by shRNA-IL8 OECs (Supporting Information Fig. S11B). This finding underscores the importance of establishing the immunogenic potential of genetically modified OECs before their usage in cell therapies and is currently being investigated by our group.

As IL8 has been previously described as a mediator of angiogenesis, its effects on the OEC angiogenic program were dissected and studied at three distinct cellular passages prior to the onset of senescence. OEC morphology varied from 1 to 100 ng/mL and proliferative potential decreased with cellular passage (Supporting Information Fig. S12A). However, IL8 did not increase colony-forming capacity in any group but significantly decreased proliferative potential when early-passage OECs were exposed to higher IL8 concentrations (p < .05) (Supporting Information Fig. S12B). Results from OEC migration studies agreed with OEC colony formation data. Late passage OEC migration rate was lower than early passage counterparts (Supporting Information Fig. S12C), and there was only a significant decrease in migration areas when early-passage OECs were exposed to high IL8 doses (p < .01) (Supporting Information Fig. S12D). In other passages, there was no significant effect when OECs were exposed to IL8. Assessment of OEC tube formation consistently indicated a clear decrease in tube forming capacity with cellular passage (Supporting Information Fig. S12E). Interestingly, while late-passage OECs do not respond to IL8 (Supporting Information Fig. S12F), we found a significant increase in tube length when early- and mid-passage OECs were exposed to lower doses of IL8 (p < .001). Exposure of OECs to exogenous recombinant human IL8 for 5 days did not affect their intrinsic senescence program (Supporting Information Fig. S12G, S12H). These findings indicate that a proangiogenic effect for IL8 is only found in early and mid-passage OECs and with low IL8 concentrations (1 ng/mL) as demonstrated by the tube formation assay. Late-passage OECs are clearly nonresponsive to IL8 pro-angiogenic effects.
DISCUSSION

In this study, we provide evidence demonstrating the markedly enhanced replicative potential of OECs in comparison to mature endothelial cells. This characteristic favors the isolation of OECs and their possible use for cell therapy, since large numbers of cells are typically required and are essential for autologous/ allogeneic stragtesms. A porcine model of acute myocardial infarction recently showed that efficient neovascularization of an individual ischemic heart required a minimum delivery of 30 million OECs [15], which is equivalent to approximately ten 75 cm² confluent cell culture flasks. If we consider that a homogeneous OEC population is generally established in vitro after passage 3 with approximately 100,000 cells, then a minimum of eight population doublings is required to amplify cell numbers for therapeutic use. Our data shows that this is feasible for both CB- and PB-derived OECs, as shown by their respective growth curves. However, our observations also indicate that OECs expanded ex vivo ultimately undergo senescence upon exhaustion of their replicative potential. Critically, the current investigation has shown that the molecular fingerprint for senescence is coupled to a dysfunctional phenotype which underlines the importance of evaluating the senescence program when expanding progenitor cells for clinical use.

The evidence demonstrating that OECs undergo replicative senescence during expansion in culture underscores their safety when applied to human cytotherapy. Very recently, it has been noted that senescence in culture is the most reliable test for tumorigenicity [16], because assays using nude mice have unacceptably low sensitivity.

Although cytotherapy for vascular repair during ischemia using patients’ own blood/bone marrow has been shown to be attainable [17], this autologous strategy faces significant limitations: (a) considering that OEC isolation and expansion takes at least 6 weeks, there is a narrow window of opportunity for treatment of patients with acute ischemic conditions; (b) patient’s clinical background and age can alter OEC functionality and numbers, for example, it has been reported that

Figure 7. shRNA lentiviral transduction-mediated IL8 depletion delayed outgrowth endothelial cell (OEC) senescence. (A): The human cytokine proteome array was used to evaluate IL8 expression by assessing conditioned media of OECs transduced with either shRNA-control or shRNA-IL8 lentiviral constructs. Quantification of spots by densitometry (n = 4) shows efficient IL8 knockdown in OECs transduced with IL8-shRNA. (B): ELISA quantification of IL8 levels in conditioned media of shRNA-control and shRNA-IL8 transduced OECs, compared to same passage nontransduced OECs (n = 3). (C): Growth curves for shRNA-control and shRNA-IL8 OECs shown in blue and red, respectively. (D): Clonogenic assay comparing colony formation potential of shRNA-control and shRNA-IL8 OECs (n = 6). Bottom panel shows representative image of cells stained with crystal violet to identify and count colonies. (E): SA-β-Gal assay 1 month after lentiviral transduction. Representative images showing higher expression (blue) in shRNA-control OECs. Scale bars = 100 μm. Quantification demonstrates significantly fewer cells positive for SA-β-Gal in the shRNA-IL8 group (n = 8). (F): Representative images of tube-like structures formed in the Matrigel 3D angiogenesis model. Cells stained in green with vital dye calcein. Quantification of number of branch points formed per field of vision (n = 12). Scale bars = 250 μm. (G): Quantitative analysis of migrated areas using the scratch wound assay shows impaired migration potential in shRNA-control OECs (blue) when compared to shRNA-IL8 OECs (red) (n = 12). The error bars indicate SEM. ***, p < .001; ns, not significant. Abbreviations: IL, interleukin; SA-β-gal, SA-senescence-associated β galactosidase; shRNA, small hairpin RNA.
involve a dynamic process whereby cells remain metabolically active and increase inflammatory cytokine production and secretion. This phenomenon has implications for the therapeutic use of OECs because these cytokines can alter tissue microenvironment conditions and affect cell engraftment, survival, and ultimately clinical outcomes. In keeping with our findings, it has been suggested that IL8 secreted by senescent cells fosters cancer progression by promoting both angiogenesis and tumor growth [27].

Among the novel findings of this study is the identification of IL8 as a major positive modulator of OEC senescence. We have shown that depletion of IL8 by shRNA in OECs leads to a significant delay in senescence, an increase in proliferation rates, and restored vasoreparative function. These data are in agreement with previous results demonstrating that knocking down the chemokine receptor CXCR2 (IL8RB) alleviates replicative and oncogene-induced senescence in fibroblasts by diminishing the DNA damage response [28]. Furthermore, a correlation between increased IL8 expression and P16INK4A immunopositivity has been reported in growth-arrested cells in human colorectal adenomas [25]. These findings alongside ours indicate an unexpected role for secreted inflammatory cytokines as autocrine regulators of senescence. Although a mechanistic model has been suggested where senescence-associated DNA damage triggers secretion of inflammatory cytokines which operate as positive-feedback loops by enhancing genes related to inflammation and cell cycle arrest [29], further studies are warranted to dissect respective molecular mechanisms. It is important to note that IL8 depletion in OECs did not fully bypass cellular senescence and this still occurred in IL8-knocked-down OECs, albeit at a significantly delayed time point. This suggests an enhancing and supporting role for IL8 in the senescence process, while indicating that p53 and pRB remain the critical components. Nevertheless, our results further advocate a prominent and clinically relevant role for regulation of IL8 and related signaling in OEC senescence, which could be a reasonable target to delay OEC senescence, without inducing uncontrolled cellular growth as has been described for other strategies targeting telomere expression or maintenance.

Identification of IL8 as one of the main components in the OEC secretome is in agreement with other reports [30-33]. It has been shown that OECs secrete significantly more IL8 than human umbilical vein endothelial cells (HUVECs) or coronary artery endothelial cells [30]. Furthermore, IL8 secretion in OECs can be modulated by PAR-1 activation [32]. In this study, we demonstrate a significant increase of IL8 expression, production, and secretion that relates to cellular passage and the ex vivo expansion process of OECs. Furthermore, as IL8 levels increase during OEC expansion and relate to senescence establishment, this cytokine could potentially be used as a reliable ageing biomarker for OECs in vitro. As OEC cell number expansion is associated with impairment in cellular function, there is a clear need for senescence and functional screenings when OECs are expanded ex vivo. Therefore, it is also clinically relevant to highlight that our data shows evidence that IL8 levels in OEC conditioned media are a consistent and reliable indicator of their passage history and relate to functionality and senescence. While early passage OECs have IL8 levels < 50pg/ml, senescent OECs have levels ranging from 150 to 1,200 pg/mL. These concentrations are very similar to the clinical parameters described for IL8 levels in human plasma when used as a biomarker for inflammation/infection [34].

Although we have evidence to indicate that IL8 acts as a provascular repair factor in the ischemic retina [35], the direct effects of IL8 on endothelial cells or OECs have not
In conclusion, this work reveals that ex vivo expansion of human OECs ultimately leads to replicative senescence linked to cellular dysfunction. An inflammatory cytokine component was unequivocally identified as part of the OEC senescence program. Silencing IL8 expression in OECs resulted in delay of senescence and extension of OECs ex vivo lifespan which could have benefits in the context of vasoparapressive or tissue engineering cell therapy.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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