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Human umbilical cord perivascular cells: A novel source of the organophosphate antidote butyrylcholinesterase

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ABSTRACT

Human butyrylcholinesterase (BChE) is a well-characterized bioscavenger with significant potential as a prophylactic or post-exposure treatment for organophosphate poisoning. Despite substantial efforts, BChE has proven technically challenging to produce in recombinant systems. Recombinant BChE tends to be insufficiently or incorrectly glycosylated, and consequently exhibits a truncated half-life, compromised activity, or is immunogenic. Thus, expired human plasma remains the only reliable source of the benchmark BChE tetramer, but production is costly and time intensive and presents possible blood-borne disease hazards. Here we report a human BChE production platform that produces functionally active, tetrameric BChE enzyme, without the addition of external factors such as polyproline peptides or chemical or gene modification required by other systems. Human umbilical cord perivascular cells (HUCPVCs) are a rich population of mesenchymal stromal cells (MSCs) derived from Wharton's jelly. We show that HUCPVCs naturally and stably secrete BChE during culture in xeno- and serum-free media, and can be gene-modified to increase BChE output. However, BChE secretion from HUCPVCs is limited by innate feedback mechanisms that can be interrupted by addition of miR 186 oligonucleotide mimics or by competitive inhibition of muscarinic cholinergic signalling receptors by addition of atropine. By contrast, adult bone marrow-derived mesenchymal stromal cells neither secrete measurable levels of BChE naturally, nor after gene modification. Further work is required to fully characterize and disable the intrinsic ceiling of HUCPVC-mediated BChE secretion to achieve commercially relevant enzyme output. However, HUCPVCs present a unique opportunity to produce both native and strategically engineered recombinant BChE enzyme in a human platform with the innate capacity to secrete the benchmark human plasma form.

1. Introduction

Butyrylcholinesterase (BChE) is a naturally occurring enzyme that circulates at low levels in human blood [1,2]. It is a non-essential gene, since *BCHE* mutant animals develop normally [3–5], and humans that lack BChE activity are generally healthy [6]. However, human BChE has some overlapping functions with the widely-expressed acetylcholinesterase (AChE). BChE can compensate for loss of AChE in *ACHE* knock-out animals, since it hydrolyzes acetylcholine [7,8] as well as several other substrates [9,10]. *BCHE* is also expressed during embryonic development, where it contributes to neural crest formation and neurogenesis [11–14].

BChE has been identified as a stoichiometric bioscavenger with significant potential as an antidote to organophosphates (OP) and other chemical agents, including pesticides, cocaine, and the nerve agents VX, soman (GD) and sarin (GB) (reviewed in Refs. [15–17]). When administered at sufficiently high doses, naturally occurring BChE purified from expired human plasma can effectively neutralize the effects of OP [18–22]. Significant effort has been devoted to developing recombinant platforms to synthesize BChE cost-effectively and on a large scale. However, human BChE has proven difficult to reproduce in recombinant systems. Monomeric BChE complexes to form dimers and tetramers [23–26], and further is subjected to extensive stereotypical post-translational glycosylation which is not coded for in the *BCHE* gene [26–28]. The benchmark BChE isoform for therapeutic use, exhibiting the longest half-life and optimal bioscavenging activity, is the

Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; BM, bone marrow; CM, conditioned media; HUCPVC, human umbilical cord perivascular cell; MSC, mesenchymal stromal cell; MSCGM-CD, Mesenchymal Stem Cell Growth Media-Chemically Defined; pAd5-*BCHE*, recombinant pAd5 adenovirus encoding human *BCHE*

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optimally glycosylated tetrameric BChE complex [26–30]. A variety of eukaryotic recombinant platforms tested to date have lacked the innate capacity to produce the properly glycosylated tetrameric complex, resulting in a BChE product with a truncated half-life and sub-optimal bioscavenging functionality [31–40]. Bacterial platforms have proven even more challenging, since the recombinant BChE becomes sequestered in inclusion bodies [41,42].

We are developing a human population of mesenchymal stromal cells (MSCs) as a platform for production of soluble therapeutic molecules. These human umbilical cord perivascular cells (HUCPVCs) are isolated from the Wharton's Jelly of umbilical cords [43,44], which are normally discarded after birth as biomedical waste. HUCPVCs exhibit numerous properties useful for clinical use. Not only are they sourced non-invasively from a plentiful and renewable source, but they are isolated at higher yields than MSCs from bone marrow, and are readily expanded to vast numbers [43–46]. MSCs also express very low levels of the major-histocompatibility class I (MHC I) receptor, and lack MHC II [45–47]; thus, they are considered immune-evasive and are being developed as allogeneic cell therapies for transplant between individuals [48–51].

The immune-evasive status of HUCPVCs [45–47] can be strategically exploited to deliver transgenes that code for therapeutically useful molecules against biological and chemical threats. In this paradigm, the cell vectors shield the exogenous genetic payload from targeted immune degradation and serve as living factories that continually synthesize and secrete the therapeutics into circulation. In our proof-of-concept study, gene-modified HUCPVCs maintained secretion of an anti-viral antibody for over 3 months after a single intramuscular (IM) administration to athymic mice [52]. Within 24 h of IM implant, the cells generated circulating antibody titers more than 4 times higher than achieved by direct injection of the purified antibody. Further, a single IM dose of these modified HUCPVCs better protected mice against lethal exposure to the viral threat Venezuelan equine encephalitis than mice traditionally treated with the purified antibody [52]. We subsequently showed that the IM delivery route is imperative to achieve longevity of the implanted MSCs [53], resulting in sustained secretion of the antibody [52]. The success of the pilot study, in which gene-modified human cells, delivered IM, rapidly produced and maintained high circulating levels of a therapeutic product, led us to investigate whether HUCPVCs might provide an alternate modality for BChE production that resolves the limited availability of human-sourced enzyme, the inappropriate glycosylation associated with non-human recombinant platforms, and the multi-dosing regimen necessary to maintain therapeutic levels of this stoichiometric bioscavenger.

Here we report that HUCPVCs naturally secrete tetrameric, glycosylated BChE in routine culture using chemically defined xeno- and serum-free media. Moreover, BChE secretion can be further increased by modifying HUCPVCs with an episomal transgene encoding human BChE. The data also reveal that HUCPVCs have an innate regulatory mechanism to limit the amount of BChE in the extra-cellular environment, which must be identified and disabled to fully maximize BChE expression. By contrast, MSCs derived from human bone marrow (BM) neither express the endogenous *BCHE* gene nor synthesize BChE at comparable levels to HUCPVCs after gene modification.

2. Materials and methods

2.1. Cell culture

HUCPVCs cryopreserved at passage (P) 2 were provided by Tissue Regeneration Therapeutics (TRT), Inc. (Toronto, Canada). HUCPVCs were thawed according to TRT's proprietary standard operating procedures and expanded in Mesenchymal Stem Cell Growth Media – Chemically Defined (MSCGM-CD; Lonza, Basel, Switzerland). Culture vessels were pre-coated with 0.75 μg/cm^2 fibronectin (Corning, Corning, NY). P1 human bone marrow-derived MSCs (BM MSCs) were also provided by TRT. BM MSCs were recovered in isolation media - Alpha-MEM (ThermoFisher Scientific, Waltham, MA) supplemented with 10% MSC-FBS (ThermoFisher Scientific) – then weaned to MSCGM-CD to facilitate direct comparison to HUCPVCs. At 70–80% confluence, MSCs were enzymatically detached from the culture vessel by brief incubation with TrypLE Select (Life Technologies (ThermoFisher Scientific)), and re-seeded at a density of 1,333 cells per cm^2. Culture conditions were maintained at 37 °C, 5% CO_2, 80% relative humidity, with media replacement every 3–4 days. MSCs were used between P3-P5 in the reported experiments.

2.2. Recombinant adenovirus transduction

MSCs were gene modified by transient transduction using a recombinant pAd5 adenovirus encoding human *BCHE* (pAd5-*BCHE*) under control of the constitutively active cytomegalovirus (CMV) promoter. A recombinant adenoviral vector bearing the sequence for Accession BC018141 was purchased from Vector BioLabs (Malvern, PA). A second adenoviral construct encoding Accession NM_000055, which is longer than BC018141 by 235 5′ nucleotides and 53 3' nucleotides, was procured from Applied Biological Materials Inc. (ABM, Vancouver, BC). Both constructs generate a *BCHE* open reading frame of 1809 base pairs. Experiments were reproduced with both constructs and generated equivalent results; only data obtained using pAd5-*BC018141* are reported here.

For adenoviral-mediated gene modification, MSCs were seeded at a density of 10,526 cells per cm^2 in 6-well plates or 75 cm^2 flasks and allowed to adhere overnight, generating 70–80% confluent monolayers. The next morning, cells from a representative well or flask were lifted and counted using a Millipore Scepter™ automated cell counter equipped with a 60 μM probe (Millipore Sigma, Burlington, MA). The desired multiplicity of infection (MOI) was calculated using the adenoviral stock concentration (particle forming units/ml) and cell count. Adenovirus was diluted in MSCGM-CD without antibiotics, and a minimal volume (0.0263–0.0316 ml/cm^2) of viral suspension was applied to the cells. Control wells were treated in parallel using media without adenovirus. MSCs were co-incubated with recombinant adenovirus for 3 h at 37C, 5% CO_2 with rocking every 10 min to prevent drying. After 3 h, the transduction media was removed and replaced with complete MSCGM-CD.

2.3. Conditioned media collection and storage

To assess BChE secretion from native HUCPVCs and BM MSCs, cells were cultured normally in T75 cm^2 culture flasks (Corning) until they reached approximately 60% confluence. Media was aspirated and replaced with half the normal volume of serum-free MSCGM-CD (4 ml instead of 8 ml) for collection of conditioned media (CM). After 24 h, cell density was near 80%. CM was collected and concentrated to approximately 100 μl in a 10 kDa molecular weight cut-off Spin-X^R 500 UF concentrator (Corning) at 15,000 × *g*. An equal volume of glycerol was added to the concentrate, and aliquots were stored at −80 °C for analysis.

CM from BChE-modified MSCs was generated in a 6-well plate format as per section 2.2, using 1 ml of MSCGM-CD per well. CM was collected every 24 h and replaced with fresh media for up to two weeks after gene modification. CM was aliquoted and stored at −80 °C for analysis.

2.4. Co-incubation with poly-L-proline

Poly-L-Proline, MW 1000–10000 (Millipore-Sigma; Cat. No. P2254), was dissolved to 1 mg/ml in deionized water then filter sterilized. This formulation contains polyproline peptides approximately 50 amino acids in length (P50) [54]. Transduction experiments were set up in a 6-well plate format as described in Section 2.2. Twenty-four hours after

modification, media was replaced with complete media supplemented with 1000 μM P50. Media was replaced every 24 h for 8 days, and BChE activity quantified as described in Section 2.6.

2.5. Native gel electrophoresis

CM samples were thawed from −80 °C storage and the total protein concentration determined using a Bio-Rad DC™ Protein Assay (Bio-Rad, Hercules, CA). 110 μg of protein from concentrated samples or 30 μg of protein from unconcentrated samples was diluted in Native Sample Buffer (Bio-Rad) according to manufacturer's instructions and loaded onto a 7.5% native Mini-PROTEAN® TGX™ gel (Bio-Rad). 13.33 μl of the human serum-derived BChE standard (Abcam, Toronto, ON) diluted in Native Sample Buffer was included as a control. Electrophoresis was performed using 1X Tris/Glycine buffer (25 mM Tris, 192 mM glycine, pH 8.3, Bio-Rad) for 35 min with a power source (Bio-Rad) set to 200 V at room temperature. After electrophoresis was complete, the apparatus was disassembled and the gels were rinsed in deionized water for 2 min.

2.5.1. Detection of functional BChE protein bands in native gels

The molecular forms of enzymatically active BChE protein were visualized in non-denaturing gels using a colorimetric detection method based on the method of Karnovsky and Roots [55]. A limitation of this approach is that band intensity does not report relative amounts of different multimeric BChE complexes, since the activity of BChE does not increase linearly with the number of BChE units per complex. Relative quantities of complexes of the same size (e.g. tetramers) in different samples can be inferred by band intensity, however. Briefly, 10 mg of butyrylthiocholine iodide (Sigma-Aldrich, St. Louis, MO) was dissolved in 13 ml of 0.1 M sodium phosphate, pH 6 (Bio Basic Canada Inc., Markham, ON). Subsequently, 1 ml of 0.1 M sodium citrate (Bio Basic Canada Inc.), 2 ml of 30 mM copper (III) sulphate (Bio Basic Canada Inc.), 2 ml of deionized water, and 2 ml of potassium ferricyanide (Bio Basic Canada Inc.) was added to the dissolved butyrylthiocholine iodide, with mixing after each addition. The working solution was prepared immediately before use. The gel was rinsed for 2 min in deionized water then submerged in the colorimetric solution. The reaction was allowed to develop at room temperature, with rocking, for 5–12 h. Gels were monitored visually during color development. The reaction was stopped by rinsing twice with deionized water as soon as background staining began to develop, or once bands were readily detectable in all sample lanes. Stained gels were imaged using a Gel Doc™ EZ System (Bio-Rad). Resulting photos were processed using Adobe Photoshop CS6.

2.6. Deglycosylation of serum-derived and HUCPVC-derived BChE

CM from native and pAd5-*BCHE* modified cells was generated by a 24 h incubation in half the normal media volume (8 ml per T150 flask) applied to 70–80% confluent monolayers. Multiple CM collections were pooled and concentrated from 32 ml to approximately 1 ml using Amicon Ultra-15 Ultracel – 3K centrifugal filters (Sigma Aldrich) according to manufacturer's instructions. Concentrated CM samples and purified human BChE (Abcam) were incubated with PNGase F (New England Biolabs Ltd, Ipswich, MA; Cat. No. P0708) to remove N-linked oligosaccharides. The manufacturer's non-denaturing protocol, incubation with PNGase F for 24 h a 37 °C, was followed in order to retain enzymatic activity. Equivalent untreated and deglycosylated samples were prepared in native sample buffer (Bio-Rad) and size fractionated on a 4–20% gradient acrylamide gel at 250 V for 18 h at 4 °C. BChE protein bands were detected by colorimetric activity assay as detailed in section 2.5.1.

2.7. Quantification of BChE activity by Ellman assay

BChE activity (μmol/minute) in CM was assayed according to the Ellman method [56] by measuring the rate of change of absorption at 405 nm. Assay parameters were optimized for analysis of CM including 0.1 M phosphate buffer, pH 7.4, 0.375 mM butyrylthiocholine iodide and 0.266 mM 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB). A single lot of human serum-derived butyrylcholinesterase with defined activity (10U/ml; Abcam) was used to generate a standard curve and for quality assurance in each Ellman assay performed.

2.8. Immunocytochemistry

HUCPVCs at P4 were seeded on polystyrene 4 chamber culture slides (Falcon, Cat. No. 354114) pre-treated with fibronectin (see section 2.1) to maximize cell adherence. Following overnight incubation, monolayers in 2 chambers were modified using pAd5-*BChE* as per section 2.2. The remaining 2 chambers were not gene-modified. Forty-eight hours after transduction, the monolayers were washed 3 × 5 min with sterile PBS, then fixed in 500 μl 10% Neutral Buffered Formalin (VWR, Mississauga, ON; Cat. No. 10790-714) at room temperature. Following 2 × 5 min washes in PBS, cells were permeabilized by 10 min incubation with PBS plus 0.25% Triton-X 100 (PBST; Millipore-Sigma) at RT. Cells were washed 3 × 5 min with PBS, and incubated 30 min in blocking buffer (PBST + 10% goat serum (Abcam). Rabbit anti-butyrylcholinesterase polyclonal antibody (AbCam, Cat. No. Ab154763) was diluted 1:200 in PBST + 10% goat serum, and 300 μl per well applied to the cells. Negative control wells received blocking buffer without primary antibody. Chamber slides were incubated overnight at 4 °C in a humidified chamber. Next day, wells were washed 3 × 5 min with PBS and then incubated with 300 μl of FITC-conjugated goat anti-rabbit detection antibody (Abcam; Cat. no: A11034), diluted 1:500 in PBS with 10% goat serum, for 1 h at RT in the dark. Excess antibody was removed by 3 × 5 min washes in PBS, and then actin structures visualized by staining with 1x Cytopainter phalloidin iFluor 555 (Abcam; Cat. No. 176760) for 30 min at RT in the dark. Following a final wash series of 3 × 5 min in PBS, well chambers were removed and the slides mounted in Prolong Diamond Antifade with DAPI (Molecular Probes; Cat. No. S36964). Confocal images were captured at 400X magnification using a Quorum WaveFX laser scanning confocal microscope (Quorum Technologies Inc.) equipped with a Hamamatsu EM-CCD camera. Images were processed using Adobe® Photoshop and Illustrator CS6.

2.9. Cell fractionation

To determine the subcellular localization of BChE, cells were seeded in T75 flasks and modified with pAd5-*BChE* as detailed in section 2.2. Equivalent unmodified cells were treated in parallel. Three days after transduction, media was replaced at half volume (4 ml instead of 8 ml) for CM collection. After 24 h, CM was collected and BChE activity quantified by Ellman assays as described in section 2.7. Each cell population was enzymatically detached using TrypLE Select. Cell suspensions were counted using a Millipore Scepter™ automated cell counter equipped with a 60 μM probe and divided into two equal aliquots. Cells were pelleted by centrifugation at 149 × *g*. To liberate cytosolic proteins, one aliquot was re-suspended in 0.5 ml of basal (unsupplemented) MSCGM-CD then subjected to 3 freeze/thaw cycles (snap freeze by submersion in liquid nitrogen followed by rapid thaw by submersion in a 37 °C water bath until a sliver of ice remained). Insoluble components were separated by centrifugation at 19,500 × *g*. The soluble fraction (supernatant) was collected and subject to Ellman assay to quantify BChE activity in the cytosol. The insoluble fraction (pellet) was subsequently lysed by suspension in 0.5 ml of native lysis buffer (Abcam; Cat. No. 156035) to liberate proteins sequestered in cellular membranes. The suspension was incubated on ice for 15 min

then centrifuged at 19,500 × *g* for 15 min. The resulting supernatant, containing liberated membrane proteins, was collected and assayed for BChE activity by Ellman assay.

To assess BChE activity in the combined membrane and cytosolic fractions, the second aliquot of cells was re-suspended in 0.5 ml of native lysis buffer (Abcam), incubated for 15 min on ice to lyse the cells, and centrifuged at 19,500 × *g* for 15 min. The resulting supernatant was then tested by Ellman assay quantify BChE activity. All samples were treated with a protease inhibitor cocktail kit (Abcam) at each step of the procedure.

2.10. miRNA modulation

HUCPVCs were seeded at 10,500 cells/cm^2 in 6-well culture plates and allowed to adhere overnight. Transfection protocols for the miRNA mimic (Dharmacon miRIDIAN microRNA Human hsa-miR-186 Mimic; GE Life Sciences, Chicago, IL) and miRNA hairpin inhibitor (Dharmacon miRIDIAN microRNA Human hsa-miR-200c Hairpin Inhibitor; GE Life Sciences) were first optimized using Dy547-labelled control oligos (Dharmacon miRIDIAN microRNA hairpin inhibitor and microRNA mimic transfection control with Dy547; GE Life Sciences). Four transfection reagents (DharmaFECT 1, 2, 3 and 4; GE Life Sciences) were tested in combination with a range of transfection control concentrations to identify the optimal combination for each construct. Toxicity was assessed by microscopic phenotypic evaluation for stress lines, increased granularity and lifting cells. Transfection efficiency was evaluated by fluorescence microscopy for the number of Dy547 positive cells, and overall intensity. Bright field and fluorescent photomicrographs were captured using an EVOS FL Imaging system (Thermo Fisher, Waltham, MA).

HUCPVCs were seeded as above and allowed to adhere overnight. The miR-186 mimic and the miR-200c hairpin inhibitor were prepared in MSCGM-CD at 50 and 100 nM with 0.263 µl/cm^2 of Dharmafect 3 (Dharmacon miRIDIAN, GE Life Sciences), as per manufacturer's instructions. Cells were cultured in 1 ml of miRNA-spiked media for 48 h. CM was collected and stored at −20 °C for future analysis, while cells were preserved in 0.5 ml RNAProtect® Cell reagent (Qiagen, Germantown, MD) to stabilize RNA for analysis by quantitative reverse transcription polymerase chain reaction (RT-qPCR).

2.10.1. RT-qPCR analyses of BCHE transcript

Cells preserved in RNAProtect® Cell reagent (Qiagen) were stored at −20 °C until processing. After thaw, total RNA was extracted using RNeasy Mini kits (Qiagen) as per manufacturer's instructions. RNA was quantified using a BioTek Synergy HR microplate reader (BioTek Instruments Inc., Winooski, VT) and 100 ng of RNA from each cell sample was prepared in triplicate for RT-qPCR. QuantiNova SYBR Green RT-PCR kits (Qiagen) were used to quantify *BCHE* gene transcription using KiCqStart primers (Sigma-Aldrich) for human *BCHE* (Oligo # 3016679585-10/0 and/1 - Forward: AAAGTCTTGGAAATGA CAGG and Reverse: ATCATGTAATTGTTCCAGCG) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Oligo# 3016854082-10/0 and/1 - Forward: ACAGTTGCCATGTAGACC and Reverse: TTTTTGGTTGA GCACAGG. Samples were run on a BioRad CFX Connect Real Time PCR Detection System (Bio-Rad, Hercules, CA) in a 20 µl reaction volume. Run conditions were 50 °C for 10 min, 95 °C for 2 min, followed by 40 cycles at 95 °C for 5 seconds, 65 °C for 10 seconds, then 95 °C for 10 seconds, followed by a melt curve at 65 °C - 95 °C for 5 min.

2.10.2. RT-qPCR analyses of miRNA transcript levels

Native and pAd5-*BCHE* modified cells preserved in RNAProtect® Cell reagent (Qiagen) were stored at −20 °C until processing. After thaw, RNA was extracted using a combination of RNEasy Mini kits and RNEasy MinElute Cleanup kits (Qiagen) according to Qiagen Supplementary Protocol RY26- "Purification of miRNA from animal cells using the RNeasy Plus Mini Kit and RNeasy MinElute Cleanup Kit". Protocol 2 was used to separately purify small RNA and larger RNA from each sample. Small RNA was eluted into 50 µl of RNase-free water.

Relative abundance of *miR-186* and *miR-200c* were assessed by RT-qPCR using QuantiNova SYBR Green RT-PCR kits (Qiagen). The following KiCqStart primers (Sigma-Aldrich) were used: human hsa-miR-186-3p (MIMAT0004612) (Oligo # 3021926585-000030 and −000040 - Forward: GCAGGCCCAAAGGTA and Reverse: GTCCAGTTTTTTTTTT TTTTTCCCA), hsa-miR-186 5p (MIMAT0000456) (Oligo # 3021926585-000070 and −000080- Forward: CGCAGCAAAGAATTCT CCT and Reverse: CCAGTTTTTTTTTTTTTTTAGCCCAA); hsa-miR200c-3p (MIMAT0000617) (Oligo # 3021926585-000050 and −000060- Forward: AGTAATACTGCCGGGTAATGA and Reverse: GGTCCAGTTTT TTTTTTTTTTCCA); hsa-miR-200c-5p (MIMAT0004657) (Oligo # 3021926585-000010 and −000020- Forward: CGTCTTACCCAGCAG TGT and Reverse: GGTCCAGTTTTTTTTTTTTTTCCA), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Oligo# 3016854082-10/0 and/1 - Forward: ACAGTTGCCATGTAGACC and Reverse: TTTT TGGTTGAGCACAGG. Reactions were performed in a 20 µl reaction volume including 2 µl of small RNA or 2 µl of the large RNA together with the appropriate primers for the miRNAs and GAPDH, respectively. Reactions were carried out on a BioRad CFX Connect Real Time PCR Detection System (Bio-Rad) set to cycle at 50 °C for 10 min, 95 °C for 2 min, then 55 cycles at 95 °C for 5 seconds, 65 °C for 10 seconds, and 95 °C for 10 seconds, ending with a melt curve at 65 °C - 95 °C for 5 min.

2.11. Atropine-mediated blockage of muscarinic cell surface receptors

HUCPVCs were seeded at 4,000 cells/cm^2 and transduced with pAd5-*BChE* at MOI 1000 18–24 h after seeding. A 2 mg/ml stock solution of atropine (Sigma-Aldrich) was prepared by dissolving the solid stock in distilled water to the desired concentration, followed by filter sterilization using a 0.2 µm syringe filter. Atropine was diluted to the final concentration in complete MSCGM-CD; stock solutions at the final concentration were prepared for each experiment, to ensure replicate dosing between samples. Atropine concentrations ranging from 0 to 2000 µM were tested. Media supplemented with atropine was changed daily to compensate for metabolized and degraded compound. The collected CM was tested for BChE activity by Ellman assay. Sample aliquots were stored at −20 °C for future analysis by native gel electrophoresis.

2.12. Microarray processing

RNA expression was analyzed using GeneChip™ U133A 2.0 arrays (Affymetrix, Waltham, MA). RNA was amplified and labeled using GeneChip™ 3′ IVT Plus kits (Affymetrix) following the manufacturer's instructions with the following modifications: 200 ng of RNA was used for the cDNA synthesis and the total amount of cRNA for the hybridization reaction was increased from 10 µg to 20 µg. Arrays were hybridized for 16 h at 45 °C, 60 RPM in a GeneChip™ Hybridization Oven 640 (Affymetrix). Samples were registered in Affymetrix GeneChip™ Control Console (AGCC) and program FS450_0002 was used with GeneChip™ Fluidics Station 450 (Affymetrix). Chips were scanned using a GeneChip™ 3000 7G Scanner (Affymetrix) and the resulting probe array images subjected to grid alignment and fluorescence intensity review in AGCC Viewer.

2.13. Statistical analyses

Two-way analysis of variance (ANOVA) and multiple t-tests were performed using GraphPad Prism (Version 6.01, GraphPad Software). Microarray. CEL files were analyzed using R [57] with packages sourced through Bioconductor [58]. Preprocessing (GCRMA background adjustment, normalization, $\log_2$ expression matrix output) was performed using gcrma [59] and Affymetrix U133A 2.0 probe affinity data. Data quality was interrogated using simpleaffy [60], following

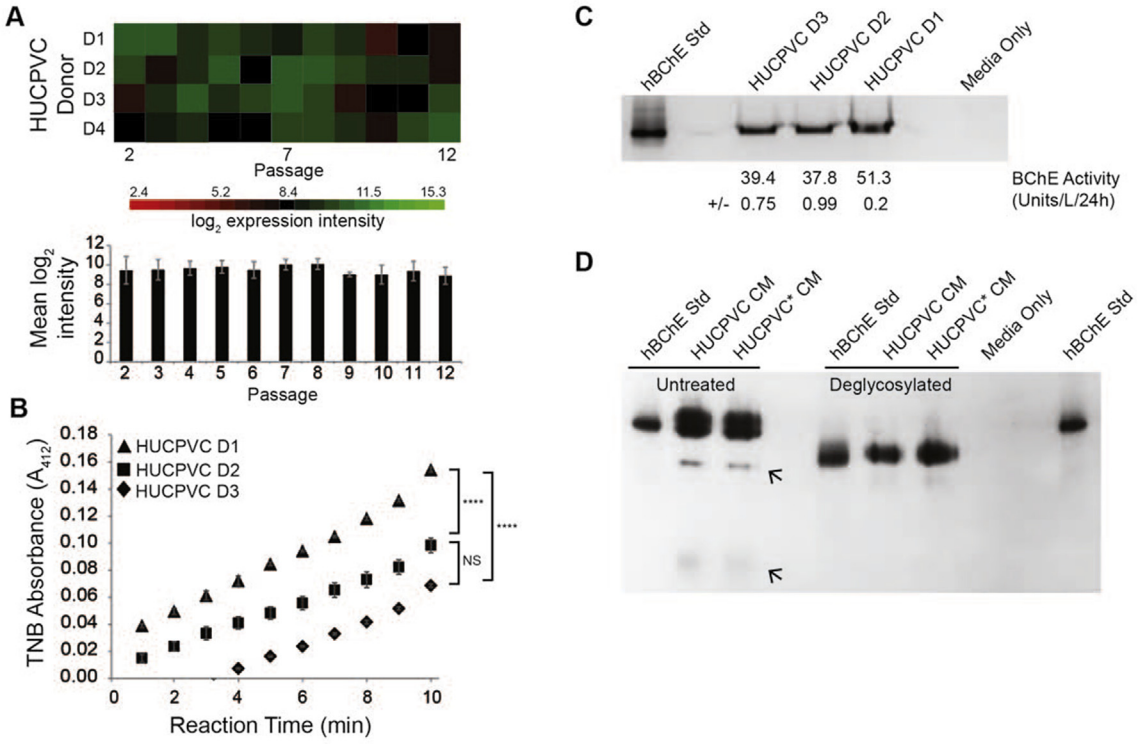

Fig. 1. HUCPVCs naturally produce BChE in culture. (A) Heat map representation of *BCHE* expression in 4 HUCPVC donor populations, detected by microarray. *BCHE* gene expression is maintained at moderate levels during *in vitro* expansion of the cells from passage 2 to passage 12, approximately 60 population doublings. (B) Representative Ellman assay data using butyrylthiocholine iodide substrate reveals BChE enzyme activity in CM from 3 HUCPVC donor populations. n = 3 independent experiments, each assayed in triplicate. (C) Native gel electrophoresis of the CM samples in (B) followed by in-gel colorimetric activity assay to visualize functional BChE enzyme. HUCPVCs secrete a BChE protein complex that migrates at a comparable rate to human serum-derived BChE. (D) Two tetrameric BChE species in untreated HUCPVC CM are revealed by 4–20% gradient gel electrophoresis. BChE monomers and dimers are weakly detected in untreated CM (arrows). Human BChE standard and concentrated CM from native (HUCPVC) and modified (HUCPVC*) cells subjected to PNGase F treatment to remove N-linked oligosaccharides exhibit comparable band shifts in non-denaturing electrophoresis, indicating that they are similarly glycosylated. Error bars represent standard deviation from the mean; NS $p > 0.05$, *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Abbreviations: BChE, butyrylcholinesterase; CM, conditioned media; D, donor; h, hour; HUCPVC, human umbilical cord perivascular cells; L, liter; NS, not significant; Std, standard.

GeneChip™ analysis guidelines [61]. Batch effects were explored by evaluating all BatchQC [62] metrics with and without ComBat [63] adjustment. No batch effects were identified.

3. Results

3.1. HUCPVCs express BCHE during routine culture expansion

We recently completed a study to assess evolution of the HUCPVC transcriptome during serial expansion in chemically defined media [64]. Four HUCPVC donor populations were serially cultured from P2 until senescence in serum- and xeno-free media, and the transcriptome assayed at each passage by microarray. We interrogated this substantial data set to determine if HUCPVCs normally express *BCHE*, since cholinergic signalling pathways have been identified in MSCs [65] and HUCPVCs have demonstrated neurogenic potential [66]. Notably, *BCHE* is among the 20% most highly expressed genes by cultured HUCPVCs. *BCHE* expression is comparable between the 4 donor populations examined, and is relatively maintained throughout *ex vivo* expansion (Fig. 1A). To test whether HUCPVCs secrete BChE protein, BChE enzyme activity was quantified by Ellman assay in a 24 h collection of CM, using a butyrylthiocholine substrate. Indeed, BChE activity was detected in the CM from all HUCPVC populations tested (Fig. 1B).

The similarity of HUCPVC-sourced BChE to the benchmark enzyme sourced from human plasma was analyzed by in-gel colorimetric activity assay [55]. CM was concentrated by centrifugal filtration, then subjected to native gel electrophoresis which leaves protein complexes intact and separates them based on molecular weight, 3-dimensional size, and charge. Functional BChE protein bands were visualized *in situ* by colorimetric activity assay. A protein band exhibiting BChE enzyme activity is evident in HUCPVC CM at a comparable position to the purified BChE standard isolated from human plasma (Fig. 1C). Extended gradient gel electrophoresis further reveals the presence of 2 BChE bands in HUCPVC CM, one of which migrates slightly slower than the plasma-derived standard (Fig. 1D). Interestingly, BChE isolated from human umbilical cord plasma also runs as 2 tetrameric bands, the heavier band identified as the C5 variant that purportedly incorporates a larger neonatal lamellipodin variant [67]. Weak bands correlating to the lower order BChE complexes were sometimes discernible in highly concentrated CM samples (Fig. 1D, arrows). These assays reveal that cultured HUCPVCs naturally secrete tetrameric BChE similar to human plasma-derived BChE.

Since glycosylation also affects electrophoretic migration, the comparable positions of the plasma- and CM-sourced BChE protein bands suggests that these proteins are also similarly glycosylated. This hypothesis was tested by incubating plasma-derived BChE standard and CM with PNGase F, which specifically removes N-linked oligosaccharides from glycoproteins. Human plasma-derived BChE includes 9 N-linked oligosaccharides [68–70]. After 24 h, BChE in all tested samples exhibited a marked and comparable band shift under non-denaturing conditions, reflecting increased mobility through the gel (Fig. 1D). Interestingly, HUCPVC-derived BChE still exhibited slightly slower electrophoretic mobility than the BChE standard, suggesting that the

observed MW difference may be attributed to incorporation of a larger polyproline peptide consistent with the umbilical cord blood-derived C5 variant [67].

The amount of BChE secreted by HUCPVCs in normal culture is not sufficient to generate clinical quantities. However, HUCPVCs appear to possess the innate cellular machinery to synthesize and secrete a functional BChE enzyme product that forms tetrameric complexes. Thus, we tested whether BChE production could be increased by genetically modifying HUCPVCs with a transgene encoding human *BCHE* under control of a constitutive promoter.

3.2. HUCPVCs can be modified with a human BCHE transgene to increase output

HUCPVCs were modified by co-incubation with a recombinant pAd5 adenoviral vector encoding human *BCHE*. We, and others, have previously shown that this modification process has minimal impact on the fundamental biology of MSCs, including their growth potential and developmental plasticity [52,53,71]. We first established the highest sub-toxic viral dose for *BCHE* modification, which was used for the remainder of the study. Integrity of the modification process and transgene product were assessed by quantifying relative *BCHE* mRNA transcript levels in native and modified cells by RT-qPCR, and normalizing against the *GAPDH* housekeeping gene to account for number of cells. Twenty-four hours after modification, the number of *BCHE* mRNA transcripts approximately doubles compared to native cells (Fig. 2A). Over 4 days in continuous culture, *BCHE* mRNA in modified cells is maintained at nearly twice the level documented in equivalent native cells (Fig. 2A).

Next, the molecular forms of BChE secreted into the culture media by modified HUCPVCs were evaluated by native gel electrophoresis followed by in-gel colorimetric activity assay. The representative gel in Fig. 2B compares equivalent un-concentrated day 7 CM samples from native HUCPVCs cultured in parallel with modified HUCPVCs treated with a range of sub-toxic viral doses. The gene-modified HUCPVCs secrete modestly higher levels of tetrameric BChE, most evident at the highest dose of virus as determined by relative protein band intensity (Fig. 2B). BChE derived from modified HUCPVCs also exhibits an equivalent band shift after PNGase F treatment as BChE derived from native HUCPVCs (Fig. 1D, asterisks). Next, the amount of BChE protein secreted daily by modified HUCPVCs compared to native HUCPVCs was quantified by Ellman assay. Representative data from 2 HUCPVC donor populations show that daily BChE output from cells modified at the high viral dose increases compared to equivalent native cells (Fig. 2C), but not substantially. Differential protein secretion was not detectable by Ellman assay prior to day 4 post modification (data not shown) despite the prompt doubling of mRNA transcript by 24 h after modification (Fig. 2A). Taken together, HUCPVCs are a previously unidentified source of tetrameric human BChE that can be genetically modified to increase enzyme output.

3.3. Investigating HUCPVC regulatory mechanisms that limit BChE synthesis

The modest increase in BChE output from modified HUCPVCs was unexpected, since modification of HUCPVCs with other secreted ([52,72] and unpublished) and cytosolic ([53] and unpublished) proteins results in robust expression of the exogenous gene product using 10–100-fold lower virus doses for modification. We hypothesized that since native HUCPVCs naturally express and secrete BChE, they may modulate BChE levels in the intracellular and/or extracellular environment by innate mechanisms. Alternatively, the constitutively overexpressed BChE might overwhelm the translational machinery resulting in improperly folded proteins that become trapped in inclusion bodies [41].

Cells regulate protein expression at multiple levels, including transcriptional control, post-transcriptional control, subcellular localization and trafficking/secretion. Since the *BCHE* transgene is an episomal construct under control of a constitutive CMV promoter, exogenous *BCHE* transcription should occur independent of endogenous *BCHE* transcriptional control mechanisms. Thus, we first investigated whether exogenous BChE protein might be sequestered in the cells. Immunocytological (ICC) staining of BChE in modified HUCPVCs reveals that most of the intracellular BChE protein is distributed throughout the cytosol, and appears to be enriched in the perinuclear region (Fig. 3A). Interestingly, actin constrictions consistent with budding vesicles positive for BChE are observed in modified cells (Fig. 3A, arrows). Native and modified HUCPVCs were next subject to cell fractionation using freeze-thaw and lysis buffer methods. BChE activity in each fraction was quantified by Ellman assay. Consistent with the cell

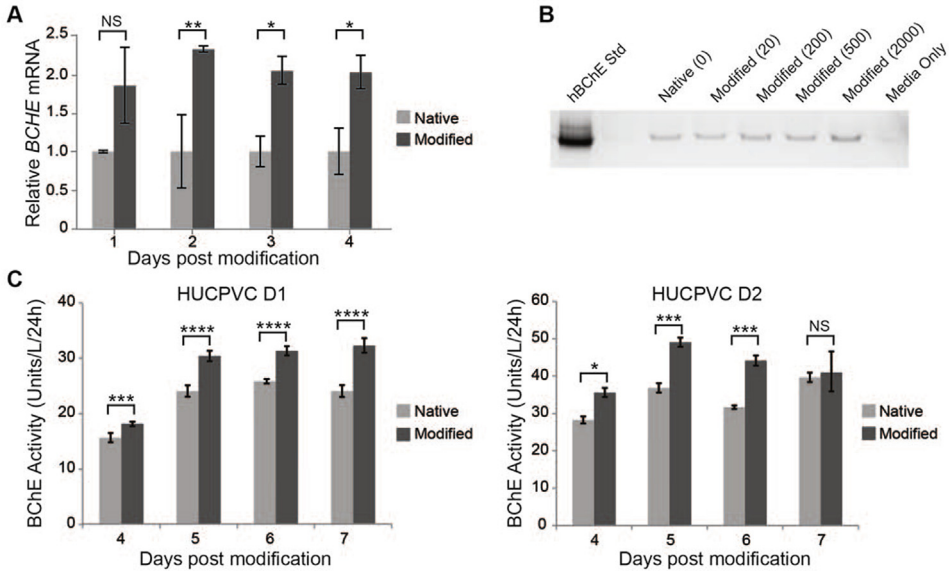

Fig. 2. HUCPVCs can be gene modified to increase BChE output. (A) Quantification of *BCHE* mRNA by RT-qPCR on days 1, 2, 3 and 4 after modification with an episomal human *BCHE* transgene, and normalized against equivalent unmodified (native) cell cultures. Gene modification doubles *BCHE* transcript levels compared to native cells. n = 3 independent tests. (B) Analysis of BChE secretion in CM from HUCPVCs modified with range of recombinant adenoviral doses using native gel electrophoresis followed by in-gel colorimetric activity assay to detect functional BChE enzyme. Intensity of the tetrameric BChE protein band increases dose-dependently, indicating that gene modification increases secreted output of functional BChE from HUCPVCs. (C) Quantification of BChE activity in CM from two representative HUCPVC donor populations shows that daily output of BChE can be modestly increased by gene modification. n ≥ 3 independent experiments, each assayed in triplicate. Error bars represent standard deviation from the mean; NS $p > 0.05$, *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Abbreviations: BChE, butyrylcholinesterase; CM, conditioned media; D, donor; h, hour; HUCPVC, human umbilical cord perivascular cells; mRNA, messenger ribonucleic acid; L, liter; NS, not signficant, Std, standard; RT-qPCR, quantitative reverse transcription polymerase chain reaction.

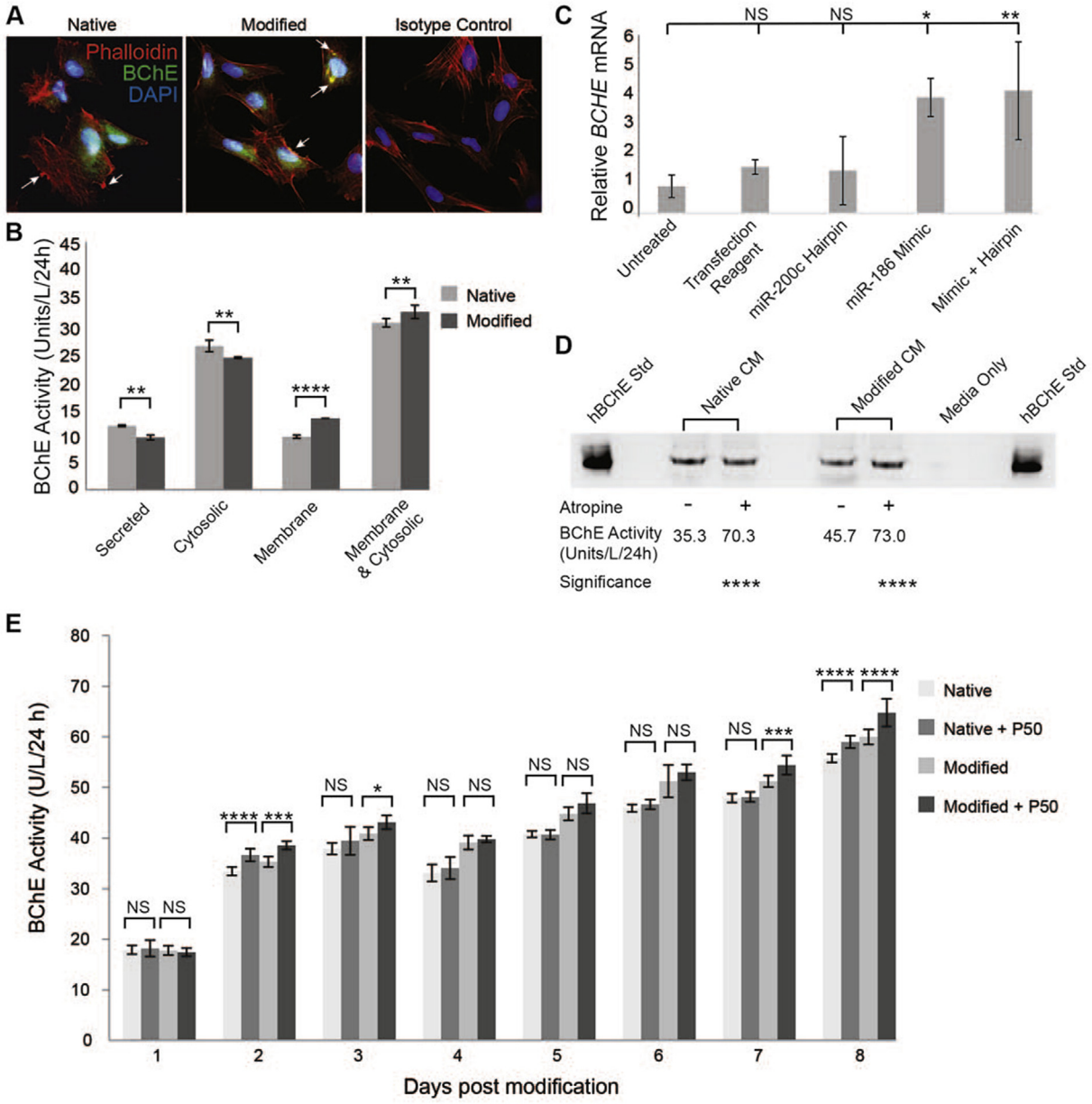

Fig. 3. HUCPVCs can modulate endogenous and exogenous BChE synthesis. (A) Confocal micrograph of native and BChE-modified HUCPVCs labeled with an antibody targeted against BChE antigen (green) and co-stained with DAPI (blue) and phalloidin (red) to visualize nuclei and F-actin, respectively. BChE is predominantly localized to the cytoplasm, and is enriched in the perinuclear region. Budding vesicles, indicated by F-actin constrictions (white arrows), are enriched for BChE in modified cells. Image was captured at 400x magnification by confocal microscopy. (B) Quantification of BChE activity by Ellman assay in secreted and subcellular fractions of native and modified HUCPVCs shows that substantial BChE activity is retained by the cells, predominantly in the cytosolic fraction. n = 3 independent tests. (C) Treatment of HUCPVCs with an miR-186 mimic results in 4x higher *BCHE* transcript levels when normalized to untreated cell levels. Addition of the miR-200c hairpin inhibitor has no apparent effect on *BCHE* transcript levels. n = 3 independent experiments, each assayed in triplicate. (D) Blocking muscarinic receptors on the cell surface by addition of 2000 μM atropine to the culture media significantly increases levels of functional BChE secreted by HUCPVCs, as shown by native gel electrophoresis followed by in-gel colorimetric assay to stain BChE protein, and by increased BChE enzyme activity in CM quantified by Ellman assay. n = 3 independent tests. Representative data is shown. (E) Co-incubation with 1000 μM P50 does not substantially increase daily BChE output from native or modified HUCPVCs, although a more significant effect is observed on modified cells at the later time points. n = 3 independent tests, each assayed in triplicate, error bars represent standard deviation from the mean. NS $p > 0.05$, *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.
Abbreviations: BChE, butyrylcholinesterase; CM, conditioned media; h, hour; HUCPVC, human umbilical cord perivascular cells; mRNA, messenger ribonucleic acid; miRNA, micro-ribonucleic acid; L, liter; NS, not significant; Std, standard.

staining, the highest BChE activity is found in the cytosolic fraction (Fig. 3B). The membrane fraction also contains substantial BChE activity, which could include BChE enzymes in the secretory pathway (Fig. 3B).

Having determined that BChE protein is not being trapped in inclusion bodies, we next examined whether HUCPVCs might regulate translation of *BCHE* messenger RNA (mRNA). Micro-RNA (miRNA) are small ribonucleic acid segments that regulate post-transcriptional gene expression. miRNA bind to partially complementary regions of the mRNA transcript and destabilize the structure. miRNA-mediated regulation would thus modulate translation of both endogenous and exogenous *BCHE* transcripts. In a mouse model of predator scent stress, increased levels of miR-186 correlated with increased BChE activity [73], while miR-200c was identified *in silico* as a putative repressor of

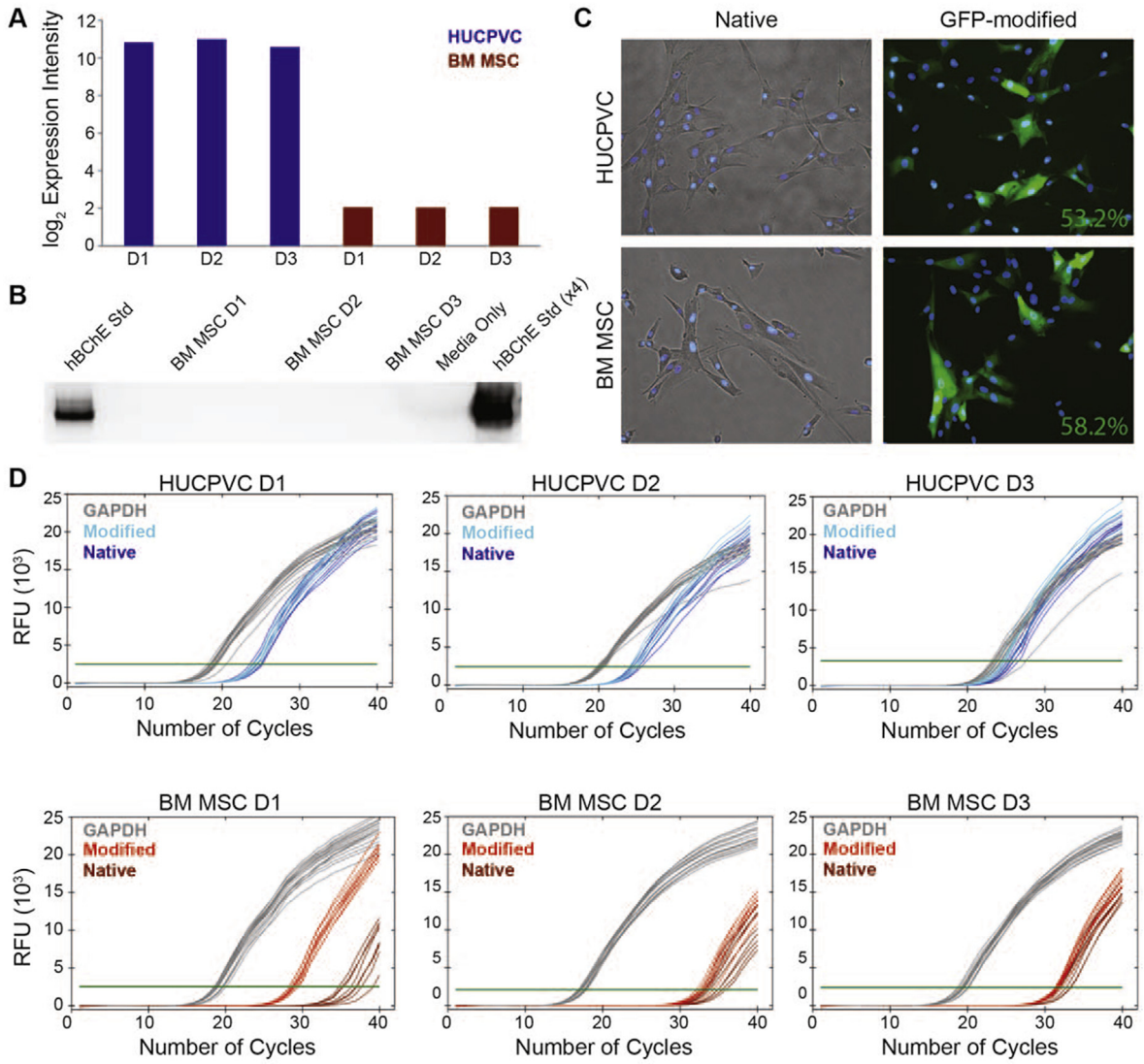

Fig. 4. Bone marrow-derived MSCs are inferior vectors for BChE production. (A) Gene expression analysis of 3 independent HUCPVC and human BM MSC donor populations shows that *BCHE* is not significantly expressed by BM MSCs (red), in contrast to robust expression by HUCPVCs (blue). (B) BChE protein is undetectable in the CM from human BM-derived MSCs by native gel electrophoresis followed by in-gel colorimetric assay to visualize enzymatically active BChE. (C) HUCPVCs and BM MSCs are gene-modified at similar efficiencies by recombinant adenovirus. Multiple donors from each MSC type were transduced using pAd5-*eGFP*. After 48 h, the number of eGFP-positive cells were visually assessed by microscopy and quantified by flow cytometry (10,000 cells/sample). Green, eGFP; blue, DAPI. (D) Comparison of *BCHE* mRNA levels in 3 native and modified HUCPVC and BM MSC donor populations by RT-qPCR. Higher levels of *BCHE* transcript in the starting sample require fewer amplification cycles to exceed the amplification threshold (green line). Levels of the housekeeping gene *GAPDH* (grey) are used to normalize for cell number. *BCHE* transcript in modified HUCPVCs (light blue) is slightly higher than native cells (dark blue). Consistent with (A) and (B), *BCHE* transcript is negligible in native BM MSCs (red) but can be slightly increased by gene modification (orange). n = 3 independent tests.

Abbreviations: BChE, butyrylcholinesterase; BM, bone marrow; CM, conditioned media; D, donor; GAPDH, glyceradehyde 3-phosphate dehydrogenase; HUCPVC, human umbilical cord perivascular cells; MSC, mesenchymal stromal cell; mRNA, messenger ribonucleic acid; NS, not significant; RT-qPCR, quantitative reverse transcription polymerase chain reaction.

BChE expression [74]. Based on these reports, we postulated that increased levels of miR-186, or decreased levels of miR-200c, might improve BChE synthesis by HUCPVCs.

The relative abundance of *hsa-miR-186* and *hsa-miR-200c* in unmanipulated cells were assessed by RT-qPCR. Only *hsa-miR-186-5p* is detectable in all HUCPVC donors, and is present at substantially higher levels in the D3 donor population (Fig. S1). By contrast, *hsa-miR-186-3p*, *hsa-miR-200c-3p* and *-5p* are undetectable in this assay (data not shown). Fluorescent reporter oligonucleotides were used to optimize the transfection reagent and protocol (not shown). Equivalent cultures of HUCPVCs were then treated with transfection reagent alone, miR-186 mimic, miR-200c hairpin inhibitor, or a combination of the 2 miRNA constructs. Twenty-four hours after addition of the miRNA constructs, cells were harvested and relative *BCHE* mRNA levels quantified by RT-qPCR normalized to *GAPDH* levels. Strikingly, treatment with the miR-186 mimic results in a 4-fold increase in *BCHE* expression (Fig. 3C). Addition of the miR-200c hairpin inhibitor does not have a measurable effect (Fig. 3C), consistent with the finding that it is not expressed at detectable levels by HUCPVCs.

We next examined whether HUCPVCs might modulate BChE expression via feedback mechanisms, in response to measured BChE levels or enzyme substrate in the extracellular environment. Nicotinic and muscarinic cholinergic signalling receptors have been detected in cultured MSCs [65]. Choline-based esters, such as acetylcholine, can bind to nicotinic and muscarinic cell surface receptors and effect numerous autocrine and paracrine signalling outcomes (reviewed by Ref. [75]).

Atropine competitively binds to muscarinic receptors and blocks their cholinergic stimulation [76]. We applied atropine to cultured HUCPVCs to disrupt cholinergic signalling and measured BChE output in daily collections of CM by native gel electrophoresis and Ellman assay. Equivalent cultures of native and modified HUCPVCs were treated normally, or supplemented with 2000 μM atropine. CM was collected every 24 h and analyzed for BChE activity by Ellman assay, and relative amounts of secreted BChE were characterized by native gel electrophoresis followed by detection using a colorimetric activity assay. Addition of atropine to the culture media substantially increases output of tetrameric BChE (Fig. 3D), with a markedly greater effect than gene modification alone (Fig. 2B and C). Although BChE secretion from both native and modified HUCPVCs increases in response to atropine treatment, comparable activity is detected in both cultures (Fig. 3D). Taken together, these results suggest that HUCPVC-mediated BChE synthesis can be increased by modulating several different regulatory mechanisms, including mRNA translation and cellular feedback via muscarinic receptors.

Previous studies have shown that the availability of polyproline can be a limiting factor in BChE complex assembly, and that addition of poly-L-proline peptides can increase higher order complex formation of monomeric BChE [24,25,29,30,54,67,77,78]. Larson et al. [54] showed that addition of synthetic 50-mer poly-L-proline peptides (P50) to cell-free media efficiently drives complex formation of recombinant human BChE. Thus, we tested whether supplementation with P50 could increase soluble BChE yields from HUCPVCs. Native and modified HUCPVCs were dosed daily with 1000 μM P50; CM was collected and replaced every 24 h for 8 consecutive days. Co-incubation with P50 had the most effect on soluble BChE activity from modified HUCPVCs, and only at statistically relevant levels at the later time points (Fig. 3E). However, the documented increases were not as profound as reported for other systems [24,25,29,30,54,67,77,78], suggesting that availability of polyproline is not a limiting factor in HUCPVC-mediated BChE synthesis.

3.4. Bone marrow-derived MSCs do not produce endogenous or exogenous *BCHE*

MSCs can be isolated from numerous tissues. Although MSCs share many core characteristics, they also possess properties unique to their tissue source. We postulated that a different MSC population might be more amenable to BChE synthesis. Human bone marrow-derived MSCs (BM MSCs), the most characterized MSC source for clinical and commercial applications, were next tested for their potential as a platform for BChE synthesis. We first evaluated whether BM MSCs innately produce BChE in culture, similar to HUCPVCs. *BCHE* mRNA is not detectable from 3 different BM MSC donors at mid-passage (P4), although it is robustly expressed in HUCPVCs from 3 different donors at the same passage (Fig. 4A). Consistent with this result, active BChE is not detectable in concentrated CM from these same donors by either native gel electrophoresis (Fig. 4B) or Ellman assay (not shown). Since HUCPVCs naturally secrete BChE, and consequently have innate mechanisms to limit BChE synthesis, we postulated that BM MSCs may be more amenable to BChE synthesis after gene modification since they do not normally express BChE.

We previously determined that BM MSCs are conducive to gene modification using the same protocols developed for HUCPVCs [53]. This data was verified by modifying HUCPVCs and BM MSCs in parallel using a pAd5-*eGFP* reporter construct using the pAd5-*BChE* modification protocol. Quantification of eGFP-positive cells by flow cytometry 48 h after transduction confirmed comparable transduction efficiency in both MSC types, which was also evident by fluorescent microscopy (Fig. 4C). BM MSCs were modified with the *BCHE* recombinant adenovirus, and relative *BCHE* mRNA determined by RT-qPCR and normalized against *GAPDH*. Higher amounts of starting transcript generate lower Cq values in the amplification curves, such that amplification of more initial transcript produces signal above the amplification threshold in fewer cycles. As described earlier (Fig. 2A), modification of HUCPVCs with pAd5-*BChE* results in only a marginal increase in *BCHE* mRNA, as evidenced by slightly earlier amplification of modified HUCPVCs (light blue) compared to native HUCPVCs (dark blue), all of which passed the amplification threshold within 25 cycles (Fig. 4D). By contrast, native (red) and modified (orange) BM MSCs do not produce enough *BCHE* mRNA to surpass the amplification threshold before 30 cycles, except for the modified donor 1 sample which crossed the threshold by 28 cycles (Fig. 4D). BChE activity was also not detectable by Ellman assay in CM from modified BM MSCs (not shown). Taken together, native and modified human BM MSCs are not suitable platforms for BChE production.

4. Discussion

4.1. HUCPVCs are a novel source of natural and recombinant human BChE

Here we identify HUCPVCs as a novel source of naturally-occurring human BChE. This discovery provides important opportunities to exploit a rich human cell source to manufacture both natural and strategically engineered recombinant BChE, without the inherent limitations associated with other production platforms. BChE-modified HUCPVCs are an ideal platform technology for conventional *in vitro* manufacturing. These primary cells are sourced non-invasively from a renewable source of biomedical waste, and exhibit robust proliferative potential [44–46]. We recently reported that a single aliquot of 1 million HUCPVCs can be expanded to over 10^{19} cells in chemically defined xeno- and serum-free media before senescing [64]. Here, we show that *BCHE* is highly expressed throughout that cell expansion, demonstrating the potential utility of HUCPVCs for large-scale BChE manufacturing without animal or human serum additives. Importantly, HUCPVCs also present a new paradigm to achieve sustained BChE synthesis directly in the recipient [52], which would maximize BChE yields without costly purification steps, and provide a sustained source of the stoichiometric bioscavenger in a single dose.

4.2. HUCPVCs do not require interventions to produce tetrameric BChE

Considerable effort has been expended on recombinant systems to produce BChE enzyme comparable to the human form. For example, plasma-derived BChE is stereotypically glycosylated [70,79]. These sugar moieties promote tetramer formation, thereby enhancing enzyme activity, and physically protect the enzyme from degradation [26,27,69] resulting in a circulatory half-life of approximately 12 days [16,30]. Since non-human systems do not process the nascent protein appropriately, steps to genetically modify the platform or the *BCHE* transgene to prevent glycosylation at abberant sites, which leads to immunogenicity, or to direct appropriate glycosylation, have been required [34,35,68,80,81]. Additional efforts to extend the half-life of insufficiently glycosylated recombinant BChE have included PEGylation to simulate the protective effects of normal *in vivo* glycosylation [82–84], expression of an albumin-BChE fusion protein [85] and nanoparticle encapsulation [16,86]. In humans, the quaternary BChE structure is driven by polyproline peptides containing a proline-rich attachment domain (PRAD) [30], which are derived from cytosolic proteins including lamellipodin [30,87]. Thus, addition of PRAD-containing polyproline peptides to culture media has been utilized to drive complex formation of monomeric recombinant BChE [24,25,29,30,54,67,77,78]. By contrast, BChE production by HUCPVCs does not appear to be limited by availability of polyproline, since supplementation with P50 only modestly increased BChE activity in CM. Human hepatocytes require addition of linoleic acid (LA) and alpha-linolenic acid (ALA) for efficient secretion of BChE into culture media [88]. Addition of LA and ALA alone or in combination had minimal effect on BChE secretion from HUCPVCs (data not shown).

4.3. HUCPVCs are ideal vectors for non-permanent gene transfer of BChE

To circumvent the challenges associated with *in vitro* production platforms, and the multi-dosing requirement to maintain protective stoichiometric BChE levels, a direct gene therapy approach has been explored in small animals. Mice treated with a recombinant adenovirus (pAd) encoding human *BCHE* maintained high levels of circulating enzyme for nearly a week [89,90], while inoculation with a pAd-vectored mouse *BCHE* produced high serum titers for at least a year [91]. Although such an approach demonstrates prophylactic utility in challenge studies [92], the likelihood that human subjects would accept long-term gene-modification for OP prophylaxis is limited. Moreover, sustained elevation of BChE has already been associated with detrimental physiological outcomes, including Alzheimer's disease [93].

Delivery of human *BCHE* transgenes using an allogeneic cell vector offers certain advantages over direct gene transfer. Allogeneic cell vectors are gene modified *ex vivo,* which allows for superior dosing control and eliminates exposure of the recipient to large doses of live virus that have been shown to elicit adverse effects in clinical trials. In 1999, Shao et al. [94] demonstrated the potential utility of cell-vectored *BCHE* gene transfer. They reported that human fibroblasts, gene modified to express human *BCHE,* produced increasing levels of serum BChE up to day 13 after subcutaneous or intraperitoneal transplant in mice [94]. However, Shao et al. cited limitations in transduction efficiency of the fibroblasts [94], which are terminally differentiated cells and thus not tolerated in human allogeneic transplants. By contrast, allogeneic MSCs have a proven clinical safety record [95] and are highly amenable to gene modification [52,53,72].

Indeed, our recent preclinical study demonstrated that a single intramuscular injection of antibody-modified HUCPVCs rapidly achieved antibody-conferred protection against lethal virus challenge, and extended this protection from 24 h to over 10 days [52]. Administration of MSCs via skeletal muscle [96] offers a practical delivery modality that supports cell survival [53] and sustained synthesis of soluble therapeutic molecules with delocalized or systemic benefits [52,96,97]. Encapsulated MSC delivery may further allow for cell retrieval, or improved survival and limited dispersion post-transplant [98–100], and is a potential avenue to further improve the cell-vectored *BCHE* paradigm.

4.4. Cholinergic signalling is a broader MSC property, but expression of BChE is not

This study revealed that expression of *BCHE* is not common to all MSC sources. Although *BCHE* is among the 20% most highly expressed genes in the HUCPVC transcriptome, and is detected both intracellularly by ICC and extracellularly in the CM, expression of *BCHE* by BM MSCs was not detectable using any of these methods. These findings are consistent with other published transcriptome data from Wharton's jelly-derived MSCs and human BM-derived MSCs [64,101,102]. Notably, HUCPVCs produce a tetrameric BChE variant with reduced electrophoretic mobility, reminiscent of the C5 variant found in umbilical cord plasma [54]. Given the perivascular origin of HUCPVCs, it is tempting to speculate that these cells may contribute to the C5 found in cord blood. Although BM MSCs do not express BChE, Hoogduijn et al. [65] have shown that cultured BM MSCs utilize acetylcholine-based cholinergic signalling. The relatively consistent expression of *BCHE* by HUCPVCs suggest that it may contribute to routine cell functions, which may include non-neuronal cholinergic processes such as proliferation [65,103], differentiation [103,104], migration [105] or immune function [106–110], since MSCs exhibit potent immune-modulatory properties [111–113]. Neonatal HUCPVCs also exhibit enhanced neurogenic potential compared to adult MSCs [66,114], and it is possible that expression of *BCHE* contributes to this effect.

4.5. Additional characterization of HUCPVC-mediated BChE expression is required

Although the data show that HUCPVCs secrete tetrameric BChE, the relative ratio of various BChE complexes secreted by HUCPVCs has not yet been elucidated. BChE monomers and dimers could be weakly detected in very concentrated CM samples visualized by activity staining of non-denaturing gels, but since the smaller complexes possess lower enzyme activity than the tetrameric form, relative amounts cannot be extrapolated based on band intensity. Commercially available antibodies targeted against BChE antigen bind to an epitope that is masked by BChE quaternary structures. Thus, western blot analysis of BChE can only be performed on the denatured monomeric form. We attempted to extract the BChE gel bands detected in native gels, but the buffers required for the process were not compatible with western blot procedures.

In the various attempted manipulations, more substantial change was documented at the level of *BCHE* mRNA compared to secreted BChE protein or activity units. The experiments reported here indicate that although BChE is not being efficiently exported from the cell, it is not being titrated out in inclusion bodies. As such, this disparity may reflect preferential formation of higher order BChE complexes in HUCPVCs. Four BChE monomers are required to generate a single tetrameric complex; if most monomers participate in the highest order complex, a 4-fold increase in BChE mRNA would only result in a 1-fold increase (or doubling) of tetrameric BChE protein as we observed.

An exploratory evaluation of the potential effect of miRNA regulation on BChE synthesis revealed that enzyme levels are likely regulated via complex mechanisms. Currently, miR-186 is the only reported miRNA with an experimentally proven effect on BChE levels [73]. We determined that miR-186 is expressed by both HUCPVCs and BM MSCs, and that addition of miR-186 mimic oligos substantially increases *BCHE* mRNA levels in HUCPVCs. However, miR-186 has multifarious roles, many of which are context-dependent, and its effects on *BCHE* are likely indirect. In addition, the one HUCPVC donor with substantially higher endogenous levels of this miRNA did not generate higher BCHE yields, either in native or modified forms. Numerous putative cholinesterase-targeting miRNAs have been identified *in silico* [73,74]. From these, miR-200c was selected as a potential candidate. However, miR-200c was not detected in any of the MSC populations, and did not have a measurable effect on BCHE expression at the transcriptional level. Thus, considerable work is still required to identify strategic and specific miRNA targets that maximize BCHE synthesis from HUCPVCs.

5. Conclusions

Gene-modified HUCPVCs are a promising new platform amenable to both conventional cell manufacturing processes and allogeneic cell-based gene transfer. Importantly, this platform is suitable for producing natural human butyrylcholinesterase, as well as functionally-enhanced recombinant BChE isoforms including G117H [31], A328Y cocaine hydrolase [115], or a future catalytic mutant. Formation of tetrameric BChE complexes occurs without modifications required by other recombinant platforms, although internal mechanisms that limit BChE output must be disabled to maximize enzyme production. Once this challenge is resolved, BChE-modified HUCPVCs are amenable to large-scale batch production processes in xeno- and serum-free media, eliminating potential sources of endotoxin or human pathogens. Alternatively, *in vivo* delivery of BChE-modified HUCPVCs would bypass the enzyme purification steps which add substantial cost and reduce protein recovery. Moreover, a persistent source of BChE also addresses the current requirement for repeat dosing to maintain sufficient levels of the stoichiometric bioscavenger to neutralize a target agent.

Authorship contributions

Participated in research design: Braid, Wood, Ford.
Conducted experiments: Braid, Wood.
Contributed new reagents or analytical tools:
Performed data analysis: Braid, Wood.
Wrote or contributed to the writing of the manuscript: Braid, Wood, Ford.

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.cbi.2019.03.022.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2019.03.022.

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