



Mesenchymal stromal cell-derived extracellular vesicles as engineered nanocarriers of HIV-1-derived angiogenic peptides

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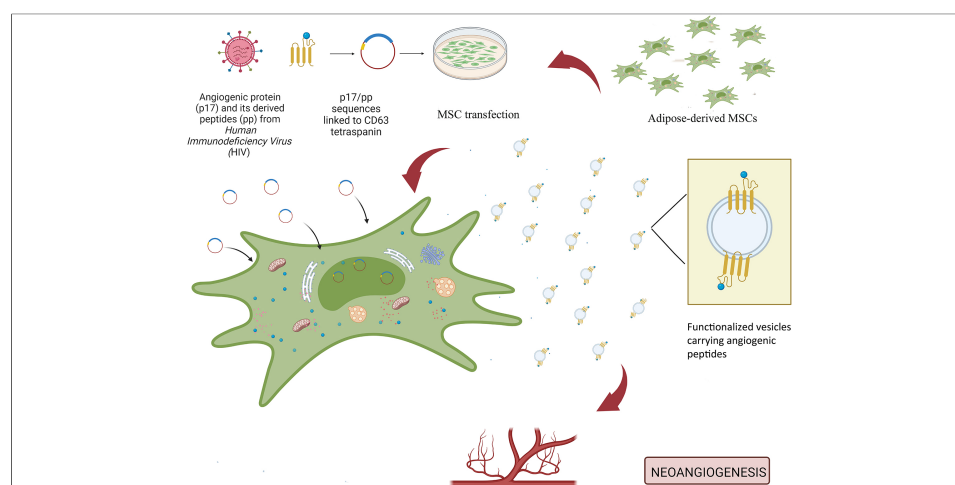
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Abstract

Aim: Therapeutic angiogenesis based on protein delivery is strongly limited by poor targeting, rapid degradation, and systemic clearance of bioactive molecules. This study aimed to develop a biologically based delivery platform exploiting canine Mesenchymal Stromal Cell-derived extracellular vesicles (MSC-EVs) engineered to expose Human Immunodeficiency Virus type 1 (HIV-1) p17-derived angiogenic peptides, and to identify a minimal functional angiogenic motif compatible with EV engineering.

Methods: We used a CD63-mediated engineering strategy to generate MSC-EVs functionalized with p17-derived peptides (F2 and F3) and shorter bioactive derivatives identified through integrated bioinformatic and structural analyses. Extracellular vesicles (EVs) were isolated from transfected MSCs and characterized by Nanoparticle Tracking Analysis, Western blotting, flow cytometry, and electron microscopy. Peptides were tested both in free form and as EV-associated cargo through *in vitro* angiogenesis assays.

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Results: CD63-mediated EV functionalization enabled effective surface display and delivery of F3-derived peptides while preserving their pro-angiogenic activity. Among the p17-derived sequences, a pentapeptide from the F3 fragment (F3 39-43) emerged as a minimal angiogenic motif that retained robust biological activity across experimental conditions. Shorter F2-derived fragments, although active as free peptides, failed to elicit angiogenic responses when associated with EVs, indicating a critical dependence on peptide length and spatial presentation for functional activity.

Conclusion: These findings identify a minimal HIV-1-derived angiogenic peptide and demonstrate the feasibility of CD63-mediated EV engineering as a platform for the presentation and delivery of p17-derived peptides, providing a rational framework for future development of EV-based applications.

INTRODUCTION

The restoration of functional blood supply remains a major clinical goal in conditions such as chronic diabetic ulcers, limb ischemia, and tissue injury, where defective angiogenesis contributes to disease progression in both humans and animals. Protein-based therapeutic angiogenesis, which relies on administering angiogenic growth factors and bioactive peptides, is a promising strategy that has been extensively investigated^[1,2].

However, despite encouraging preclinical outcomes, its clinical translation remains hampered by major limitations, including poor targeting efficiency, rapid systemic clearance, and susceptibility to proteolytic degradation in the hostile inflammatory environment, ultimately compromising bioavailability and therapeutic efficacy^[3,4]. Advanced drug delivery systems have been developed to address these limitations. Synthetic nanocarriers such as liposomes, polymeric nanoparticles, and micelles can improve the stability of therapeutic proteins; however, their clinical application is often constrained by limited targeting capability, rapid uptake by the mononuclear phagocyte system, and potential cytotoxicity or immunogenicity. Extracellular vesicles (EVs) have emerged as a promising alternative: their intrinsic biocompatibility, low immunogenicity, stability in circulation, and ability to cross biological barriers make them particularly attractive as natural nanocarriers for therapeutic delivery^[5-7].

Among EV-producing cells, Mesenchymal Stromal Cells (MSCs) are especially relevant due to their well-established regenerative and immunomodulatory properties. MSC-derived EVs (MSC-EVs) have been shown to recapitulate many beneficial effects of parental cells, including promoting angiogenesis and tissue repair. In addition, they display a natural tropism toward injured or inflamed tissues, making them suitable candidates for potential targeted delivery of therapeutic molecules^[8-11].

Beyond their natural cargo, EVs can be further engineered to carry or display specific proteins and peptides, enabling controlled delivery and improved targeting^[12]. This is commonly achieved through EV-associated scaffold proteins, such as tetraspanins, which allow bioactive molecules to be displayed on the vesicle surface while preserving vesicle integrity^[13,14]. Among these, CD63 is particularly well suited as an engineering platform: it tolerates genetic modification while maintaining correct membrane topology and efficient incorporation into vesicles, and its large extracellular loop provides a suitable insertion site for displaying bioactive peptides in direct contact with target-cell receptors^[13-21]. Notably, Zhang and colleagues^[22] comprehensively reviewed and catalogued 192 studies on EV engineering using various carrier proteins, concluding that tetraspanins represent the most widely employed class of carrier proteins, with CD63 being the most frequently used and extensively studied member.

Building on this platform, we previously introduced the HACK-EV concept - the reprogramming of MSC-EV biogenesis, conceptually inspired by the principles of computer hacking, to generate biologically

compatible nano-shuttles for the delivery of proteins and peptides^[23]. In the present study, we selected peptides derived from the Human Immunodeficiency Virus type 1 (HIV-1) matrix protein p17 as cargo for this platform, a unique source of angiogenic bioactive sequences. p17 and its derived peptides have evolved to mimic endogenous regulatory molecules, such as cytokines and chemokines, enabling efficient interaction with host receptors involved in processes such as inflammation and angiogenesis through molecular mimicry.

Accordingly, p17 promotes endothelial cell migration and angiogenesis via the chemokine receptors C-X-C motif chemokine receptor 1 (CXCR1) and CXCR2. Structure-function analyses have identified two functional regions responsible for this activity: F2, which acts through CXCR1/CXCR2 signaling under stress conditions, and F3 37-52, which mimics erythropoietin (EPO) and induces angiogenesis in a β common chain (β c) receptor-dependent manner under both basal and stress conditions^[24,25].

Several shorter F2- and F3-derived fragments have further been shown to retain this pro-angiogenic activity as soluble peptides^[26]. The identification of minimal bioactive motifs represents an attractive strategy for the development of peptide-based therapeutics, as shorter sequences may facilitate synthesis, reduce production costs, and improve their integration into engineered delivery platforms. Whether such minimal motifs remain active once physically constrained on a vesicle surface, however, is a separate question: membrane topology and steric hindrance may interfere with receptor recognition in ways that do not apply to a free peptide in solution. We hypothesized that MSC-derived EVs could serve as a platform for the surface display and delivery of p17-derived angiogenic peptides, while preserving their biological activity and that systematic shortening of the known F2 and F3 regions could help identify a minimal functional motif suitable for EV engineering. Accordingly, the present study was designed to address two main questions: (i) whether minimal angiogenic motifs can be identified within the HIV-1 p17 protein while retaining biological activity; and (ii) whether these motifs remain functional when displayed on the surface of MSC-derived EVs through a CD63-mediated engineering strategy. To this end, the previously characterized F2 and F3 regions were subjected to a rational structure-function analysis, novel shortened peptides were generated and functionally evaluated, and selected sequences were subsequently tested as EV-associated cargos.

METHODS

Production of recombinant HIV-1 p17 protein

Highly pure (> 98%) recombinant p17 protein (clade B isolate BH-10, amino acids 1-132, GenBank accession number M15654)^[27] was produced in lipopolysaccharide (LPS)-free and biologically active monomeric form, as previously described^[28]. Briefly, the coding sequence of p17 was cloned into the BamHI and EcoRI sites of the prokaryotic expression vector pGEX-2T (GE Healthcare / Cytiva, Marlborough, MA, USA), which ensures the production of large amounts of biologically active recombinant proteins. The glutathione S-transferase (GST)-linked p17 protein was expressed in BL21(DE3), an appropriate *Escherichia coli* strain for high-level protein production, by adding 0.8 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 4 h at room temperature, once the OD₆₀₀ reached a value of 0.6. The viral protein was purified by using Glutathione Sepharose 4B beads and cleaved from GST by thrombin. The p17 protein was further purified by reverse-phase Fast Protein Liquid Chromatography (FPLC). We analyzed protein fractions for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and identified them by Western blot using monoclonal antibodies to p17, such as MBS-3 [recognizing amino acid(s) (aa) 9-22]. Protein fractions were quantified by bicinchoninic acid (BCA) assay. The absence of endotoxin contamination in the recombinant p17 batches (< 0.125 EU/mL) was assessed by Limulus Amebocyte Lysate (LAL) assay (Associates of Cape Cod, Inc., East Falmouth, MA, USA).

Table 1. Amino acid and corresponding oligonucleotide sequences of p17-derived peptides analyzed in this study

Oligopeptide	Amino acid sequence	Oligonucleotides (5'-3')	Status
F2	EKIRLRPGGKKKYKLKHIVW	For: CCGGCAGAAAAATTCGGTTAAGGCCAGGGGAAAGAAAAATATAAATTAACATATAGTATGGGTAC Rev: CCATACTATATGTTTTAATTATATTTTCTTCCCTGGCCTTAACCGAATTTTTCTG	Previously reported (parental fragment) ^[26]
F3	KHIVWASRELERFAVNPGLLE	For: CCGGCAAAACATATAGTATGGGCAAGCAGGGAGCTAGAACGATTGCGAGTTAATCCTGGCCTGTTAGAAGTAC Rev: TTCTAACAGGCCAGGATTAAGTGCGAATCGTTCTAGCTCCCTGCTTGCCATACTATATGTTTTG	Previously reported (parental fragment) ^[26]
F3 (37-52)	ASRELERFAVNPGLLE	For: CCGGCAGCAAGCAGGGAGCTAGAACGATTGCGAGTTAATCCTGGCCTGTTAGAAGTAC Rev: TTCTAACAGGCCAGGATTAAGTGCGAATCGTTCTAGCTCCCTGCTTGCTG	Previously reported and named F3 ^[26]
F3 (37-44)	ASRELERF	For: CCGGCAGCAAGCAGGGAGCTAGAACGATTGCTAC Rev: GAATCGTTCTAGCTCCCTGCTTGCTG	Previously reported ^[26]
F2 (17-22)	EKIRLR	For: CCGGCAGAAAAATTCGGTTAAGGGTAC Rev: CCTTAACCGAATTTTTCTG	New in this study
F2 (26-33)	KKKYKLKH	For: CCGGCAAGAAAAATATAAATTAACATGTAC Rev: ATGTTTTAATTATATTTTCTTTG	New in this study
F3 (39-43)	RELER	For: CCGGCAAGGGAGCTAGAACGAGTAC Rev: TCGTTCTAGCTCCCTG	New in this study Refined from F3 (40-44)
F3 (40-44)	ELERF	For: CCGGCAGAGCTAGAACGATTGCTAC Rev: GAATCGTTCTAGCTCTG	New in this study

For: Forward; Rev: reverse.

Bioinformatic analysis and design of p17-derived peptides

An integrated bioinformatic approach was employed to design p17-derived peptides and identify minimal functional epitopes with pro-angiogenic activity. Starting with the HIV-1 matrix protein p17 (clade B isolate BH-10), the sequences corresponding to fragments F2 (amino acids 17-36: EKIRLRPGGKKKYKLKHIVW) and F3 (amino acids 32-52: KHIVWASRELERFAVNPGLLE) were analyzed.

Electrostatic and physicochemical properties, including molecular charge and hydrophobicity, were assessed using online resources such as UniProtKB (<http://www.uniprot.org/>), BIOPEP (<http://www.uwm.edu.pl/biochemia/index.php/en/biopep>), and PeptideRanker (http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptideranker.php). Hydrophobic (Ile34, Val35, Trp36) and nonpolar residues (Pro23, Gly24, Gly25), considered less likely to be involved in receptor interactions, were excluded during peptide refinement.

Structural accessibility of selected residues was verified using the p17 crystal structures available in the Protein Data Bank (PDB entries 1TAM for monomeric p17 and 1HIW for trimeric p17) to ensure extracellular exposure. Additionally, phylogenetic analysis and sequence alignment of HIV-1 p17 F3 with human EPO, and with the corresponding HIV-2 p17 fragment, were performed to further shorten the F3 sequence.

All peptides were chemically synthesized to > 98% purity (Thermo Fisher Scientific, Waltham, MA, USA). We calculated theoretical solubility and stability parameters, including isoelectric point (pI) and net charge at pH 7.0, using the ExPASy “Compute pI/Mw” tool and the Peptide Property Calculator (Peptide 2.0 Inc., Chantilly, VA, USA).

Previously described p17-derived peptides and those identified through bioinformatic analysis and used for EV engineering in this study are listed in Table 1; their functional characterization is detailed in the RESULTS section.

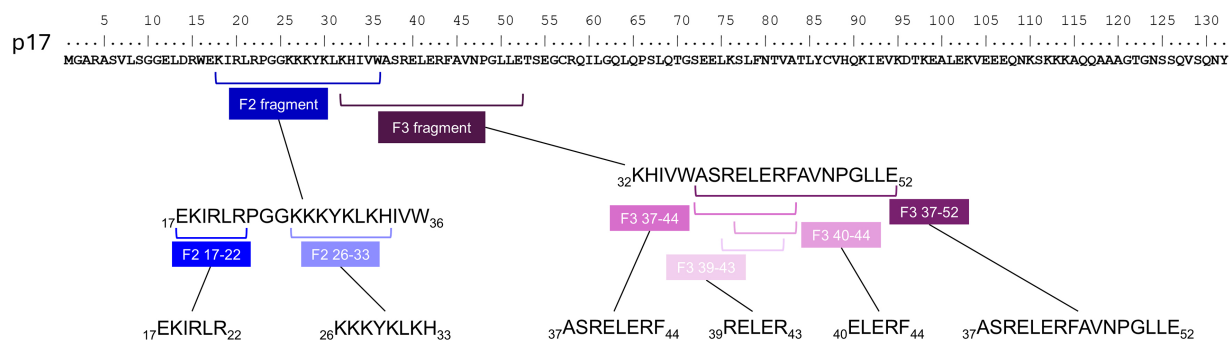


Figure 1. Schematic representation of the aa sequence of the HIV-1 p17 matrix protein and its derived synthetic fragments. The primary sequence of the full-length p17 protein (132 aa) is shown at the top. Two main regions, designated as the F2 (blue) and the F3 (purple) fragments, were further dissected into shorter sub-fragments to map specific functional sequences. The F2 region (aa 17-36) was subdivided into two smaller peptides: F2 17-22 (EKIRLR) and F2 26-33 (KKKYKCLKH). The F3 region (aa 32-52) was truncated into shorter sequences, including F3 37-52 (ASRELERFAVNPGLLE), F3 37-44 (ASRELERF), F3 40-44 (ELERF), and F3 39-43 (RELER). The aa positions are numbered according to the p17 reference sequence. The original illustration was created with Microsoft PowerPoint. aa: Amino acid; HIV-1: Human Immunodeficiency Virus type 1.

A schematic representation of the amino acid sequence of the HIV-1 p17 matrix protein and its derived synthetic fragments is shown in [Figure 1](#).

Plasmid design and generation of CD63-based constructs

The coding sequence of canine CD63 was amplified by high-fidelity polymerase chain reaction (PCR) from a previously described plasmid^[23] using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA). Primers were designed to introduce *Hind*III and *Xma*I restriction sites (forward: GGCAAGCTTCCATGGCGGTGGAAGG; reverse: AAAACCCGGGATGACTTCATAGCCACTTCGG). PCR amplification was performed according to the manufacturer's instructions, and the resulting amplicon was purified using a PCR Clean-Up kit (Zymo Research, Irvine, CA, USA). The purified CD63 fragment was then subcloned into the pTagGFP-2N expression vector (Evrogen, Moscow, Russia).

Mutated CD63 constructs were generated using a two-step overlap extension mutagenic PCR strategy. Briefly, two separate PCR reactions were carried out to amplify the N-terminal and C-terminal portions of CD63 using primers introducing *Age*I and *Kpn*I restriction sites. PCR products were purified, mixed in equimolar amounts, and subjected to an extension PCR to obtain the full-length mutated CD63 sequence. Amplified products were purified, quantified by spectrophotometry (NanoDrop™, Thermo Fisher Scientific), digested with the appropriate restriction enzymes (New England Biolabs), and ligated into the pTagGFP-2N vector using T4 DNA Ligase (New England Biolabs).

Ligation products were transformed into chemically competent *Escherichia coli* XL1-Blue cells by heat shock and plated on Luria-Bertani agar supplemented with kanamycin (50 micrograms/mL; Sigma-Aldrich, St. Louis, MO, USA). Bacterial colonies were screened by PCR, and positive clones were verified by Sanger sequencing.

Recombinant constructs were generated to express CD63 fused to green fluorescent protein (GFP) and functionalized with p17-derived peptides. GFP served as a reporter to detect functionalized chimeric constructs by fluorescence-based techniques (flow cytometry and fluorescence microscopy) and as a target for immunological detection (Western blotting and dot blot). Indeed, the short p17-derived peptides could not be reliably used as detection targets themselves, and CD63 was equally unsuitable, being a natural, endogenous component of EVs; the GFP tag therefore provided the only reliable means of distinguishing the

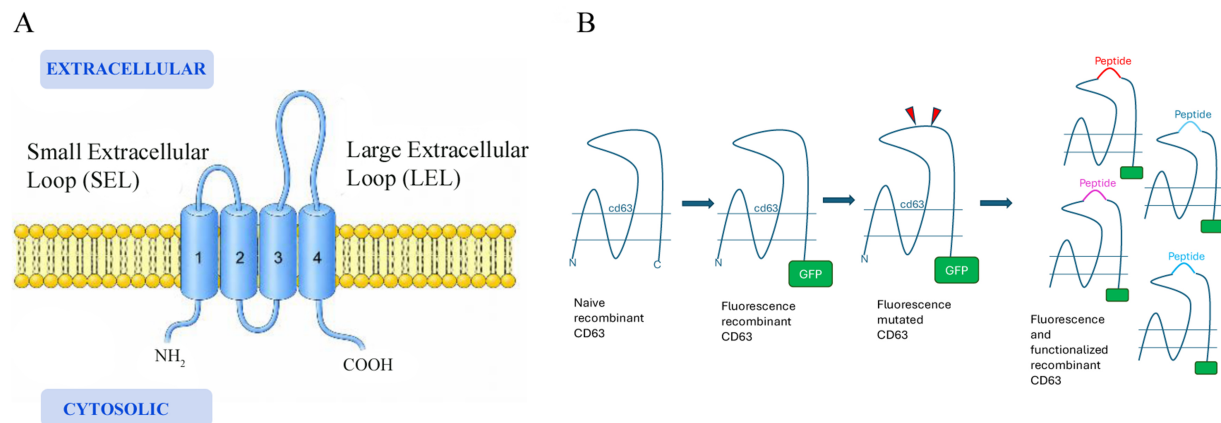


Figure 2. (A) Schematic representation of CD63 tetraspanin topology. Tetraspanins consist of four transmembrane domains (TM1-TM4) connected by a SEL and a LEL. The N- and C-terminal regions are located on the cytosolic head; (B) schematic representation of the stepwise engineering strategy used to evaluate different peptide sequences on the same scaffold protein (CD63). Native CD63 was first fused to GFP and then modified to introduce a peptide insertion site. Different peptides were subsequently displayed on the extracellular domain, enabling comparative functional analysis while maintaining a constant structural backbone. CD63: Cluster of differentiation 63; GFP: green fluorescent protein; LEL: large extracellular loop; SEL: small extracellular loop; TM: transmembrane domain.

engineered constructs from endogenous vesicle components. The empty backbone vector encoding CD63 fused to GFP without any peptide insert (pTagGFP2N-CD63) was used to generate control EVs (EV-CD63-GFP). This construct served as an engineered baseline control to account for potential non-specific effects associated with genetic manipulation and GFP tag expression.

The second extracellular loop of CD63 was selected as the insertion site for peptide exposure, based on structural topology analysis [Figure 2]. Plasmids encoding CD63 fused to p17-derived oligopeptides were obtained by ligating annealed complementary oligonucleotides into the *KpnI* and *AgeI* restriction sites previously introduced into the mutated CD63 backbone. Briefly, forward and reverse oligonucleotides were mixed at equimolar concentrations, heated to 80 °C, and slowly cooled to room temperature to allow correct base pairing before ligation. Bacterial transformation, clone selection, and sequence verification were performed as described above. Oligonucleotide sequences encoding p17-derived peptides are reported in Table 1. As the F3 40-44 peptide failed to show biological activity *in vitro* (see the RESULTS section), the corresponding construct was generated, but EVs exposing this peptide were not produced.

Isolation, culture and transfection of MSCs

MSCs were isolated from canine adipose tissue collected for therapeutic purposes. Surplus cells not required for clinical application were used for this study with the informed consent of the owner. The study protocol was reviewed and approved by the Comitato Universitario di Bioetica dell'Università degli Studi di Perugia (Bioethics Committee of the University of Perugia), protocol number 6/2026.

MSC isolation was performed according to a protocol previously described by Scattini *et al.*^[29] and further standardized for EV production and genetic manipulation^[23].

Briefly, adipose tissue samples were enzymatically digested with 0.1% Collagenase I (Worthington Biochemical Corp., Lakewood, NJ, USA) and plated to allow stromal cell adhesion. MSCs were expanded in Dulbecco's Modified Eagle Medium- low glucose (DMEM-LG, Euroclone S.p.A., Milano, Italy) supplemented with 10% fetal bovine serum (FBS; Euroclone S.p.A., Milan, Italy) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) in TPP tissue-culture flasks (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and maintained at 37 °C in a humidified atmosphere.

with 5% CO₂. Culture medium was replaced every 2-3 days, and cells were used between passages 3 and 5. MSCs were seeded to reach approximately 60% confluence at the time of transfection. Cells were transfected using Lipofectamine™ Stem Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) with plasmid DNA at a final amount of 250 ng/cm², complexed with the transfection reagent at a 1:4 (DNA: reagent) ratio in DMEM-LG. After 6 h of incubation, FBS was added to restore a final concentration of 10%. 24 h after transfection, the medium was replaced with serum-free DMEM-LG to reduce contamination by FBS EVs, and cells were incubated for an additional 48 h to allow the release of engineered EVs.

Isolation and characterization of EVs

EV isolation and characterization were performed in accordance with the Minimal Information for Studies of Extracellular Vesicles 2023 (MISEV2023) guidelines^[30]. Conditioned media from MSCs transfected with different constructs were collected 72 h after transfection and processed. Briefly, culture supernatants were centrifuged at 2,000 × g for 15 min at 4 °C to remove detached cells and cellular debris. The clarified supernatant was subsequently concentrated using Vivaspin® ultrafiltration units with a 100 kDa molecular-weight cut-off (Sartorius, Göttingen, Germany), centrifuging at 2,000 × g at 4 °C for 30 min, repeating centrifugation cycles as needed to reach a final concentration factor of approximately 100-150-fold. Concentrated samples were washed twice with sterile 0.1 micrometer-filtered phosphate-buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA, USA) and subjected to a second ultrafiltration step.

EV quantification and size distribution

Concentrated conditioned media were analyzed by Nanoparticle Tracking Analysis (NTA) using a NanoSight NS300 system equipped with a 488 nm laser (Malvern Panalytical, Malvern, UK) to determine EV concentration and size distribution.

Prior to analysis, 5-10 µL of each sample was diluted 1:100 in 0.1 micrometer filtered ultrapure water. Samples were infused using a syringe pump at a constant flow rate of 30 microliter/min. For each sample, five 60 s videos were recorded at a frame rate of 25 fps. Video acquisition was performed with the Camera Level set at 13. Data were subsequently analyzed using NTA software v3.4, with a Detection Threshold set at 5.

Western blotting and dot blot

Detection of CD63-GFP carrying p17-derived peptides in MSCs and EVs was performed by Western blotting. MSCs were lysed in radioimmunoprecipitation assay (RIPA) buffer, and 10 micrograms of total protein was mixed with Laemmli buffer and heated at 95 °C for 5 min; EV samples (10⁹ particles per sample) were mixed directly with Laemmli buffer and heated at 95 °C for 5 min for Alix, SDCBP, Mitofilin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) analysis, or at 37 °C for 30 min for GFP blotting. Samples were loaded onto NuPAGE® Novex® 10% Bis-Tris Protein Gel (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA), followed by electrophoresis at 120 V for 2 h. Proteins were then transferred onto a nitrocellulose membrane and blocked with 5% non-fat milk for 1 h. The dot blot was performed by depositing a suspension of 2 × 10⁸ EVs in a final volume of 5 microliter onto a nitrocellulose membrane and allowing it to air-dry for 2 h; the membrane was then blocked and incubated with antibodies, following the same procedure as for Western blot membranes.

Membranes were incubated overnight at 4 °C with primary antibodies diluted in 5% non-fat milk with gentle shaking. Subsequently, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Target proteins were detected using an ImageQuant LAS 500 system (Cytiva, Marlborough, MA, USA) after adding ECL solution. Raw chemiluminescence images were analyzed using ImageJ to perform densitometric analysis.

Antibody list: anti-GFP diluted 1:7,500 (A6455, Life Technologies, Carlsbad, CA, USA), anti-Alix diluted 1:500 (sc-271975, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Mitofilin diluted 1:8,000 (ab109424, Abcam, Cambridge, MA, USA), anti-GAPDH diluted 1:10,000 (GTX627408, GeneTex, Irvine, CA, USA), anti-Syntenin diluted 1:8,000 (SDCBP) (ab62530, Abcam, Cambridge, MA, USA), anti-CD81 diluted 1:200 (sc-166029, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-mouse immunoglobulin G (IgG) HRP-conjugated diluted 1:10,000 (A90-116P, Fortislife, Boston, MA, USA), and goat anti-rabbit IgG HRP-conjugated diluted 1:10,000 (A120-101P, Fortislife, Boston, MA, USA).

Flow cytometry

To compare samples, an equal amount of starting material was used, and EVs isolated from transfected and untransfected MSCs were analyzed by flow cytometry to assess the presence of GFP-positive events. Based on particle count data, we diluted EV preparations in 0.22 micrometer-filtered PBS to achieve an identical final concentration of 10^7 particles/mL across all samples. For Triton X-100 treatment, vesicles were pre-incubated with 1% (v/v) Triton X-100 on ice for 1 h before dilution. Samples were acquired on a flow cytometer equipped with 405 and 488 nm lasers (CytoFlex; Beckman Coulter, Brea, CA, USA). The flow cytometer was calibrated with a mixture of FITC-positive polystyrene beads (Megamix-Plus FSC and SSC beads; BioCytex, Marseille, France) to identify particles in the nanometric (100, 160, 200, 240, 300, 500 and 900 nm) range in the Violet SSC-H and FITC-H channels. For each diluted sample, 50 μ L of each sample was acquired at a constant flow rate of 10 μ L/min for 5 min. After exclusion of background signal in untransfected controls, GFP-positive events were identified by applying a defined fluorescence gate in the FITC channel. Data were expressed as the number of GFP-positive events within the FITC-positive gate. Details on the gating strategy, instrument calibration, and Triton X-100 controls are provided in [Supplementary Figure 1](#).

Electron microscopy

EV preparations were assessed by electron microscopy. Aliquots of the vesicle suspensions (10 μ L) were deposited as individual droplets onto a sheet of Parafilm, and a formvar-coated nickel grid (Electron Microscopy Sciences, Hatfield, PA, USA) was laid face-down onto each droplet and left for 30 min to allow adsorption inside a humidified chamber.

For scanning electron microscopy (SEM), grids were subsequently mounted onto metal stubs. Samples were sputter-coated with a 10 nm chromium layer and observed with a ZEISS-LEO 1525 scanning electron microscope (Laboratorio Universitario di Nanomateriali, University of Perugia).

For transmission electron microscopy (TEM), after adsorption, grids were rinsed sequentially in PBS and distilled water, negatively stained with 2% uranyl acetate for 5 min, and air-dried. Samples were examined using a Philips EM208 transmission electron microscope equipped with a digital camera (CUMEF - University Centre of Electron Microscopy and Fluorescent Microscopy, University of Perugia).

Endothelial cell culture and angiogenesis assays

Cell cultures

Human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell GmbH (Heidelberg, Germany). Cells were cultured in Endothelial Growth Medium (EGM) (PromoCell GmbH) supplemented with 10% (v/v) FBS (Thermo Fisher Scientific Inc., Waltham, MA, USA) and maintained at 37 °C in a humidified atmosphere with 5% CO₂. All experiments were performed using cells between passages 5 and 7.

Wound healing assay

HUVECs were seeded at a density of 10^5 cells per well in 24-well plates in EGM containing 10% FBS. Confluent monolayers were serum-starved for 16 h in Endothelial Basal Medium (EBM) (PromoCell GmbH) supplemented with 0.5% FBS. Monolayers were then scratched using a sterile 200 μ L pipette tip.

After washing to remove cellular debris, cells were left untreated or treated with 10 ng/mL of full-length p17, a scrambled control peptide, or p17-derived fragments (F2, F2 17-22, F2 26-33, F3, F3 37-52, F3 37-44, F3 40-44, and F3 39-43) in complete medium (EGM supplemented with 10% FBS), and maintained at 37 °C in humidified atmosphere with 5% CO₂ for 12 h.

For experiments performed under standard conditions, confluent monolayers were scratched without prior serum starvation and treated as described above, except that wound closure was assessed after 10 h.

In vitro tube formation assay

HUVECs were cultured under standard conditions (EGM containing 10% FBS) and serum-starved in medium containing 0.5% FBS for 16 h. Harvested cells were resuspended in EGM supplemented with 10% FBS and seeded at a density of 5×10^4 cells per well onto 48-well plates previously coated with Cultrex Reduced Growth Factor Basement Membrane Extract (RGF BME; TemaRicerca, Castenaso, BO, Italy).

Cells were then left untreated or treated for 8 h with 10 ng/mL of full-length p17, a scrambled control peptide, or p17-derived fragments (F2, F2 17-22, F2 26-33, F3, F3 37-52, F3 37-44, F3 40-44, and F3 39-43) in a humidified atmosphere with 5% CO₂.

For experiments performed under standard conditions, cells were seeded without prior serum starvation and treated as described above.

For assays using MSC-EVs, cells were serum-starved for 4 h in EBM supplemented with 0.5% FBS before treatment. EVs tested included those engineered to carry p17-derived peptides (EV-F2, EV-F2 17-22, EV-F2 26-33, EV-F3, EV-F3 37-52, EV-F3 37-44, and EV-F3 39-43), EVs engineered with the empty backbone CD63-GFP (EV-CD63-GFP) as a negative control, and untreated EVs (EV-UT).

Harvested cells were then resuspended in EGM supplemented with 1% FBS and seeded at a density of 2.8×10^4 cells per well onto black 96-well μ -plates with round-bottom wells for high-throughput imaging (Ibidi GmbH, Gräfelfing, Germany), previously coated with RGF BME. Cells were subsequently treated with different EV preparations for 14 h.

Tube formation was monitored using an inverted microscope, and images were acquired for each experimental condition. Tube formation was quantified manually from phase-contrast microscopy images by counting closed mesh structures per well. To avoid observer bias, an operator blinded to the treatment groups quantified the images. Quantitative analysis of angiogenic potential was expressed as the number of tubes formed per well.

Statistical analysis

All wound healing and tube formation assays were performed in three independent biological replicates (cell passages 5-7), with each condition tested in triplicate. Data are expressed as mean $\pm$ standard deviation (SD). Statistical significance among multiple treatment groups was evaluated using one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test for pairwise comparisons. All statistical analyses were

performed using GraphPad Prism software (version 8.0, GraphPad Software, La Jolla, CA, USA). In accordance with international reporting guidelines, exact *P*-values are reported throughout the text and figure legends. Differences were considered statistically significant at $P < 0.05$. Statistical significance with a calculated *P*-value lower than 0.0001 is explicitly indicated as **** $P < 0.0001$.

RESULTS

Identification of HIV-1 p17 F2- and F3-derived peptides with potential angiogenic activity

The HIV-1 p17 F2 fragment (aa 17-36: EKIRLRPGGKKKYKLKHIVW) has been shown to be involved in angiogenic activity under stress conditions^[26]. To identify the smallest peptides derived from the F2 sequence capable of inducing angiogenesis, a structure-function analysis was performed. This analysis identified two motifs that are critical for receptor binding: an ELR-like motif (RLR aa 20-22), known to mediate CXCR1 interaction^[31], and a highly polybasic region (KKKYKLKHI aa 26-34) involved in binding to heparan sulfate proteoglycans (HSPGs) and CXCR1^[32].

Accordingly, the F2 fragment was refined by excluding non-essential hydrophobic/nonpolar residues (Ile34, Val35, Trp36; Pro23, Gly24, Gly25), which are less likely to participate in receptor interactions, and by focusing on the acidic (Glu) and basic (Arg, Lys) residues that typically drive such interactions. Based on these considerations, two sub-fragments were derived as potential candidates for triggering angiogenesis under stress conditions: F2 17-22 (EKIRLR) and F2 26-33 (KKKYKLKH) [Figure 1]. Structural analysis further confirmed that the proposed sub-fragments are exposed to the extracellular environment in both the monomeric and trimeric p17 protein structures (PDB entries 1TAM and 1HIW), supporting their potential for receptor interaction.

Previous studies on the HIV-1 p17 F3 fragment (aa 32-52: KHIVWASRELERFAVNPGLLE), which induces angiogenesis under both stress and non-stress conditions through molecular mimicry with EPO, led to the identification of two angiogenic fragments, F3 37-52 and F3 37-44 (an 8-amino-acid sequence ASRELERF)^[26] [Figure 1].

To identify the minimal functional sequence, a comparative analysis of HIV-1, HIV-2, and EPO sequences was performed, focusing on conserved residues and conservative amino acid substitutions with similar physicochemical properties.

The HIV-2 sequence was included in the analysis because the p17 proteins of both HIV-1 and HIV-2 contain a functional angiogenic motif within their respective F3 37-44 regions (ASRELERF for HIV-1 and AANELDRF for HIV-2), both of which promote angiogenesis in HUVECs despite sequence divergence^[26]. This comparative sequence alignment highlighted a conserved V/ELERx motif (aa 40-44) [Figure 1]. Consequently, the pentapeptide F3 40-44 (ELERF) was selected as a promising candidate motif for promoting angiogenesis. The C-terminal phenylalanine (Phe44) was initially retained because it is structurally conserved across viral variants^[26], suggesting a key functional role in mediating biological activity.

Finally, theoretical solubility profiling indicated that these shortened, hydrophilic peptides, characterized by a high proportion of charged residues (> 25%) and a low hydrophobic content (< 25%), exhibit good solubility in aqueous solutions, a critical feature for their potential application as angiogenic factors.

Pro-migratory and angiogenic activity of p17 F2- and F3-derived peptides

The biological activity of the identified p17-derived peptides was evaluated *in vitro* in HUVECs at 10 ng/mL using wound healing and tube formation assays.

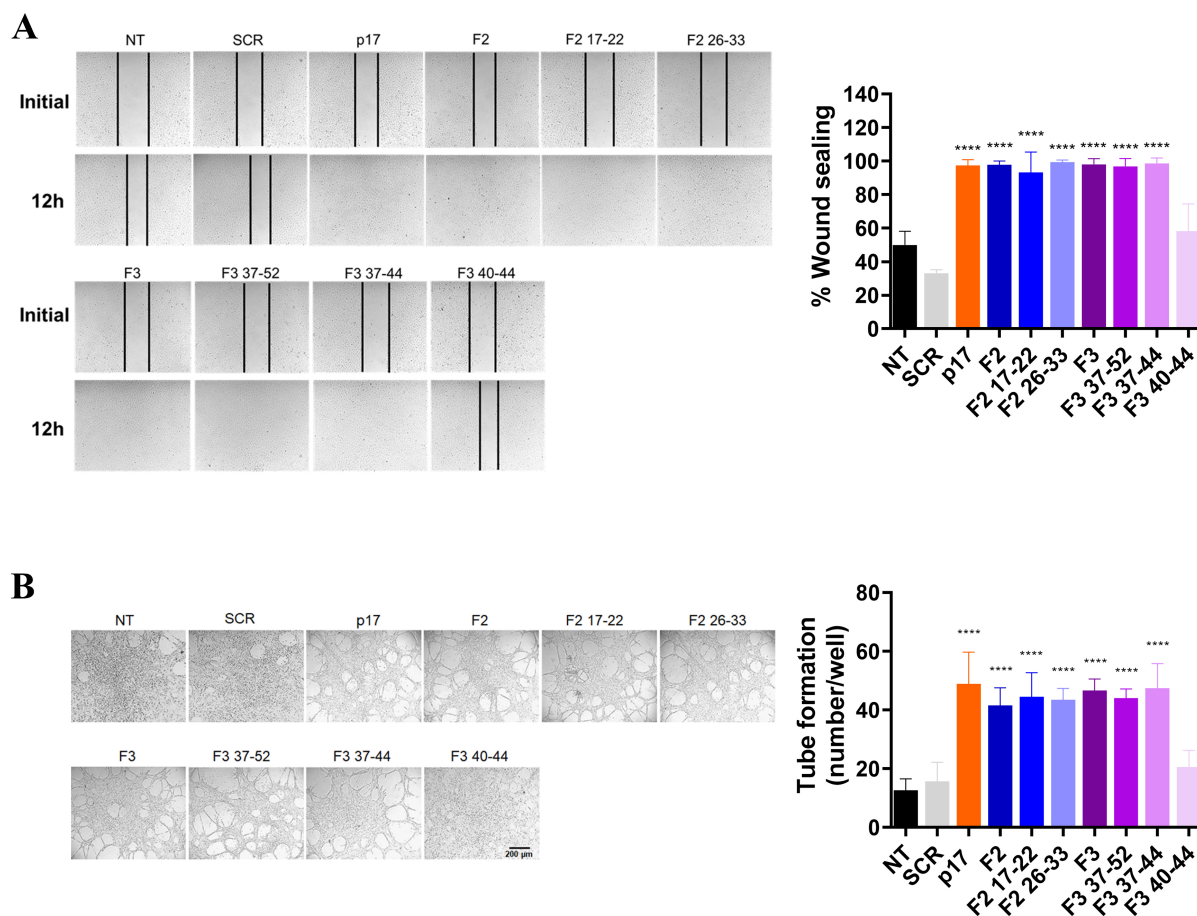


Figure 3. Effects of different p17 F2- and F3-derived peptides on migration and tube formation of HUVECs under stress conditions. (A) Pro-migratory activity evaluated by a wound-healing assay in serum-starved HUVECs treated with NT, SCR, full-length p17, or the indicated p17-derived peptides; (B) angiogenic activity evaluated by tube formation assay in HUVECs, treated as indicated. Scale bar: 200 μ m. (A and B) Images are representative of three independent experiments with similar results (original magnification, $\times 10$). Data are expressed as mean $\pm$ SD of three independent experiments performed in triplicate. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post hoc test ($^{****}P < 0.0001$). ANOVA: Analysis of variance; HUVEC: human umbilical vein endothelial cell; NT: no treatment; SCR: scrambled peptide; SD: standard deviation.

In the wound healing assay, HUVECs starved under stress conditions for 16 h and treated with the reference peptide F2, as well as with its derived fragments F2 17-22 and F2 26-33, showed enhanced wound closure within 12 h of treatment. The full-length p17 protein, used as a positive control, induced a significant pro-migratory effect on HUVECs relative to untreated cells, comparable to that observed with F2 and its derived fragments. As expected, the scrambled peptide negative control did not promote wound closure [Figure 3A].

In contrast, the candidate peptide ELERF (F3 40-44), derived from F3, failed to exhibit pro-migratory activity in wound healing assays under stress conditions. Conversely, the peptides F3, F3 37-52, and F3 37-44, used as positive controls, significantly promoted endothelial repair and complete wound closure, similar to p17, F2, and F2-derived fragments [Figure 3A]. The angiogenic activity of the F2-derived (F2 17-22 and F2 26-33) and F3-derived peptides was then evaluated using tube formation assays in HUVECs cultured under stress conditions for 16 h. F2-derived peptides, similar to F2, significantly induced tubular structure formation after 8 h of treatment compared with untreated control cells. In contrast, F3 40-44 was unable to promote tube formation, like the scrambled peptide, whereas F3, F3 37-52, and F3 37-44 exhibited angiogenic activity comparable to that of the full-length p17 protein [Figure 3B].

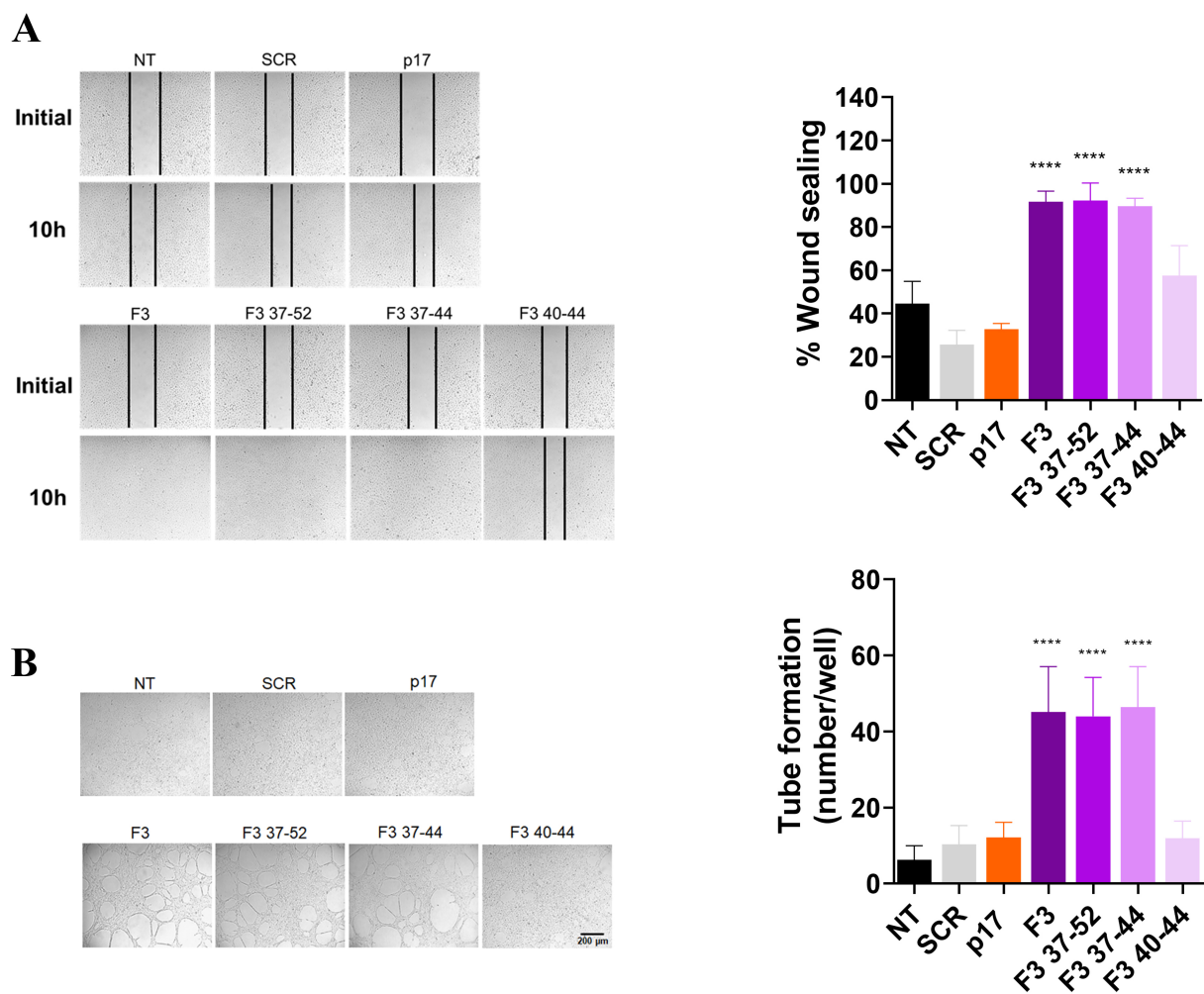


Figure 4. Effects of p17 F3-derived peptides on migratory and tube formation activity of HUVECs under standard conditions. (A) Pro-migratory activity evaluated by wound healing assay in HUVECs cultured under standard conditions, treated with NT, SCR, full-length p17, or the indicated F3-derived peptides (F3; F3 37-52; F3 37-44; F3 40-44); (B) angiogenic activity evaluated by tube formation assay in HUVECs cultured under standard conditions, treated as indicated. Scale bar: 200 μ m. (A and B) Images are representative of three independent experiments with similar results (original magnification, $\times 10$). Data are expressed as mean $\pm$ SD of three independent experiments performed in triplicate. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni's post hoc test (**** $P < 0.0001$). ANOVA: Analysis of variance; HUVEC: human umbilical vein endothelial cell; NT: no treatment; SCR: scrambled peptide; SD: standard deviation.

Consistent with these findings, wound healing and tube formation assays were also performed under standard culture conditions to further assess the lack of pro-migratory and angiogenic activity of the F3-derived fragment F3 40-44. Under these conditions, F3 40-44 did not exhibit any detectable activity [Figure 4], showing results comparable to untreated cells and cells treated with the scrambled peptide.

While the pentapeptide F3 40-44 (ELERF) was initially selected based on its conservation across HIV-1, HIV-2, and EPO, its subsequent lack of *in vitro* activity prompted us to evaluate the adjacent pentapeptide F3 39-43 (RELER). We hypothesized that the new peptide would benefit from both the introduction of the basic arginine 39 (Arg39) and the removal of the bulky hydrophobic phenylalanine 44 (Phe44)-despite the conservation of Phe44 in HIV-1 and HIV-2-to generate a palindromic sequence. This sequence adjustment allowed us to test whether shifting the sequence by a single residue, while maintaining a 5-amino-acid length, could restore the pro-angiogenic activity. Treatment with 10 ng/mL F3 39-43 resulted in a significant increase in endothelial cell migration, leading to complete wound closure, and in the formation of tubular

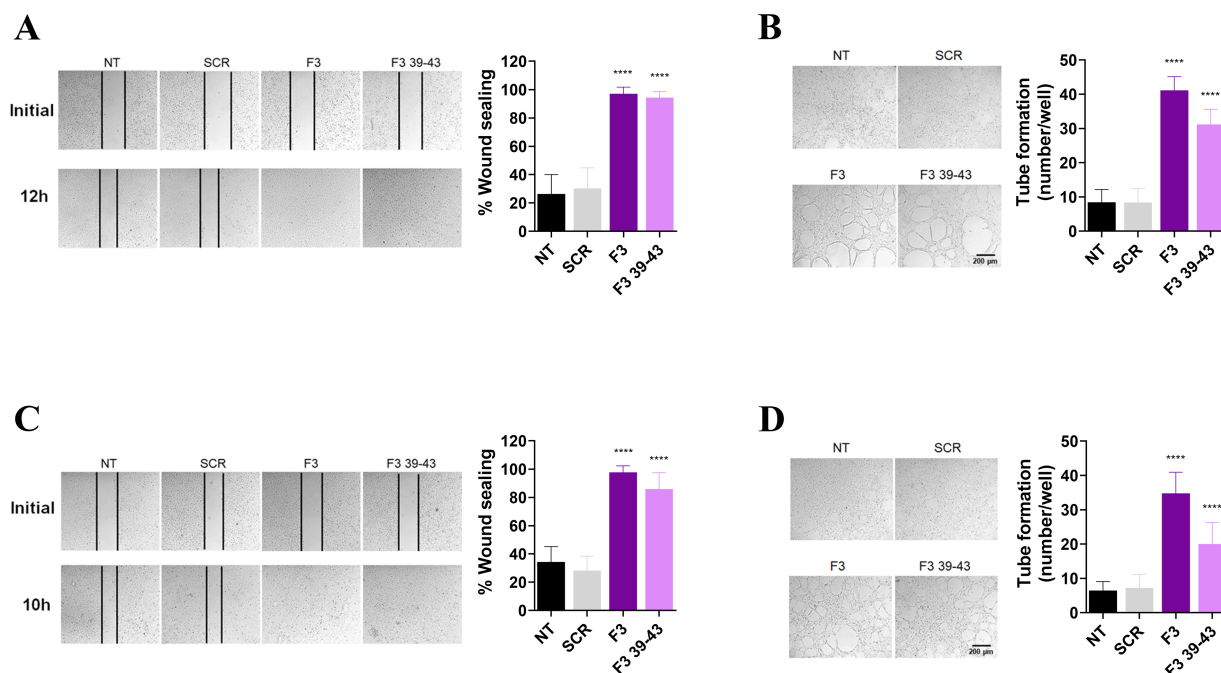


Figure 5. Effects of the p17-derived peptide F3 39-43 on pro-migratory and angiogenic activity of HUVECs under stress and standard conditions. (A and C) Pro-migratory activity evaluated by wound healing assay in HUVECs cultured under serum-starved conditions (A) and under standard conditions (C), treated with NT, SCR, F3, or F3 39-43; (B and D) angiogenic activity was evaluated by tube formation assay in HUVECs cultured under serum-starved conditions (B) and under standard conditions (D), treated as indicated. Scale bar: 200 μ m. (A-D) Images are representative of three independent experiments with similar results (original magnification, $\times 10$). Data are expressed as mean $\pm$ SD of three independent experiments performed in triplicate. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post hoc test (**** $P < 0.0001$). ANOVA: Analysis of variance; HUVEC: human umbilical vein endothelial cell; NT: no treatment; SCR: scrambled peptide; SD: standard deviation.

structures. These effects were observed under both starved [Figure 5A and B] and standard culture conditions [Figure 5C and D].

The activity of F3 39-43 was comparable to that of the reference peptide F3 and significantly higher than that observed in untreated cells or in cells treated with the scrambled peptide used as a negative control.

MSC morphological features before and after transfection

Canine MSCs exhibited a typical fibroblast-like morphology and strong adherence to plastic throughout the culture period [Figure 6A and B]. Cells expanded efficiently under standard conditions and maintained a homogeneous morphology within the passage range used for EV production (P3-P5). No evident cytotoxic effects or major morphological alterations were observed following transfection with CD63-based constructs [Figure 6C].

Replacement of the culture medium with serum-free DMEM-LG 24 h after transfection did not visibly affect cell morphology and enabled the collection of EV-enriched conditioned media after an additional 48 h. Fluorescence microscopy confirmed the expression of CD63-GFP and peptide-functionalized variants in transfected MSCs, showing a localization consistent with vesicular compartments [Figure 6C].

Characterization of MSC-EVs

EVs were successfully isolated from conditioned media by ultrafiltration. NTA showed that both untransfected MSC-EVs (UT) and engineered EVs displayed size distributions consistent with small EV populations. Most particles were detected within the 80-150 nm range, with comparable modal diameters

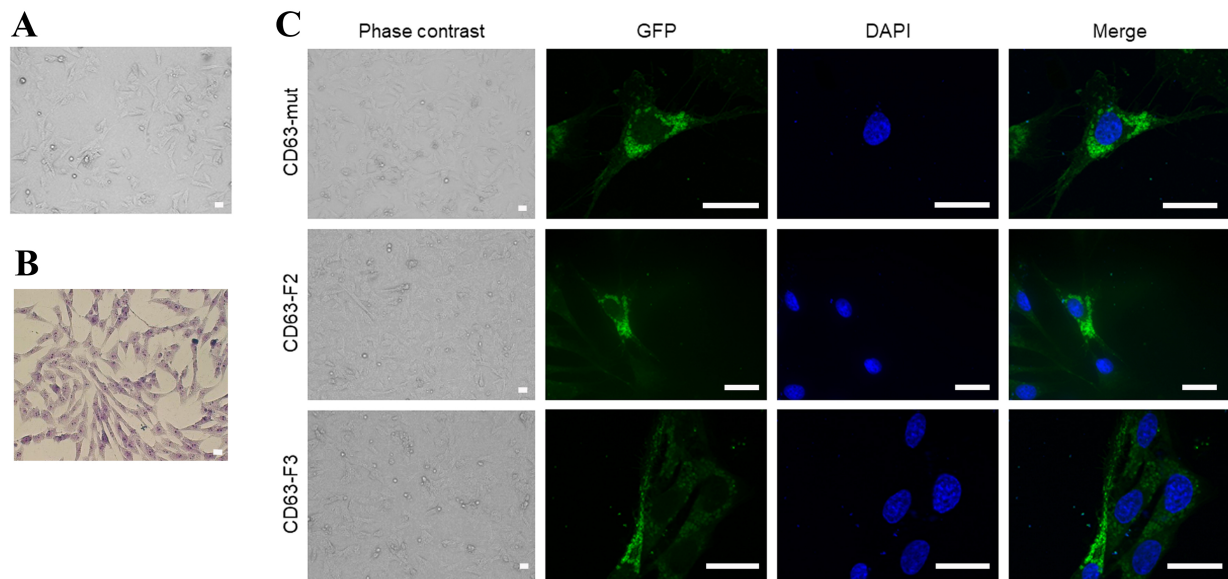


Figure 6. Canine MSC morphology and expression of CD63-based constructs. (A) Phase-contrast image; (B) representative Giemsa-stained image of untransfected canine MSCs in culture, both images showing a typical fibroblast-like morphology and strong adherence to plastic. Scale bar: 20 micrometer; (C) Distribution and localization of CD63-GFP, CD63-F2-GFP, and CD63-F3-GFP in transfected MSCs. Phase-contrast images; GFP, DAPI, and merged images. Scale bar: 20 micrometer. CD63: Cluster of differentiation 63; DAPI: 4',6'-diamidino-2-phenylindole; GFP: green fluorescent protein; MSC: mesenchymal stromal cell.

among untransfected EVs, EV-CD63-GFP, and EVs functionalized with p17-derived peptides [Figure 7A]. Quantitative NTA revealed that overall EV yields were not substantially affected by MSC transfection or by expression of CD63-based fusion constructs. Similar particle concentrations were obtained from untransfected MSCs, MSCs expressing CD63-GFP, and MSCs engineered to express CD63-peptide constructs. NTA revealed particle concentrations in the range of 10^{10} - 10^{11} particles/mL across all concentrated samples, corresponding to $2\text{--}7 \times 10^8$ particles/mL in the MSC-conditioned media, with an average yield of $0.8\text{--}1.4 \times 10^9$ particles per million cells [Figure 7B], supporting the suitability of the obtained EV preparations for downstream analyses.

Western blot analysis confirmed enrichment of EV-associated markers (Alix) and depletion of cellular contaminants (Mitofilin, a mitochondrial marker, and GAPDH, a cytosolic marker) in the EV fraction compared with the corresponding cell lysates, consistent with a *bona fide* EV preparation largely free of cellular debris. GFP detection in cell lysates confirmed expression of the CD63-GFP fusion constructs, while its presence in the EV fraction - restricted here to representative populations (CD63-mut, F2, F3) - further validated the successful sorting of the engineered constructs into EVs [Figure 7C].

Electron microscopy further confirmed the identity of the isolated particles. Both SEM and TEM analyses revealed round-shaped vesicles consistent with the typical morphology of EVs, free of major aggregates or cellular debris, with sizes in agreement with the range determined by NTA [Figure 7D].

Flow cytometry analysis was performed to assess EV-associated GFP signal across conditions. Due to the application of a fluorescence threshold on the FITC channel, the acquisition was biased toward GFP-positive events, while only a fraction of non-fluorescent particles was detected. As a result, the percentage of positive events could not accurately reflect the true proportion of fluorescent EVs and was therefore not used for quantitative comparisons [Figure 8A]. Under identical dilution and acquisition settings for all samples, the relative number of GFP-positive EVs was instead used for comparative analysis. As shown in Figure 8B,

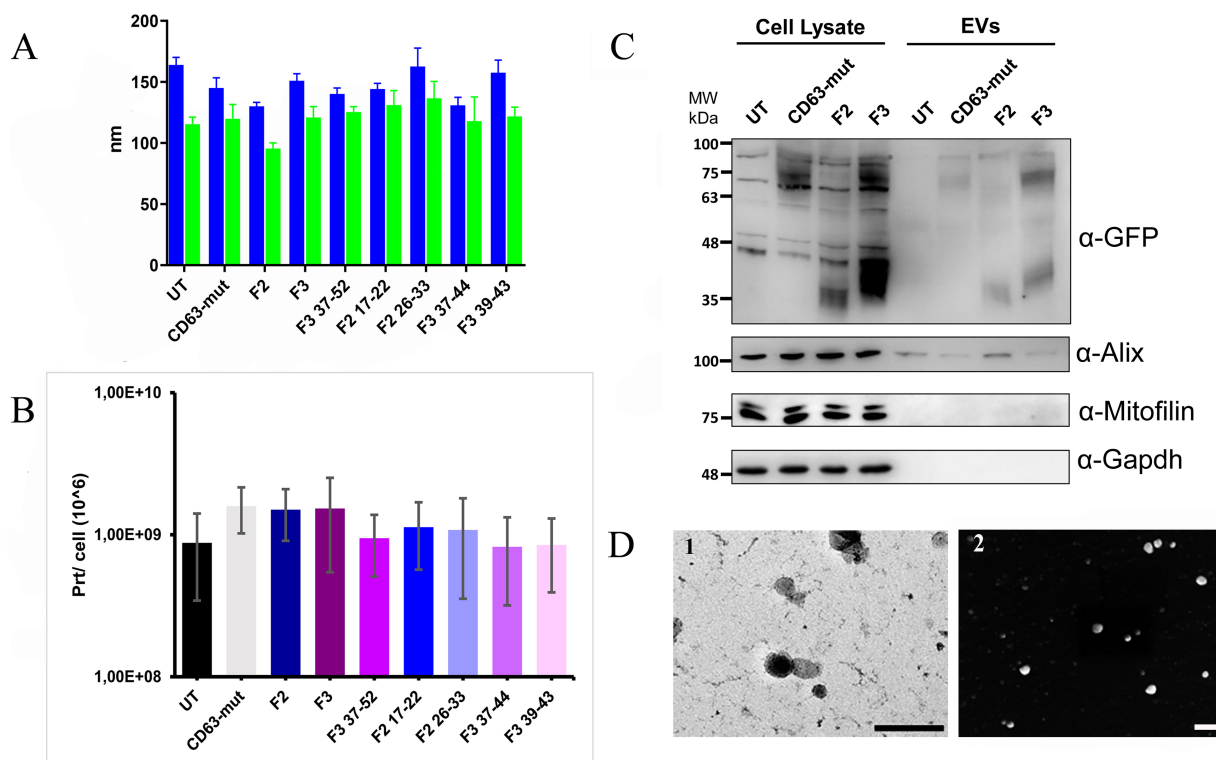


Figure 7. Characterization of MSC-EVs. (A) Size distribution of EVs derived from UT MSCs and MSCs transfected with the different plasmids, presented as a bar histogram; blue bars show the mean size, and green bars show the mode size; (B) EV recovery yield from conditioned medium of UT MSCs and MSCs transfected with different plasmids, normalized to the number of cells counted after conditioned medium collection and expressed as particles per million cells; (C) Western blot analysis of cell lysates and corresponding EVs from UT cells and cells expressing CD63-mut, F2-, or F3-engineered constructs, probed for GFP (construct expression/EV loading), Alix (EV marker), Mitofilin (mitochondrial contamination marker), and GAPDH (cytosolic contamination marker). The EV populations shown are representative of the engineered constructs and are not intended to depict the full panel of peptide variants characterized in this study; they confirm EV identity and purity across the engineering strategy rather than validate each individual peptide construct. Molecular weight markers are indicated on the left and expressed in kDa. Raw, uncropped blots corresponding to (C) are shown in [Supplementary Figure 2A-E](#); (D) Representative electron microscopy images of EV preparations used throughout the study. In D1, a TEM image of MSC-derived EVs, showing their characteristic size range and morphology (scale bar = 200 nm). In D2, a SEM image of MSC-derived EVs, showing their typical rounded morphology. CD63: Cluster of differentiation 63; EHT: extra high tension; EV: extracellular vesicle; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GFP: green fluorescent protein; kDa: kilodalton; KX: kilo-times; MSC: mesenchymal stromal cell; NTA: nanoparticle tracking analysis; SEM: scanning electron microscopy; TEM: transmission electron microscopy; UT: untransfected; WD: working distance.

CD63-GFP and F3-tagged CD63 samples exhibited the highest number of GFP-positive EVs. Notably, the F3 39-43 variant also showed relatively high levels of GFP-positive EVs, approaching those observed for the CD63-GFP control. In contrast, F2 constructs displayed generally lower counts, indicating reduced or variable labeling efficiency, a finding also confirmed by Western blotting and dot blot results on EVs carrying the F2 peptide [Figure 8C and D]. Details on the gating strategy, instrument calibration, and Triton X-100 controls are provided in [Supplementary Figure 1](#).

Western blotting analysis confirmed the presence of peptide-tagged CD63 in the EVs derived from transfected MSCs [Figure 8C]. The CD63-mut construct (CD63-GFP lacking peptide insertion) appeared as a single signal at approximately 75 kDa, whereas the peptide-tagged constructs appeared as two distinct signals, one at 75 kDa and one at approximately 40-45 kDa, with the relative proportion of the two bands varying between samples. Positivity to Alix, Syntenin (SDCBP), and CD81 confirmed the identity of the EVs, while the GFP dot blot confirmed the presence of GFP-tagged, engineered EVs, consistent with the flow cytometry results [Figure 8A], and allowed quantification of the GFP signal used in Figure 8D. The relative

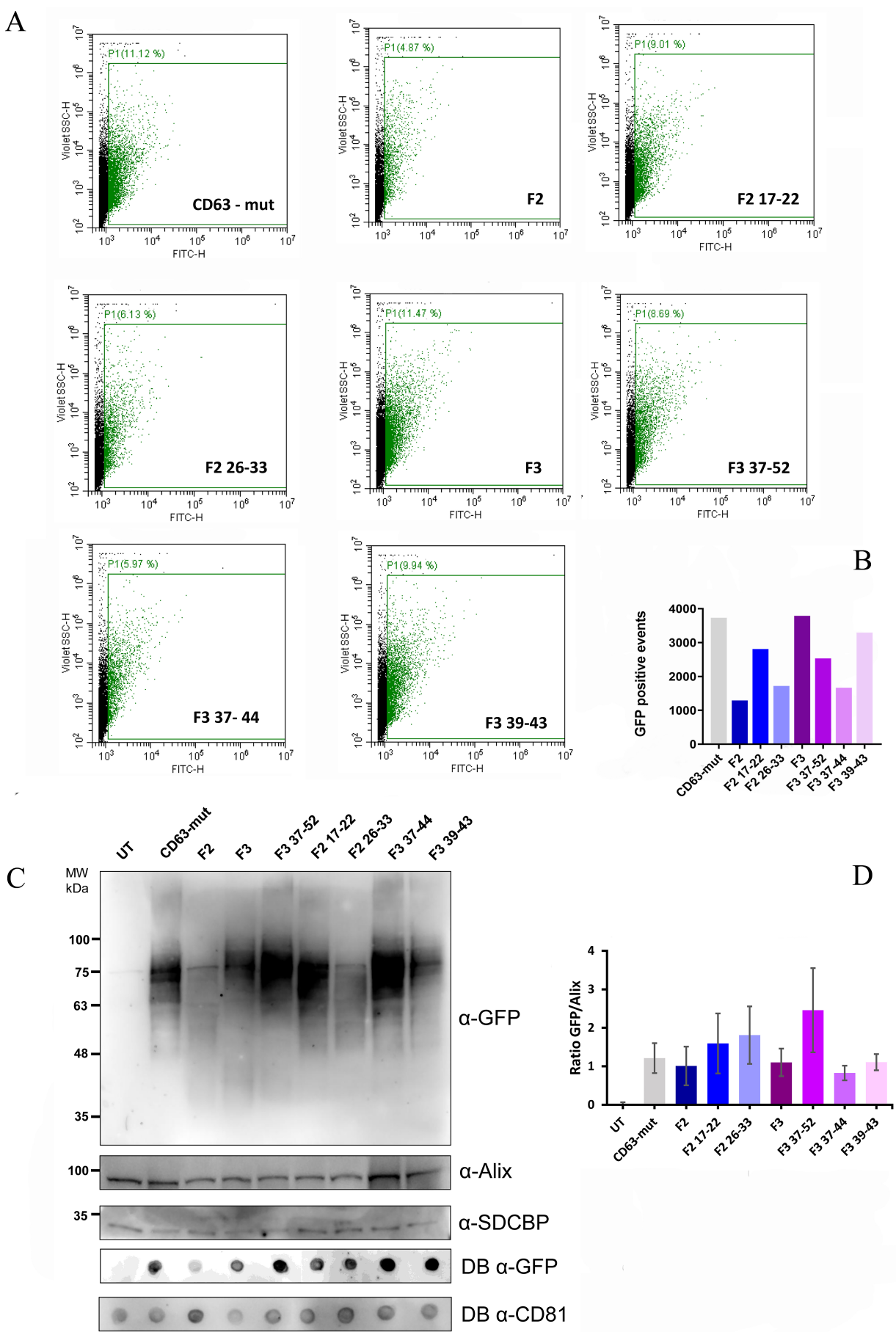


Figure 8. p17-peptide incorporation in MSC-EVs. (A) Representative dot plots from flow cytometry analysis of EVs; samples were analyzed in the FITC channel to detect CD63-p17-peptide-GFP-positive EVs; (B) The bar graph summarizes the relative abundance of

GFP-EVs across the different CD63-based p17-derived peptide constructs, indicating construct-dependent variability in GFP signal while confirming the presence of GFP-positive EV subpopulations; (C) Western blotting of MSC-derived EVs from UT cells and cells expressing CD63-GFP constructs. GFP blotting reveals the peptide-tagged CD63 constructs, migrating as a single ~75 kDa band for CD63-mut and as an additional ~40-45 kDa band for peptide-tagged variants. Alix and SDCBP blotting, together with CD81 dot blot, confirm the EV identity of the samples; GFP dot blot confirms the presence of engineered EVs and quantifies the GFP signal used in (D). Molecular weight markers are indicated on the left and expressed in kDa. Raw, uncropped blots corresponding to (C) are shown in [Supplementary Figure 2F-K](#); (D) Bar histogram of the relative GFP abundance across constructs, expressed as the GFP/Alix ratio (mean $\pm$ SD). CD63: Cluster of differentiation 63; DB: dot blot; EV: extracellular vesicle; FITC: fluorescein isothiocyanate; GFP: green fluorescent protein; kDa: kilodalton; MSC: mesenchymal stromal cell; MW: molecular weight; SDCBP: syndecan-binding protein; SD: standard deviation; SSC: side scatter; UT: untransfected.

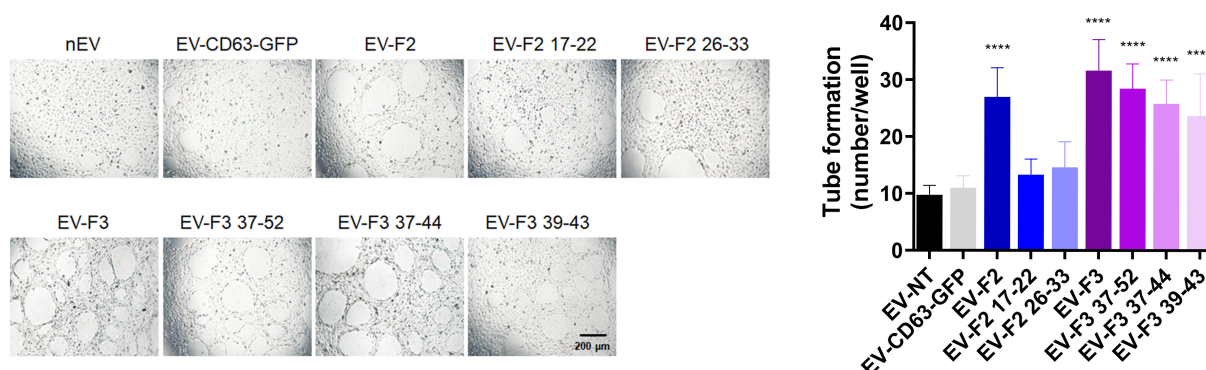


Figure 9. Effects of EVs functionalized with p17-derived peptides on angiogenesis in HUVECs. Angiogenic activity evaluated by tube formation assay in HUVECs treated with nEVs, EV-CD63-GFP, or EVs functionalized with the indicated p17-derived peptides. Images represent three independent experiments with similar results (original magnification 10 $\times$). Scale bar: 200 μ m. Data are expressed as mean $\pm$ SD of three independent experiments performed in triplicate. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni's post hoc test (**** P < 0.0001). ANOVA: Analysis of variance; CD63: cluster of differentiation 63; EV: extracellular vesicle; GFP: green fluorescent protein; HUVEC: human umbilical vein endothelial cell; nEV: native extracellular vesicle; SD: standard deviation.

GFP abundance in EV samples was calculated as the ratio between the GFP signal obtained by dot blot and the Alix signal obtained via Western blotting [[Figure 8D](#)].

Evaluation of the angiogenic potential of functionalized EVs carrying p17-derived peptides

The angiogenic potential of EVs functionalized with p17-derived peptides (EV-F2, EV-F2 17-22, EV-F2 26-33, EV-F3, EV-F3 37-52, EV-F3 37-44, and EV-F3 39-43) was assessed in HUVECs using a tube formation assay. Native EVs (nEVs) and EV-CD63-GFP were used as negative controls.

As shown in [Figure 9](#), functionalization of EVs with F2 and F3 fragments significantly enhanced the formation of capillary-like structures compared with the nEV and EV-CD63-GFP control groups (**** P < 0.0001). Notably, both shorter sub-fragments derived from F2 (EV-F2 17-22 and EV-F2 26-33) failed to induce a significant angiogenic response. In contrast, all F3-derived sub-fragments (EV-F3 37-52, EV-F3 37-44, and EV-F3 39-43) retained strong pro-angiogenic activity, comparable to that of the parental F3 fragment [[Figure 9](#)].

DISCUSSION

Protein-based therapeutic angiogenesis remains limited by poor stability, rapid degradation, and inefficient delivery of bioactive molecules *in vivo* [[3,33](#)]. EVs have emerged as promising biological delivery systems because of their biocompatibility, ability to interact with target cells, and capacity to carry therapeutic cargo [[34,35](#)]. Building on these properties, the present study investigated whether MSC-derived EVs could be engineered to display HIV-1 p17-derived angiogenic peptides while preserving their biological activity, and sought to identify minimal functional motifs suitable for EV-based delivery. These findings demonstrate the

feasibility of CD63-mediated EV engineering as a strategy for the surface display of angiogenic peptides. Consistent with previous reports, our molecular characterization - including Western blot analysis and flow cytometry - confirmed the successful incorporation of CD63-peptide fusion proteins into MSC-EVs, supporting the suitability of this platform for the delivery of bioactive peptide cargos. Beyond demonstrating the feasibility of CD63-mediated MSC-EV engineering, a central finding of this study was the identification of F3 39-43 (RELER) as a minimal angiogenic motif that retained biological activity both as a soluble peptide and when displayed on the EV surface. In particular, the F3 39-43 pentapeptide retained potent biological activity at 10 ng/mL, comparable with that of the reference F3 peptide, stimulating endothelial cell migration and tube formation under both starved and standard conditions. These data support the concept that short, well-defined peptide sequences can preserve angiogenic function when appropriately presented. The inclusion of the arginine (R39) residue provides a strongly basic group, known to facilitate high-affinity protein-protein interactions through salt bridges and hydrogen bonding^[36,37]. Moreover, this perfectly palindromic pentapeptide sequence eliminates bulky hydrophobic interference, maximizing the exposure of the ExE/DR-charged motif. This structural symmetry is of particular interest, since palindromic motifs often exhibit unique conformational stability, enhanced resistance to proteolytic degradation, and a propensity for ordered self-organization. It should be further emphasized that very short peptides, such as F3 39-43, typically exhibit lower immunogenicity, despite an increased susceptibility to proteolytic degradation. These observations support the concept that the rational miniaturization of bioactive ligands, combined with biologically compatible carriers such as EVs, represents an effective strategy for achieving controlled and targeted therapeutic responses.

Importantly, our findings revealed a marked difference between F2- and F3-derived peptides following EV functionalization. While the parental F2 fragment retained robust pro-angiogenic activity in its free form, its shorter derivatives (F2 17-22 and F2 26-33) failed to induce angiogenesis when displayed on the EV surface. In contrast, all F3-derived peptides, including progressively shortened variants, consistently maintained angiogenic activity after EV presentation. This differential behavior between F2- and F3-derived peptides can be explained by structural and spatial constraints associated with peptide presentation on EV membranes. One possible explanation is that the reduced length of the F2-derived sub-fragments (6-8 amino acids) limits their accessibility to endothelial cell receptors, as they remain near the bulky extracellular loops of the CD63 scaffold protein^[16]. Under these conditions, the peptides may be unable to achieve the spatial orientation required for productive receptor engagement, despite retaining intrinsic activity in their free form. In contrast, the F2 fragment (20 amino acids) might provide a natural spacer effect, extending the active domain sufficiently far from the EV surface to allow proper molecular recognition. The preserved activity of F3-derived peptides suggests that the F3 39-43 sequence RELER represents an intrinsically stable, minimal functional core of p17-mediated angiogenesis. The ability of this short motif to remain active even in a constrained membrane environment indicates favorable structural or physicochemical properties, such as charge distribution, flexibility, or receptor-facing conformation, that facilitate receptor recognition independently of peptide length. The F3 37-52 fragment has been shown to promote angiogenesis through molecular mimicry of EPO in a β c receptor-dependent manner^[26], suggesting that its shortened derivatives may preserve this mechanism when displayed on EVs. This non-canonical signaling axis suggests that the EV-F3 constructs studied here can promote functional vascularization through a highly specific pathway, offering a previously unexplored avenue for further investigation. Beyond peptide-specific effects, these findings highlight a broader principle relevant to EV engineering. The biological activity of EV-displayed ligands depends not only on the intrinsic properties of the cargo itself, but also on how the cargo is presented on the vesicle surface. EV membranes constitute complex biological interfaces in which ligand accessibility, orientation, and local microenvironment can substantially influence receptor engagement and downstream signaling. Consequently, successful EV functionalization cannot be predicted solely from the activity of soluble molecules; instead, the compatibility between a bioactive sequence and its mode of presentation on

the EV surface should be considered a critical design parameter when developing engineered EV-based delivery platforms.

From a translational perspective, these findings support the development of modular EV-based delivery platforms, in which peptide size, orientation, and mode of presentation can be optimized according to the intended biological function. The identification of a minimal angiogenic motif that remains active after EV incorporation may facilitate the design of more controllable and standardized EV formulations.

Study limitations and future perspectives

In this proof-of-concept study, our primary objective was to demonstrate *in vitro* the technological feasibility of displaying engineered functional peptides on the EV surface to drive angiogenesis.

While the long-term goal of this platform is its translational application as a therapeutic nanocarrier, a limitation of the present study is that it remains confined to an *in vitro* setting. Several intermediate steps will therefore be required before *in vivo* application can be considered. Mechanistic studies addressing receptor engagement, binding kinetics, and internalization dynamics of the EV-displayed peptides, as discussed below, will be essential to substantiate the proposed mode of action. In addition, more complex, physiologically relevant models - such as 3D co-culture or *ex vivo* tissue explant systems - could help bridge the gap between simplified 2D endothelial assays and the multicellular, dynamic environment of native tissue. Indeed, the wound healing (scratch) and tube formation assays employed here, while widely used and informative, remain surrogate, single-cell-type readouts of angiogenesis; they do not capture the contribution of pericytes, immune cells, or extracellular matrix remodeling that shape the angiogenic response *in vivo*, and the present results should be interpreted within this constraint.

Moreover, the functional characterization of the engineered EVs was restricted to HUVECs; potential off-target effects on other cell populations present in the wound microenvironment, such as fibroblasts or resident immune cells, were not investigated and should be addressed in future safety-oriented studies.

Dose-response and pharmacokinetic characterization, together with assessment of EV stability and reproducibility across production batches, and robustness of the engineering strategy across different MSC biological backgrounds, will also be necessary before translational application can be considered.

Closely related to this, the manufacturing scalability of this EV-engineering approach has not been addressed in the present study; translating peptide-displaying EVs from small-scale laboratory production to a large-scale, GMP-compliant process suitable for clinical development represents an additional, non-trivial technical hurdle.

Moreover, *in vivo* validation will require the prior identification of a disease-relevant setting - including the specific pathological condition and animal model best suited to test this platform. Extending the platform in this direction could represent a valuable line of future work.

The rational design of EV-based delivery platforms relies on translating known bioactive motifs onto a nanovesicular scaffold. We previously demonstrated that angiogenesis induced by the p17-derived fragments F2 and F3 37-52 requires distinct receptors - CXCR1, CXCR2, and the β c receptor^[24,26]. Consequently, each engineered construct may exhibit unique surface docking, entry kinetics, and downstream crosstalk. Although the interaction and internalization of MSC-derived EVs by endothelial cells are well-established phenomena^[38-40] and our functional readouts confirm that EV-displayed peptides successfully trigger angiogenic responses, a current limitation of this study is the lack of precise molecular mechanisms and receptor-mediated dynamics for the EV-displayed constructs.

In particular, receptor-blocking or neutralization experiments, which previously confirmed the involvement of CXCR1, CXCR2, and the β c receptor for the free peptide counterparts, were not performed for the EV-displayed constructs, and therefore cannot yet formally establish that the same receptors mediate the angiogenic activity observed in the engineered EV format.

Mapping the spatio-temporal dynamics of EV internalization and determining whether these interactions occur exclusively at the plasma membrane or dynamically persist within the endosomal network represent an intriguing area for future investigation, building on a fully characterized baseline of the binding dynamics and signaling of the soluble, free peptide counterparts, which is currently under active investigation.

Future studies may also explore alternative scaffold proteins, optimized linker sequences, or different insertion sites to further improve peptide accessibility and functional density on the EV surface. In addition, while the present *in vitro* results are promising, future *in vivo* translation will need to account for the complexity of the wound microenvironment. In particular, the formation of a biomolecular corona and the presence of proteolytic enzymes may hinder peptide bioavailability and receptor recognition, warranting further investigation for clinical tissue-repair applications^[41].

Direct molar equivalence between free peptides and EV-displayed constructs was not applied in our bioassays, as concentrations were chosen based on optimal biological responsiveness. Owing to the high surface density of displayed peptides on the vesicle membrane, restricted spatial orientation, and increased local avidity, EV-bound ligands may engage target-cell receptors differently than freely soluble, monovalent counterparts^[42-45]. In addition, the absolute surface density of the displayed peptides and the fraction of EVs effectively carrying the engineered constructs were not quantitatively determined. Therefore, differences in biological activity among EV formulations cannot be unequivocally separated from potential differences in peptide copy number or display efficiency. Quantitative studies using advanced biophysical tools will be needed to precisely map peptide-per-vesicle stoichiometry, binding kinetics, spatial stoichiometry, and dose-response dynamics of functionalized EVs.

The cross-species experimental design, combining canine MSCs, previously optimized for EV production and engineering, with human HUVECs as a standardized model for angiogenic assessment, introduces a degree of biological heterogeneity that cannot be fully controlled. While EV-mediated intercellular communication is thought to be largely conserved across species, species-specific differences in receptor structure, signaling components, or membrane composition cannot be excluded and may have influenced the magnitude of the responses observed. Results obtained in this setting will therefore need to be confirmed using matched-species models before any translational extrapolation.

Finally, using GFP as a reporter and detection tag, while methodologically necessary to track and quantify the engineered constructs, introduces a protein sequence that is itself potentially immunogenic and is not part of the intended therapeutic cargo. Future iterations of this platform aimed at translational applications should consider alternative, non-immunogenic detection strategies, or removal of the reporter tag once construct validation has been achieved.

Conclusion

In conclusion, this study advances the understanding of structure-function relationships governing peptide activity in EV-based delivery systems and highlights the critical role of peptide structure, orientation, and accessibility in determining functional outcomes. Taken together, these observations support the use of

p17-derived peptides as a source of unconventional bioactive sequences and provide generalizable design principles for future EV engineering strategies targeting angiogenesis-related applications.

DECLARATIONS

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Authors' contributions

Conceptualised the study: Pascucci L, Giagulli C, Alessandri G, Caruso A

Designed the study and supervised the work: Pascucci L, Giagulli C

Developed the EV protocols and assessment tools: Pascucci L, Giagulli C, Alessandri G, Scattini G

Isolated and characterized MSCs: Scattini G

Isolated and characterized EVs: Scattini G

Performed the bioinformatic analysis and designed the p17-derived peptides: Giagulli C, Caruso A, Uggeri M

Performed the *in vitro* angiogenic assays: Venneri G, Bugatti A

Performed the flow cytometry analysis: Ragni E

Performed the Western blotting: Scattini G, Dojchinovska K

Led the drafting of the manuscript: Pascucci L, Giagulli C, Venneri G

Acquired the funding: Pascucci L, Giagulli C

Availability of data and materials

Raw data are available from the corresponding author upon reasonable request.

AI and AI-assisted tools statement

During the preparation of this manuscript, the AI tool Claude (Anthropic) (Sonnet 5, released 2026-06-30) was used solely for language editing and improvement of readability without unsupervised automatic generation. In addition, the AI tool Microsoft Copilot Image Creator (DALL-E 3, released 2023-09-20) was used solely to generate Figure 2A, which was subsequently edited by the authors using Adobe Photoshop in May 2026 (first submission) and in August 2026 (manuscript revision). The tool did not influence the study design, data collection, analysis, interpretation, or the scientific content of the work. All authors take full responsibility for the accuracy, integrity, and final content of the manuscript.

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PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) - Missione 4 "Istruzione e Ricerca" - Componente C2, Investimento 1.1, "Fondo per il Programma Nazionale di Ricerca e Progetti di Rilevante Interesse Nazionale (PRIN)" Project title: HACKING EXTRACELLULAR VESICLES (HACK-V): ENGINEERED EXTRACELLULAR VESICLES AS EMPOWERED NANO-SHUTTLES FOR ANGIOGENIC PROTEINS (2022BJB5FP) - Finanziato dall'Unione europea - Next Generation EU. CUP J53D23006190006.

Conflicts of interest

Ragni E is an Editorial Board Member and a Guest Editor of the Special Topic "GISM Annual Meeting 2025: From MSCs to Extracellular Vesicles" of the journal *Extracellular Vesicles and Circulating Nucleic Acids*. Ragni E was not involved in any steps of the editorial process, notably including reviewer selection, manuscript handling, and decision-making. Uggeri M is affiliated with Lifescience Innovation Good Healthcare Technology. The other authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

The Comitato Universitario di Bioetica dell'Università degli Studi di Perugia (Bioethics Committee of the

University of Perugia) reviewed and approved the study protocol (protocol number 6/2026). The approval covered the use of surplus canine adipose-derived MSCs, obtained as excess material from procedures independently indicated for clinical treatment of the donor dogs. Informed consent for the use of surplus cells was obtained from the owners of the donor dogs. The approval also covered collecting endothelial cells from umbilical cord tissue discarded after full-term delivery. However, the latter material was not used in the present study, in which commercially sourced HUVECs were employed instead (see METHODS).

Consent for publication

Not applicable.

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Supplementary Materials

[Supplementary Materials](#)

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