Stem Cells and Cloning: Advances and Applications

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ORIGINAL RESEARCH

Novel, Reproducible, Consortia Factors Derived from Adipose Stem Cells for Burn Wound Treatment

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Video abstract

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Introduction: The therapeutic application of adipose-derived stem cells (ASCs) for wound healing has been reported. However, consistently controlling ASC secretory-factor levels, scaling up, and their mechanism of action have been poorly understood, which are critical steps for developing biological drugs to treat second-degree burns.

Objective: Our goal is to develop a biological product, named consortia factors-δ2 (CFx-δ2), derived from cell-to-cell interactions between human immortalized ASCs using our patented technology and to evaluate the product consistency and in vitro biological effects to enhance the wound healing process.

Methods: To assess product consistency, three batches of CFx-δ2 were analyzed and compared using immunomicroarray and metabolomics. The biological effects of these batches were studied using an in vitro wound healing assay and ex vivo human skin explants subjected to burn wounds. Anti-inflammatory effects were analyzed by inhibition of Nuclear Factor- κB (NF-κB) nuclear translocation in human dermal endothelial cells (HMEC-1). Finally, pro-angiogenesis was evaluated by the induction of tube-like structures and capillary networks in HMEC-1 cells promoted by CFx-δ2.

Results: Our technology enabled the production of CFx-δ2 with enhanced efficiency and quality. When the three batches were compared, we found high consistency in the pattern and levels of cytokines as well as a strong correlation in the metabolite pattern. Accelerated wound healing was observed after in vitro and ex vivo wound treatment with CFx-δ2 in comparison to controls. The nuclear translocation of NF-κB, induced by Lipopolysaccharides (LPS), was 50% inhibited by CFx-δ2. Induction of more tube-like structures and networks was observed in endothelial cells treated with CFx-δ2 in comparison to controls.

Discussion: These findings demonstrate that our technology is efficient to derive and scale up consistent levels of CFx-δ2 from immortalized adipose stem cells. The product accelerates wound healing by increasing fibroblast proliferation and migration, inhibiting inflammation, and promoting angiogenesis.

Keywords: wound healing, burn treatment, stem cells

Introduction

Adipose stem cells (ASCs) are capable of self-renewal and differentiation into multiple cell lineages.[1] Recently, more attention has come to ASC's secretory factors that have several beneficial biological effects such as the promotion of skin wound healing.[2–7] Wound healing is a complex process involving various signals and cell types to repair damaged tissue.[2] Several growth factors have been identified as key regulators of wound healing such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), and insulin-like growth factor (IGF).[8–14] Recombinant EGF has been used clinically to accelerate epidermal regeneration in second-degree burns.[15,16] In the first stage of wound healing, transcription factors such as nuclear factor kappa B (NF-κB) promote changes in

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ASC and endothelial-cell secretory factor profiles.[17] Although ASC secretory factors show significant potential for the treatment of burn wounds, the ability to produce such products with consistency has not been reported. The effectiveness of these ASC-derived factors has been widely studied in preclinical experiments.[7] These authors reviewed the therapeutic effects of different mesenchymal cells on burn wound management and found some studies where ASCs improved skin graft survival, blood flow, and accelerated wound epithelialization in a full-thickness burn model.[7] Additionally, some experiments demonstrate the safety in the use of ASC to treat burn wounds as well as the benefit in reducing the formation of post-burn scars.[8] Although the benefits of ASC in the treatment of burn wounds have been widely explored in animals, there are still challenges in cytokine consistency for biopharmaceutical preparations to be used in humans. One reason for this inconsistency is the ASC biological variability. For instance, in previous work, we found that the pattern and levels of secretory factors released by stromal vascular fraction (SVF) from adipose tissue, varies according to the body region where these cells are harvested.[6] Other factors that produce such variability are related with technical aspects such as culture conditions, cellular yield, the source of cells, and the cell processing method.[6,10] Therefore, the use of secretory factors to prepare effective biopharmaceutical formulations is mainly limited by variability.

We have developed a novel process for culturing stem cells to produce a unique, reproducible, and scalable set of ASC secretory factors, termed consortia factors-δ2 (CFx-δ2), utilizing our patented platform technology.[5] In this paper, we present our results demonstrating the consistency of CFx-δ2 across multiple batches and highlighting its beneficial biological effects as a potential treatment for human skin burns.

Materials and Methods

Cell Lines

Ethics

The immortalized Human Adipose Cell Lines and immortalized Human Dermal Fibroblasts Cell Lines were obtained from a method previously published.[18] The Human Adipose tissue and Human Dermal Fibroblasts used to develop the immortalized cell lines, were obtained from medical waste after written informed consent was obtained from all participants or their legal guardians. The skin samples were purchased from Genoskin (Salem, MA). All specimens, including cell lines and human skin explants, were then transferred to a laboratory in a manner wherein the laboratory could not ascertain the identity of the individual donors, all in compliance with the U.S Department of Health and Human Services' Office of Human Research Protections ("OHRP"), policies and regulations.

Adipose Stem Cells (ASCs)

We developed an immortalized human ASC line 6614–5 (Rinati Skin, LLC, Hawthorne, CA), that was used at passages 14–20 to produce consortia factors (CFx-δ2).[18] These cells were plated at a density of 1×10^5 cells/mL in T-75 and reached confluence after 7 to 10 days. For cell maintenance, serum-free base media (SFM), developed in our laboratory (Rinati Skin, LLC, Hawthorne, CA),[18] was supplemented with 10% serum-free human fibroblast conditioned media (HF-CM, Rinati Skin, LLC, Hawthorne, CA). The ASC was tested for any pathogen including mycoplasma (third party-lab, ATCC, Manassas, VA), staphylococcus aureus, E. coli and coliforms, and Salmonella spp. (In-house microtesting). The media for cell growth as well as the cell expansion was done under strict standard operating procedures (SOPs) and strict and precise regulatory requirements. We have a cGMP facility registered and approved by the Department of Public Health with the capacity to produce culture media and handle several cell lines. Manufacturing instructions, validation methods, and specific protocols are followed to ensure consistency, purity, and quality of CFx-δ2.

Human Dermal Microvascular Endothelial Cells (HMECs)

Confluent monolayers of human dermal microvascular endothelial cells (HMEC-1, American Type Culture Collection [ATCC], Manassas, VA) at passage 15–20 were cultured in MCDB131 (Life Technologies, Carlsbad, CA) supplemented with 10 ng/mL of EGF (Sigma-Aldrich, St. Louis, MO), 1μg/mL of hydrocortisone (Alfa Aesar, Haverhill, MA), 10 mm of L-Glutamine (Sigma-Aldrich, St. Louis, MO), and 10% of fetal bovine serum (ATCC, Manassas, VA).

Human Dermal Fibroblasts (HF)

The human dermal fibroblast (HF) cell line 7868 was developed in our laboratory.[18] The cells were plated at 1×10^6 cells/mL in T-75 flasks in SFM media supplemented with 10% human serum (HS). Following attachment, the cells were washed with 1X PBS, and the media was replaced with SFM supplemented with 10% human fibroblast conditioned media (HF-CM).

Human Adipose Stem Cell Consortia Factors (CFx-δ2) and Human Fibroblasts Conditioned Media (HF-CM) Production

Immortalized human ASC line 6614–5 was used at passages 14–20. The ASCs were expanded in tissue culture flasks at a density of 3×10^6 cells/mL before being transferred to spinner bottles to induce cell shear stress. The spinner bottles contained 10 grams of Fibra-Cel® Disks as scaffolds (Eppendorf, Enfield, CT). To produce CFx-δ2, the ASCs were re-inoculated weekly into the same spinner bottles over three weeks using the same cell line at different harvesting stages. The CFx-δ2 were then harvested every 72- or 96-hrs. During the fourth week, there was no re-inoculation, and only CFx-δ2 collection. The harvested product was pooled to generate a batch for microbiological testing and for in vitro and ex-vivo experiments. To validate the consistency of ASC secretory factors, three batches of CFx-δ2 were manufactured at different dates (Batch 11, 12, and 13).

To produce human fibroblast conditioned media (HF-CM), human fibroblasts (HF) at passages 3–10 were inoculated into spinner bottles with Fibra-Cel® Disks (Eppendorf, Enfield, CT). The conditioned media was collected or harvested after 72 hrs., and samples were collected for microbiological testing and multiplex immunomicroarray analysis (Eve Technologies, Calgary, AB, Canada).

Immunomicroarray

All samples treated with CFx-δ2, or control medium were collected from either 500 mL spinner bottles or 5-liter bioreactors to show consistency with up scaling, frozen at –20°C, and sent for immunomicroarray analysis to a third-party laboratory (Eve Technologies, Calgary, AB, Canada).

Metabolomics

Untargeted metabolomics was performed by the Carver Metabolomics Core, University of Illinois Urbana-Champaign Roy. J. Carver Biotechnology Center. For detailed procedures, refer to the Supplementary Text.[19–22]

Wound Healing Assay

An in vitro wound healing assay (ab242285, Cambridge, MA) was conducted to evaluate the effects of different batches of CFx-δ2 (Batch 11, 12, and 13) derived from the same ASC cell line 6614–5 (Rinati Skin, LLC, Hawthorne, CA). Each well of a 24-well plate was precoated with collagen (R&D Systems, Minneapolis, MN). The precoated plate can be stored at 4°C for up to one week before the assay. An insert was placed into each well using sterile forceps and a cell suspension containing 1×10^5 human fibroblasts (HF) was added to each well, evenly distributed on the insert. After 24 hrs., the inserts were removed using sterile forceps and a 0.9 mm-wound was generated in vitro in each well. Then we treated each other well with DMEM/F12, as negative control, and 30% CFx-δ2 of each batch. The wound areas were analyzed at multiple time points: 0, 24, and 48 hrs. The cells were fixed in 4% paraformaldehyde (PFA; Santa Cruz Biotechnology, Dallas, TX) and stained using Crystal Violet Solution (Abcam, Cambridge, UK). Then, each wound area was imaged, and the wound length was analyzed using ImageJ software v. 1.52a (National Institute of Health [NIH], Bethesda, MD).

Inflammatory Assay and Immunocytochemistry

HMEC-1 cells were plated on Nunc Lab-Tek II 4-chamber slide (Thermo Fisher Scientific, Waltham, MA) with 100,000 cells per well. The cells were incubated for 24–48 hrs and then treated with 5 µg/mL Lipopolysaccharides (LPS) (Sigma-Aldrich, St. Louis, MO) to induce pro-inflammatory phenotype by stimulating Nuclear Factor-κB (NF- κB) nuclear

translocation. For the experiments, cells were treated for 1 hour with either regular culture media only (negative control), media containing LPS (positive control), media containing LPS plus 1 μM TAK-242 (Resatorvid) (anti-inflammatory effect by inhibition of toll-like receptor 4) (MedChemExpress, Monmouth Junction, NJ), or media containing LPS plus 30% CFx-δ2 from Batch 11, 12, or 13.

After treatment, cells were fixed with 4% PFA for 20 minutes, and permeabilized with 0.3% Triton-X for 5 minutes (Sigma-Aldrich, St. Louis, MO). Cells were then stained with the primary antibody, anti-NF-kB p65- E379 (ab32536, Abcam, Cambridge, MA) (1:100 dilution) overnight at 4°C. The next day, the cells were incubated with a goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 555 (Thermo Fisher Scientific, Waltham, MA) (1:500 dilution), for 1 hour in the dark. After washing, coverslips were mounted onto slides using ProLong™ Diamond Antifade Mounting with DAPI (Thermo Fisher Scientific, Waltham, MA). The slides were imaged and analyzed using Image J software (NIH, Bethesda, MD). Quantification of the inflammatory effect was done by counting the pink nuclei (inflammatory cells) and divide this number by the number of blue nuclei (non-inflammatory) to obtain the fraction (percent) of inflammatory cells.

Angiogenesis Assay

Angiogenesis was evaluated by treating endothelial cells (HMEC-1) embedded in collagen-fibronectin gels to form tube or capillary-like structures. To prepare 1 mL of the gel, the following ingredients were added: 10X MEM (Gibco, Brooklyn, NY), HEPES, NaHCO3 (Caisson Labs, Smithfield, UT), and NaOH (LabChem, Zelienople, PA). Fibronectin was added to the final concentration of 1 ng/mL. The mixture was kept at 4°C or in ice. Meanwhile, endothelial cells were harvested using trypsin, and a cell suspension of 5×10^6 cells/mL was prepared. Then, Type I Collagen (Santa Cruz Biotechnology, Dallas, TX) was added to the gel mixture (kept at 4°C or in ice). The cells were added to achieve a final concentration of 1×10^5 cells/mL. Several 100 μL drops of this cell-gel mixture were dispensed into Y-Plate Petri dishes and incubated at 37°C for 15–20 min. After incubation, the gels containing cells, were placed in the hood, and 5 mL of testing media (regular media/DMEMF12, VEGF 10 ng/mL, or 30% CFx-δ2 Batch 11, 12, and 13) was added to each compartment of a Y-Plate Petri dish. After 48–72 hrs of treatment, the conditioned media was collected for future immunomicroarray analysis (Eve Technologies. Calgary, AB, Canada). The cells embedded in the gels were fixed with 4% paraformaldehyde and permeabilized with TritonX100. A 100 μL solution of 1X Phalloidin conjugated with a red fluorochrome was added to the fixed cells for 20 min. The cells were then imaged, and three different fields were analyzed using an epifluorescent microscope (Leica, DMIL LED inverted with phase contrast, Buffalo Grove, IL).

Antioxidant Assay

The total antioxidant capacity (TAC) of CFx-δ2 Batch 11, 12, and 13 was measured using OxiSelect TAC kit according to manufacturer's Instruction (Cell Biolabs, Inc, San Diego, CA). The assay is based on the reduction of copper II to copper I by antioxidant compounds present in the samples. The TAC was expressed as Vitamin C Equivalent Antioxidant Capacity (VCEAC).

Burn Wound of ex-vivo Skin Samples

NativeSkin access® (Genoskin, Salem, MA) were used to evaluate the wound healing effects of CFx-δ2 on ex-vivo human skin burn injuries. Once the skin samples arrived, the plate was allowed to equilibrate with media in the incubator at 37°C and 5% CO_2 for 1 hour. A second-degree burn was inflicted on the skin tissue using a soldering iron at 300°C for 15 seconds. Images of the burns were taken using an AmScope dissection microscope (AmScope MD35, Irvine, CA). The skin samples were then placed in the incubator for 2 hrs. DMEM/F12 or CFx-δ2 were applied topically on the skin tissue and left for 24 hrs. The media was replaced daily for 4 and 7 days. The samples were cut in half and fixed in 10% formalin (Sigma-Aldrich, St. Louis, MO) for 7 days. After the fixation, all samples were transferred to 70% ethanol (Electron Microscopy Sciences, Hatfield, PA). The formalin-fixed tissue was sent for histological analysis at Histowiz, Brooklyn, NY. This analysis included H&E staining to observe the different components of the skin.

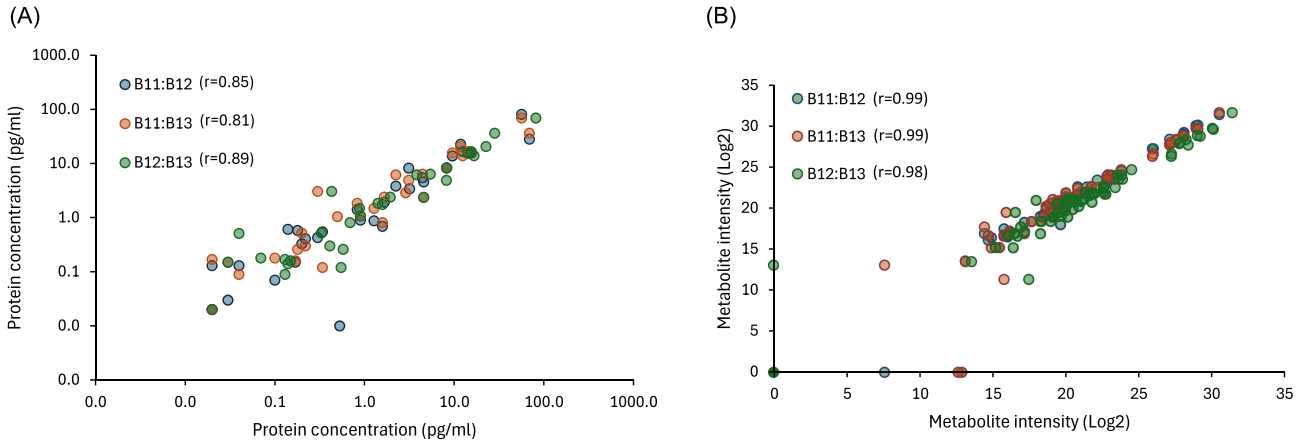

Figure 1 Consistency in secretory factors between CFx-δ2 batches. Scatter plots of 71 cytokine/chemokine concentrations in pg/mL (**A**) and 64 (MSI1) metabolite abundancies or intensities (**B**) in three CFx-δ2 batches (Batch 11, 12, and 13.) r = Pearson's correlation coefficients between indicated batches. All "r" values are significant, ie, P-values <0.00001.

Statistical Analysis

For functional experiments, three replicates from each batch were analyzed. The statistical test used to determine the P-value in Figure 1 is called Pearson correlation significance test. Each statistical test was done between two separate batches, ie, Batch11 vs Batch12, Batch11 vs Batch13, and Batch12 vs Batch13. All statistics and significance for experiments to test biological activity, were calculated using unpaired Student's *t*-test with GraphPad Prism (v.5.0.1). P-value thresholds were defined as follows: *P ≤0.05; **P ≤0.01 and ***P ≤0.001.

Results

Production of Consistent Levels and Profiles of ASC Consortia Factors (CFx-δ2) from Human Immortalized ASCs

The consistency between different batches of *ASC Consortia Factors (CFx-δ2*) was tested by profiling the abundance of proteins and metabolites in three different manufactured CFx-δ2 batches. A selected group of 71 cytokines and chemokines were profiled using immunomicroarray (Supplementary Table 1 and Supplementary Figure 1) and metabolomics. The immunomicroarray analysis showed 44 out of 71 cytokines, chemokines, and growth factors detected by the system. The rest were out of range (OOR) where the value fell below the standard curve. From 44, 28 showed more consistency (23 with standard deviation (SD) < 1 and 5 with SD >1 but < 2). We observed that some cytokines have variation in a range and decided to evaluate consistency using also metabolomics and functional tests. The metabolites have been profiled using untargeted metabolomics (LC-MS) (see Metabolomics Method with more detail in Supplement). Untargeted metabolomics identified 64 metabolites at the Metabolomics Standards Initiative level 1 (MSI1 level). Comparing the protein concentrations and metabolite abundancies between the three CFx-δ2 batches provided high correlation coefficients (Figure 1A and B). Pearson's r ranges between 0.81–0.89 for proteins and 0.98–0.99 for metabolites, P-values <0.0001.

CFx-δ2 Accelerated Wound Healing

Wound healing potential of CFx- δ 2 was evaluated using an in vitro wound healing assay by measuring the migration of cells into the wound area. DMEM/F12 did not promote wound closure at 48 hrs. (Figure 2A). In contrast, 100% HF-CM, used as positive control, effectively promoted wound closure within this time frame. All three batches of CFx-δ2 also promoted wound healing at 24 and 48 hrs. (Figure 2A). The quantification of the distance between the borders of in vitro wounds after CFx-δ2 treatment in several experiments is shown in Figure 2B.

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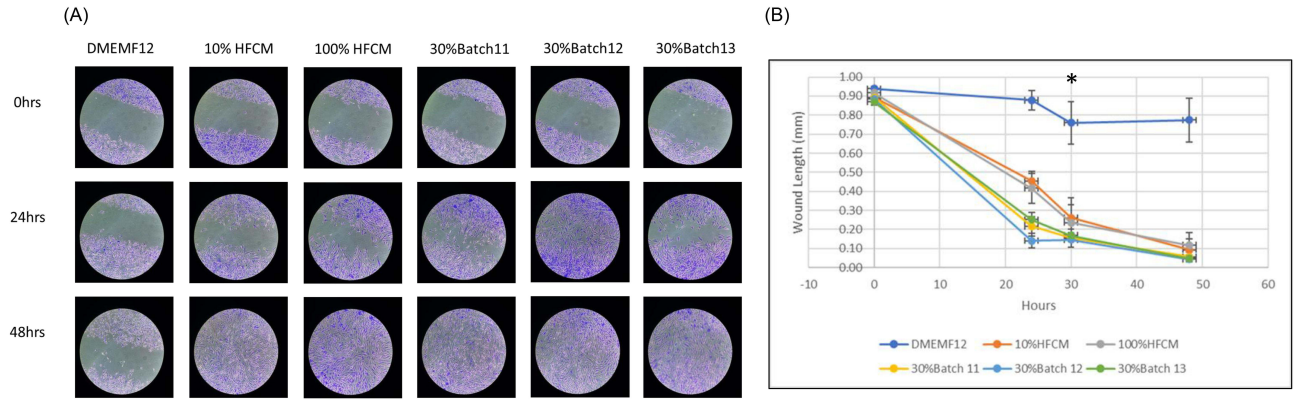

Figure 2 Wound healing assay with human dermal fibroblasts (HF) treated with different batches of CFx-δ2. (**A**) In vitro wounds on HF monolayers were treated with DMEM/F12, DMEM/F12 + 10% HF-CM, DEMEF/12 + 100% HF-CM, DMEM/F12 + 30% CFx-δ2 Batch 11, DMEM/F12 +30% CFx-δ2 Batch 12, and DMEM/F12 + 30% CFx-δ2 Batch 13. (**B**) The graph shows the average wound measurements from three independent experiments (n = 3) by treating human dermal fibroblasts (HF) with the media described in "A". *P < 0.05.

Anti-Inflammatory Effects of CFx-δ2

To evaluate the anti-inflammatory effects of CFx-δ2, the NF-κB nuclear translocation was analyzed in endothelial cells (HMEC-1). Non-inflammatory endothelial cells (HMEC-1) showed perinuclear distribution of NF-κB (Figure 3A i, blue nuclei). Following treatment with Lipopolysaccharide (LPS) for one hour, NF-κB translocated into the nucleus (Figure 3A ii, pink nuclei). This translocation was inhibited by Resatorvid (TAK242), a known anti-inflammatory agent that blocks the toll-like receptor (Figure 3A iii). Important and statistically significant anti-inflammatory effects were observed after treatment with 30% CFx-δ2 (Figure 3A iv). The quantification of these effects shows a consistent anti-inflammatory effect for all batches (Figure 3B).

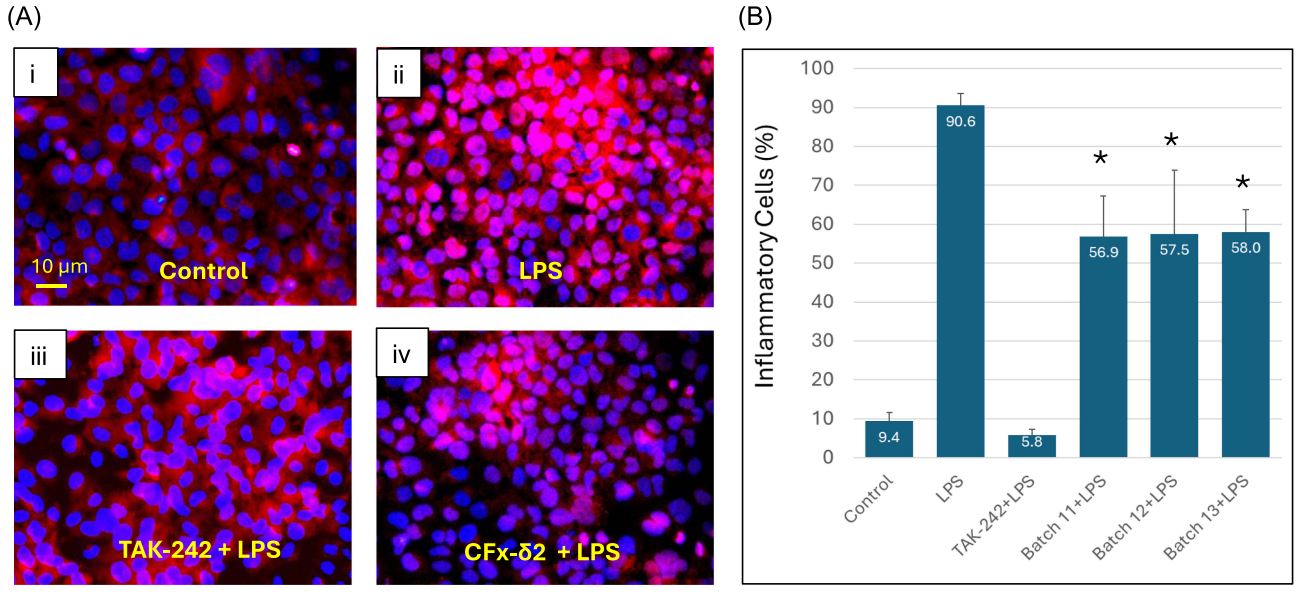

Figure 3 Anti-inflammatory effects of CFx-δ2. Endothelial cells (HMEC-1) were cultured in 4-chamber slides. After reaching confluence, cells were treated for one hour with different test media. Cells were fixed, permeabilized, and stained with anti-NF-κB, Alexa Fluor 555 (red), and DAPI (blue). (Ai) Endothelial cells (HMEC-1) are growing in regular media. (Aii) HMEC-1 treated with 5 µg/mL Lipopolysaccharide (LPS). (Aiii) HMEC-1 treated with 5 µg/mL LPS + 1 µM TAK242. (Aiv) HMEC-1 treated with 5 µg/mL LPS + 30% CFx-δ2. (**B**) Inflammatory HMEC-1 showed pink nuclei after LPS treatment and NF-κB nuclear translocation. The nuclei were counted, and the percentage calculated. 100% is the maximum of inflammatory cells. As negative control, the cell grew in regular media and, as positive control of inflammation, the cells were treated with Lipopolysaccharides (LPS). Resatorvid (TAK-242) was used as an anti-inflammatory control. Then, different batches of CFx-δ2 (Batch 11, 12, and 13) were used to treat the cells. *P < 0.05.

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CFx-δ2 has Robust Antioxidant Capacity

The total antioxidant capacity of CFx-δ2 was calculated from a calibration curve using Vitamin C equivalent antioxidant capacity (VCEAC) as control (Figure 4A). Then, the antioxidant capacity was obtained and compared between batches. A slightly lower antioxidant capacity was found in Batch 11 (107 VCEAC) in comparison to Batch 12 (138.1 VCEAC) and Batch 13 (129.2 VCEAC) (Figure 4B).

CFx-δ2 Promotes Angiogenesis and Re-Epithelization

The angiogenic potential of CFx- δ2 was assessed by examining the tube/capillary-like structure formation in endothelial cells. DMEM/F12 medium can promote proliferation of endothelial cells without apparent remodeling (Figure 5A i). Proliferation with some tube/capillary-like formation tendency was observed after treatment with 10 ng/mL VEGF (Figure 5A ii). More evident tube/capillary-like formation was induced by 30% CFx-δ2 Batch 11 (Figure 5A iii), Batch 12 (Figure 5A iv), and Batch 13 (Figure 5A v).

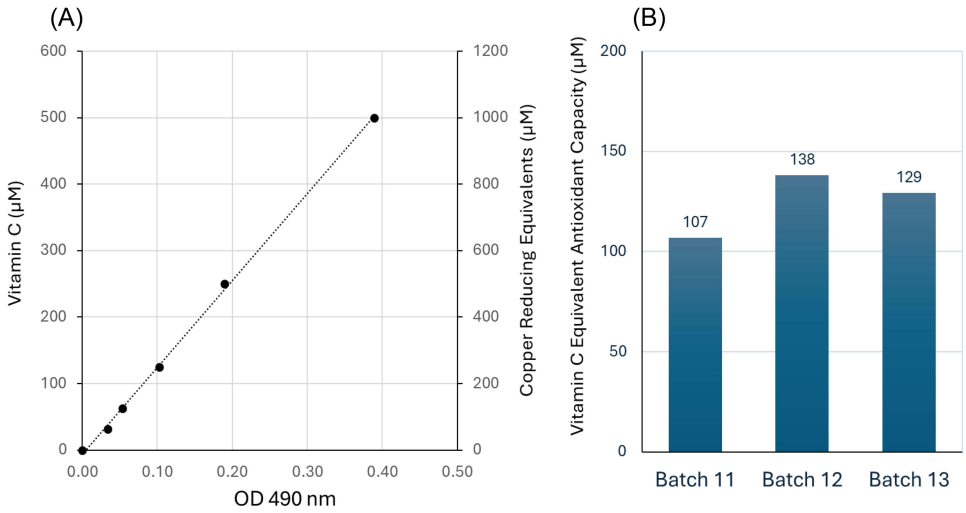

Figure 4 Quantification of antioxidant effects of different CFx-δ2 batches. (**A**) Standard curve showing Vitamin C Equivalent Antioxidant Capacity (VCEAC) (1 µM VCEAC equals 2.2 µM of copper reducing [Cu2+ to Cu+] equivalents). (**B**) The total antioxidant capacity of three batches (11, 12, and 13) expressed as VCEAC.

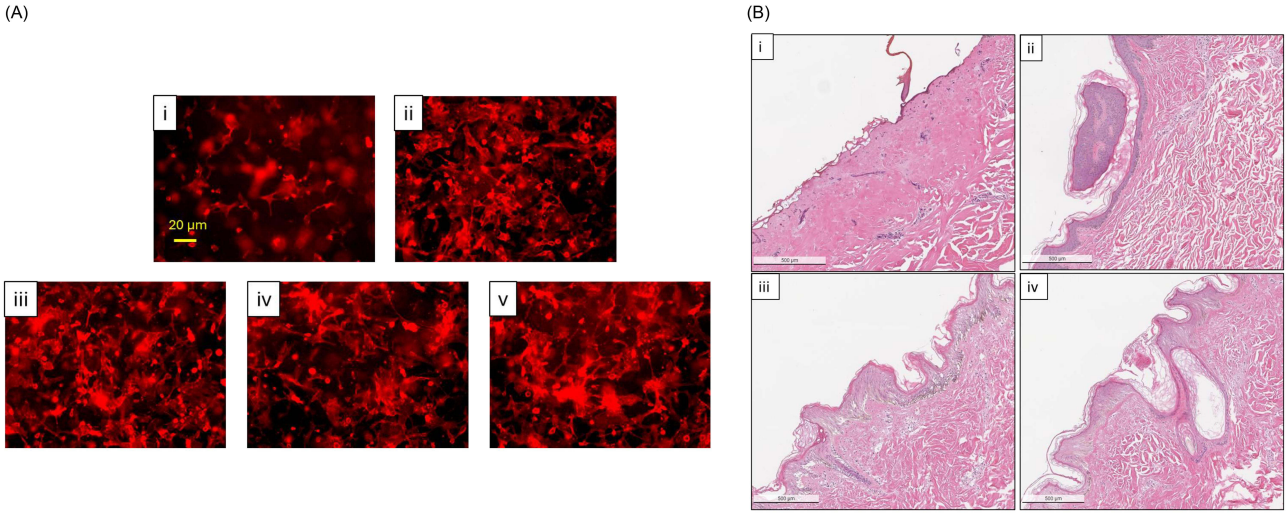

Figure 5 Angiogenesis and effects of different batches of CFx-δ2 on human endothelial cells and skin explants. (**A**) Human dermal microvascular endothelial cells (HMEC-1) were plated on collagen-fibronectin gels to form tube-like structures and stained with phalloidin (red) to identify the actin filaments. (i) Cells growing in DMEM/F12, (ii) DMEM/F12 + 10 ng/mL VEGF, (iii) DMEM/F12 + 30% CFx-δ2 Batch 11, (iv) DMEM/F12 + 30% CFx-δ2 Batch 12, and (v) DEMEM/F12 + 30% CFx-δ2 Batch 13. (**B**) Skin explant at 4 days after burn treated with (i) DMEM/F12 (control), (ii) 15% CFx-δ2, (iii) 30% CFx-δ2, or (iv) 100% CFx-δ2.

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Wound healing effects of CFx-δ2 were also evaluated using ex vivo human skin samples. Four days after inflicting a second-degree burn, the skin treated with DMEM/F12 as negative control showed blister formation and a deep area of inflammation (Figure 5B i). In contrast, those ex-vivo skin explants treated with CFx-δ2 for four days at concentrations of 15%, 30%, and 100% (Figures 5B ii–iv, respectively), showed accelerated re-epithelization and reduced inflammation.

Discussion

The cornerstone of FDA-approved burn therapy since 1973 has been silver sulfadiazine, valued for its broad-spectrum antimicrobial properties.[23] However, no new medications specifically targeting burns have been approved since then. Instead, advancements in burn care have come from innovative devices, including biosynthetic wound dressings, autologous cultured epidermal grafts, and devices for spray-on cell suspension using autologous skin cells—each with distinct indications and benefits. Despite these advances, no off-the-shelf acellular products or drugs have been introduced.

Over the last two decades, regenerative medicine and stem cell technology have advanced rapidly. While early research focused on cellular therapies, there is now a growing interest in using secretome—cell-derived signaling molecules—as they are easier to scale and can be broadly applied.[24] Such off-the-shelf products could be easily administered. However, one of the primary challenges in the biomedical industry has been the production of biological products with consistent quality, as live cell cultures can vary in response to environmental stimuli, making it difficult to produce a product that remains consistent across batches.

Achieving high batch-to-batch consistency is crucial for ensuring the safety and efficacy of biological drugs. In this study, we utilized immunomicroarray, metabolomic, and functional testing to demonstrate that our ASC lines, through our patented cell culturing process, produced consortia factors—specifically CFx-δ2—with consistent levels and patterns of high-quality, physiologically balanced factors and metabolites. These factors displayed similar biological activity in in vitro wound healing assays, indicating that CFx-δ2 could reliably influence key processes such as immunoregulation, inflammation, wound healing, and tissue regeneration. When introduced into tissues, consortia factors (CFx) trigger a targeted paracrine cascade that promotes rapid and safe wound healing.[25]

It is known that ASC-derived factors enhance cellular proliferation, migration, and survival during wound healing.[24] In our study, CFx-δ2 was found to contain cytokines that have been previously identified as crucial for wound healing.[25] Although our results did not detect some of these factors, such as EGF, we still demonstrated robust wound healing effects. This fact suggests that other cytokines, pathways or mechanisms can compensate for the function of EGF if it is absent or secreted in very low amounts. For instance, detected factors in our experiments such as FGF-2 play a key role in angiogenesis, granulation tissue formation, re-epithelialization, and remodeling.[26] Other cytokines found in CFx-δ2, such as GROα, MCP-1, MCP-2, MCP-4, 6Ckine, BCA-1, ENA-78, I-309, and IL-16, play a significant role in wound healing by chemoattracting leukocytes and enhancing keratinocyte proliferation and migration.[27] Additionally, some cytokines also activate macrophages, which produce additional secretory factors that promote fibroblast survival, proliferation, angiogenesis, and keratinocyte migration.[28] Our results suggest that CFx-δ2 accelerates proliferation and remodeling by reducing inflammation and enhancing cell recruitment to the wound, as demonstrated by its anti-inflammatory effects, accelerated wound healing, and promotion of angiogenesis in vitro.

28 cytokines showed more consistency in cytokine levels and patterns; however, some cytokine values vary within ranges, we used metabolomic analysis and in vitro assays to evaluate consistent biological effects. These tests demonstrated a high degree of consistency across batches in terms of metabolites, anti-inflammatory, pro-angiogenic activity and antioxidant capacity. To our knowledge, this is the first report that demonstrates reproduction of biological products.

Inhibition of NF-κB nuclear translocation and antioxidant effects are critical to accelerating the wound healing process, as NF-κB is a transcription factor that induces the expression of pro-inflammatory cytokines.[17] The inhibition of NF-κB nuclear translocation suggests that CFx-δ2 possesses strong anti-inflammatory activity. While hemostasis and inflammation are necessary in the initial stages of skin injury, prolonged inflammation without subsequent cell proliferation and remodeling can delay or prevent wound repair. Therefore, proinflammatory effects should be abolished by anti-inflammatory effects to initiate tissue repair.[29,30]

Other cytokines found in CFx-δ2, such as IFNγ and IL-28A, have antiviral effects, while IL-6, IL-10, IL-17F, and IL-18 exhibit immunoregulatory effects.[30,31] These secretory factors may play a role in cell-mediated immune responses and antiviral activities, aiding wound healing by regulating inflammation and the immune response to potential infections.

Additionally, CFx-δ2 batches tested on human dermal endothelial cells showed consistent effects on the pattern and density of tube/capillary-like structures in three-dimensional cultures, indicating that CFx-δ2 consistently promotes angiogenesis and contains essential components for blood vessel regeneration. Notably, unlike VEGF, which primarily induced rudimentary isolated tube-like structures, CFx-δ2 promoted the formation of blood vessel networks, suggesting a more comprehensive signaling for revascularization of damaged tissues.[32,33]

The early re-epithelialization process observed in in vitro skin explants was also consistent across batches, indicating strong effects on fibroblasts and keratinocyte activation induced by the secretory factors and metabolites in CFx-δ2. The factors identified in CFx-δ2 have been previously found in wound fluid and are known to promote the proliferation of epithelial and endothelial cells, which are essential for wound healing. Importantly, the wound healing observed in the ex vivo skin explants primarily involved healthy tissue rather than scar formation, suggesting that early intervention with CFx-δ2 may prevent scarring and allow for more effective tissue regeneration.[34]

Conclusion

To our knowledge, we are the first to be able to demonstrate consistency in reproduction of biological products. Our novel and patented methodology enabled the production and scaled up of CFx-δ2 with consistent cytokine and metabolite patterns and levels across batches. These batches also demonstrated consistency in functional effects in vitro. CFx-δ2, derived from immortalized ASCs, contains a combination of physiologically balanced factors that may drive in vivo signaling toward desired anti-inflammatory and wound-healing actions at the cellular level, potentially leading to improved treatment for burn wounds. CFx-δ2 can be formulated into an off-the-shelf acellular burn and wound product for clinical testing. Further studies should be done in-vivo using CFx-δ2 to support our in vitro results.

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Disclosure

All authors report a Patent No.: US 11,473,117 B2. The author (s) report no other conflict of interest.

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