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Novel nanopolymer RNA therapeutics normalize human diabetic corneal wound healing and epithelial stem cells

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Abstract

Human diabetic corneas develop delayed wound healing, epithelial stem cell dysfunction, recurrent erosions, and keratitis. Adenoviral gene therapy modulating c-Met, cathepsin F and MMP-10 normalized wound healing and epithelial stem cells in organ-cultured diabetic corneas but showed toxicity in stem cell-enriched cultured limbal epithelial cells (LEC). For a safer treatment, we engineered a novel nanobiopolymer (NBC) that carried antisense oligonucleotide

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(AON) RNA therapeutics suppressing cathepsin F or MMP-10, and miR-409–3p that inhibits c-Met. NBC was internalized by LEC through transferrin receptor (TfR)-mediated endocytosis, inhibited cathepsin F or MMP-10 and upregulated c-Met. Non-toxic NBC modulating c-Met and cathepsin F accelerated wound healing in diabetic LEC and organ-cultured corneas *vs.* control NBC. NBC treatment normalized levels of stem cell markers (keratins 15 and 17, ABCG2, and ΔNp63), and signaling mediators (p-EGFR, p-Akt and p-p38). Non-toxic nano RNA therapeutics thus present a safe alternative to viral gene therapy for normalizing diabetic corneal cells.

Abstract

Schematics of nanobioconjugate action on diabetic cell and corneal wound healing and epithelial stem cells. Left, effects on cultured LEC: the lead NBC causes faster wound healing than control NBC without therapeutic AON. Stem cell marker expression is increased. Right, similar effects are observed on epithelial wound healing and limbal stem cells in organ-cultured diabetic corneas

Keywords

diabetic cornea; gene therapy; RNA therapeutics; miRNA; nanobioconjugate; wound healing; limbal stem cells

BACKGROUND

Diabetes mellitus (DM) affects all parts of the body including the eye and has reached pandemic proportions (1). A vision-threatening DM manifestation, diabetic retinopathy, mainly affects retinal microvasculature. However, 40%–70% of diabetic patients develop corneal alterations in subbasal corneal nerves (neuropathy) and epithelium (keratopathy) (2–5). Corneal stromal and endothelial cells in diabetes also show some alterations (e.g., increased stromal stiffness and abnormal endothelial morphology) but they appear to be functionally minor (2,4,5). Diabetic keratopathy is manifested by impaired epithelial wound healing, barrier function, and tear production. Long-term diabetics may develop epithelial erosions, ulcers, and keratitis (2,4,6–8), with only symptomatic treatments currently available (2,5).

We previously described specific markers of human diabetic keratopathy including decreased c-Met proto-oncogene/hepatocyte growth factor receptor and increased proteinases, cathepsin F and matrix metalloproteinase-10 (MMP-10) (9,10). We also found dysfunction of diabetic limbal epithelial stem cells (LESC), with downregulation of putative LESC markers (11,12). As a model for studying diabetic keratopathy, we are using human corneal organ cultures that retain diabetic corneal traits including slow wound healing, protein marker changes, and LESC dysfunction (11,13,14), possibly due to epigenetic metabolic memory (15,16). Marker alterations and slow wound healing are also retained in cultured LESC-enriched limbal epithelial cells (LEC) (14). Adenoviral (AV) gene therapy increasing c-Met and/or decreasing MMP-10 and cathepsin F can significantly normalize organ-cultured diabetic corneas (2, 17, 18). However, this therapy was toxic for LEC (12). To circumvent this problem, we developed here nano RNA therapy using new nanobioconjugates (NBCs) carrying antisense oligonucleotides (AONs) against diabetes-

associated genes. NBCs were engineered on natural, non-toxic and biodegradable polymalic acid (PMLA) scaffold (19, 20) and carry covalently attached AONs to suppress cathepsin F (or MMP-10) and to c-Met-inhibiting miR-409, to increase c-Met. They also have cell-targeting monoclonal antibody (mAb) to transferrin receptor (TfR) for receptor-mediated endocytosis that directs NBCs to the endosomes (20). A trileucine (LLL) moiety that ruptures endosomal membrane at low pH is also conjugated to PMLA for NBC release from endosomes into the target cell cytoplasm where AON-PMLA disulfide bonds are cleaved by glutathione and free AONs exert their action (20,21). Similar non-toxic NBCs were successfully used for targeted preclinical therapy of brain and breast tumors (19–23). The NBCs used here were hierarchically synthesized and extensively characterized by chemical and physico-chemical methods. They provided effective and safe AON delivery to cultured stem cell-enriched human LEC where they efficiently modulated target protein levels, accelerated epithelial wound healing and upregulated stem cell markers. Similar effects were observed in organ-cultured human diabetic corneas. Non-toxic NBCs may be efficiently used for normalizing diabetic corneal wound healing and stem cell functions.

METHODS

Organ-cultured corneas

Donor human non-diabetic and diabetic postmortem whole globes and corneas were from National Disease Research Interchange (Philadelphia, PA) that operates under National Institutes of Health oversight. Discarded corneoscleral rims from non-diabetic donors were provided by Drs. Rabinowitz and Maguen after corneal transplantation under approved IRB protocol Pro00019393 from Cedars-Sinai Medical Center. A total of 6 non-diabetic (mean age 57.17 years) and 20 diabetic cases (2 type 1 diabetes, 18 type 2 diabetes; mean age 70.05 years; disease duration 4–46 years, mean 19.75 years) were used (Table 1). Non-diabetic and diabetic groups were age-matched (p=0.0696). Tissues were used within 48 hours after donor death.

The corneas were organ-cultured over agar-collagen gel filling corneal concavity (12, 13). Cultures were kept at a liquid-air interface, in serum-free low glucose DMEM medium supplemented with insulin-transferrin-selenite (Sigma-Aldrich, St. Louis, MO), antibiotics and antimycotic (Thermo Fisher Scientific, Canoga Park, CA) covering the limbal area. Hundred μL medium was added daily to moisten the epithelium.

LEC isolation

LEC were isolated from corneas and rims using dispase II (2.4 U/mL, Roche Life Science, Pleasanton, CA) for 2 h at 37°C (12,24). Corneal endothelium was removed with a cotton swab. Epithelial cells after easing off from the rims were dispersed using 0.25% trypsin – 0.02% EDTA (Thermo Fisher) for 30 min at room temperature. LEC were cultured in EpiLife medium with 60 μM Ca^{2+} containing N2, B27 and HKGS supplements (Thermo Fisher), and 10 ng/mL epidermal growth factor (EGF) (16). Cells were seeded on substrata precoated with a mixture (FCL) of human fibronectin (BD Biosciences, San Diego, CA), type IV collagen (Sigma-Aldrich), and limbal laminin-521 (BioLamina, Matawan, NJ) at 0.5–1 μg/cm^2 (12,24).

Adenoviral transduction of cultured LEC

LEC cultures were used for transduction at 70–80% confluency and passages 1–3. AV construct harboring green fluorescent protein gene (AV-GFP) was added to the cells at multiplicity of infection (MOI) 80 pfu/cell in small volume of medium containing 2 ng/mL EGF and 1 μg/mL poly-L-lysine enhancer, for 24 h at 37°C (12). Cells were then incubated for 4 days in fresh medium, with GFP expression followed microscopically.

Nanobioconjugate synthesis and characterization

NBC synthesis (Figure 1A) followed hierarchical and well controlled steps ensuring high reproducibility and correct action of all functional moieties (20, 21). Pre-conjugate was first synthesized with 40% LLL, 2% mPEG5000 and 10% MEA. It was sequentially conjugated with (a) mixture of Mal-PEG3400-TfR mAb, (b) mixture of PDP-AON miR-409, PDP-AON CF, and (c) PDP to block free thiol groups to obtain final product (Figure 1A). AON MMP-10 was conjugated separately to PMLA. Alexa Fluor 488 fluorescent dye was optionally conjugated to NBC for imaging. The complete NBC based on PMLA scaffold is schematically presented on Figure 1B.

Reagents.—Polymalic acid (PMLA) of 50,000 Da molecular mass (SE-HPLC/polystyrene sulfonate standards, polydispersity 1.2) was isolated from *Physarum polycephalum* M3CVII culture supernatant using Bioreactor BIOSTAT® Bplus (Sartorius, Bohemia, NY) and fractionated on a size exclusion (SE) column (23). It was characterized by NMR, and final products/intermediates by SE-HPLC and reversed-phase-HPLC (25). Trileucine was from Bachem (Torrance, CA). Polyethylene glycol (PEG) derivatives, maleimide-PEG3400-maleimide (Mal-PEG3400-Mal) and mPEG5000-NH2, were from Laysan Bio (Arab, AL). Superdex G-75 and PD-10 columns were from GE Healthcare (Pittsburgh, PA).

Antisense drugs.—Morpholino AONs with 3’ modified with 3-(2-pyridyldithio)-propionate (PDP) were designed by GeneTools (Philomath, OR) as follows: Cathepsin F, 5’-ACCCAACAGACGCTCCACCGACCCA-3’; and MMP-10, 5’-TACCTTCTTTGTCTACTGGGCTTCT-3’. We also used two negative control AONs with the same results, one standard as per GeneTools, 5’-CCTCTTACCTCAGTTACAATTTATA-3’, and ineffective MMP-10, 5’-GCATCATTCTCACTGCCCTTACCTT-3’. To increase c-Met, we used AON to specific miRNAs that suppressed its expression (26–28). AONs to three corneal miRNAs, hsamiR-409–3p (increased in diabetic cornea), hsa-miR-410 and hsa-miR-206 (M. Saghizadeh, unpublished) were tested. AONs against these miRNAs were: hsa-miR-409–3p, 5’-GGGTTCACCGAGCAACATTCGTCGT-3’; hsa-miR-410, 5’-ACAGGCCATCTGTGTTATATTCGTC-3’; and has-miR-206, 5’-ACCACACACTTCCTTACATTCCATA-3’. The AON to miR-409 increased in diabetes was somewhat more efficient in enhancing c-Met expression in cultured LEC compared to AONs to miR-410 and miR-206 (Supplementary Figure S1) and was used in all experiments.

Synthesis of pre-conjugate.—PMLA-based NBC was synthesized in two steps. First, a PMLA pre-conjugate PMLA/mPEG/LLL/MEA (Figure 1A, top) containing LLL, mPEG and 2-mercaptoethylamine (MEA) was prepared. PMLA was first fully activated with N-

hydroxy succinimide (NHS) in the presence of dicyclohexylcarbodiimide. Functional groups including mPEG, LLL and MEA were added sequentially after the completion of each amidation. Thin layer chromatography (Ninhydrin test) was used to confirm that the reaction was complete. Then, the unreacted polymer-bound NHS group was decomposed with phosphate buffer pH 6.8. The pre-conjugate was purified on PD-10 column, lyophilized and stored at −20°C.

Synthesis of full NBCs PMLA/mPEG/LLL/TfR mAb/AON(CF+miR-409–3p or MMP-10).—The full NBC (Figure 1A, bottom; Figure 1B) has several functional moieties that were conjugated by reacting with the thiol group of the PMLA pre-conjugate through bifunctional linkers: Mal-PEG3400-Mal for mAb, and succinimidyl-3- (2-pyridyldithio)-propionate (SPDP) for AON.

Susceptible internal disulfide bonds of cell-targeting mouse anti-human TfR mAb OKT-9 (Bio × Cell, West Lebanon, NH) were first reduced in phosphate buffer containing 5 mM Tris(2-carboxy ethyl) phosphine hydrochloride for 30 min at room temperature (RT), followed by PD-10 column purification (22). The reduced mAb was conjugated with Mal-PEG3400-Mal and purified on Sephadex G-75. mAb(S-succinimidyl-PEG3400-maleimide) was concentrated by diafiltration (30 kDa cutoff). Conjugation of Mal-PEG3400-Mal to mAbs was verified by SE-HPLC.

Pre-conjugate in 100 mM phosphate buffer pH 6.3 was added to TfR mAb-PEG3400-Mal with one mAb molecule per one PMLA molecule, in the same buffer at RT. mAb conjugation was verified by SE-HPLC. AONs were conjugated to PMLA through a disulfide bond that can be cleaved from the scaffold by disulfide exchange with cytosolic glutathione. PMLA/mPEG/LLL/TfR mAb/MEA was added to equimolar mixture of PD-PAONs, and unreacted SH-groups blocked with PDP, followed by purification on Sephadex G-75. For imaging, PMLA was labeled on pendant sulfhydryls with Alexa Fluor 488 maleimide (21). The nanocomplexes were analyzed by wet chemistry for the kind and amount of conjugated functional ligands (29). Estimated average Mw was 412 kDa for NBC with 50 kDa PMLA, 1 mAb molecule, 9 AON molecules, 172 LLL molecules and 9 PEG molecules. Detailed physico-chemical characterization of NBCs is described in Supplementary material.

AON and nanobioconjugate transfection of cultured LEC and cornea

LEC cultures were transfected with free Morpholino AON or with NBCs. For free AONs, cells were treated at 5–10 μM AON in the presence of 4 μM Endo-Porter peptide (Gene Tools) twice for 48 h with medium change (total 96 h), then harvested for western analyses. LEC cultures were treated with NBCs at 5–10 μM AON the same way. Because of targeting antibody to TfR on the NBC, no enhancer was needed for efficient transfection. Organ-cultured human diabetic corneas were transfected with NBCs using Lipofectamine STEM (Thermo Fisher), because without it NBCs could not reach corneal basal epithelial cells. Corneas were transfected similar to LEC, at a concentration of 5–10 μM AON for 96 h, with medium change after 48 h. In wound healing experiments, wounds were made after full incubation period with NBCs.

Cell viability was tested after NBC transfection at 5–30 μM AON of cultured normal and diabetic LEC using Alexa Fluor 488 Annexin V Apoptosis Kit (Thermo Fisher). Annexin V-positive cells were counted using inverted fluorescent microscope DML LED Fluo (Leica Microsystems CMS, Germany). For both normal and diabetic cells, cultures from three separate age-matched donor corneas were used. On average, 900 cells were counted for each AON dose in each LEC culture. For organ-cultured corneas, activated caspase-3 (BD Pharmingen) was assessed by corneal section immunohistochemistry.

Corneal epithelial wound healing

Epithelial removal was achieved using a 5-mm paper disc soaked in n-heptanol (1-heptanol; Sigma-Aldrich) placed on the corneal surface for one min (13). Both fellow healing corneas treated with therapeutic or control NBC were monitored every 24 h until complete healing of the created defect (17). Corneas were frozen in liquid nitrogen for western analysis or embedded in OCT compound (Sakura Finetek USA, Torrance, CA) for cryosectioning.

Cultured LEC wound healing assay

Scratch wounds were created with a 200-μL pipette tip in sub-confluent LEC cultures treated with control and therapeutic NBCs (AONs at 10 μM). The removed cells were washed out and cultures were incubated in EpiLife medium (Thermo Fisher) supplemented with 2 ng/mL EGF. Twenty hrs later, the healing was quantitated using ImageJ software (NIH, Bethesda, MD) on digitized pictures of wound areas and statistically analyzed. All experiments were performed in triplicate.

Immunostaining

LEC cultures were fixed in 1% *p*-formaldehyde for 10 min at 4°C and permeabilized with 0.25% Triton X-100 for 5 min at room temperature, or with cold methanol for 10 min at 4°C. Five-μm thick corneal sections from NBC-treated organ-cultured corneas were fixed in 1% *p*-formaldehyde for 5 min at room temperature and stained for putative diabetic and LESC markers. Primary antibodies are listed in Table 3. FITC or TRITC conjugated or Alexa Fluor 488 conjugated secondary antibodies were from Thermo Fisher. Cell nuclei were visualized with 4’,6-diamidino-2-phenylindole (DAPI). Photography of stained sections was performed using the same exposure time for each marker. Negative controls with omission of primary antibody were routinely included.

Western blot analysis

4–20% or 8–16% gradient Tris-glycine SDS polyacrylamide gels (Thermo Fisher) were used, with gel loading normalized by β-actin content (33). Primary antibodies are listed in Table 3. Reactive protein bands revealed with fluorescent secondary antibodies were visualized using Odyssey Clx System (all from LI-COR, Lincoln, NE).

Flow cytometry

Various cultured cells were trypsinized, washed in cold PBS with 2% FBS, and blocked with 1% BSA in PBS for 10 min at 4°C. They were incubated with rhodamine-labeled anti-TfR mAb OKT-9 for 45–60 min at 4°C. Cells were washed twice using cold PBS with 2% FBS,

fixed with 2% *p*-formaldehyde and run on a FACS BD LSRFortessa (BD Biosciences). Quantitative analysis was performed using BD FACSDiva software 6.0 (BD Biosciences). Negative controls included unstained diabetic LEC and D2F2 mouse breast cancer cells. Positive controls included normal human brain microvascular endothelial cells, BT-474 human breast cancer cells and LN229 human glioma cells.

Statistical analysis

Quantitative data were statistically analyzed with Prism7 software (GraphPad, San Diego, CA). Data from two groups were analyzed by a two-tailed Student *t* test, and from three or more groups, by one-way ANOVA with Dunnett's multiple comparisons posttest. $p<0.05$ was considered significant. Values are expressed as mean ± SEM.

RESULTS

Toxicity evaluation of adenovirus to cultured LEC and of NBC to LEC and corneas

In preliminary experiments, AV constructs safely transduced telomerase-immortalized corneal epithelial cells (33) (data not shown here). However, in primary LEC cultures, AV-GFP transduction even at a relatively low MOI produced cytotoxicity manifested by marked increase of Annexin V-positive apoptotic cells (Figure 2A). This prompted us to use a PMLA-based NBC because a similar construct was previously found to be non-toxic *in vivo* (19). To assess NBC toxicity, cultured normal and diabetic LEC were transfected with control (PMLA/mPEG/LLL/TfR mAb/AON Standard) and therapeutic (PMLA/mPEG/LLL/TfR mAb/AON miR-409+CF) NBCs at a wide AON dose range (5–30 μM). The numbers of Annexin V-positive apoptotic cells varied from 2 to 5%. No significant dose-dependent differences in the level of apoptosis were detected either in normal or diabetic LEC treated with control and therapeutic NBC (Figure 2B). When diabetic corneal sections after control and therapeutic NBC treatment (n=5) were stained for activated caspase-3, the expression was found only in occasional cells mostly in the limbus; this was similar to *ex vivo* (n=3) corneas (Figure 2C).

Structure and properties of the new NBCs

The lead NBC was designed to suppress the expression of diabetes-upregulated cathepsin F and of miR-409–3p that inhibits diabetes-downregulated c-Met (to increase c-Met expression). Due to the platform constraints, inhibition of another diabetes-upregulated component, MMP-10, was achieved using a separate NBC. Morpholino AON as specific, stable and long-lasting inhibitors (34, 35) were chosen to suppress the expression of diabetes-related components. NBC synthesis (Figure 1A) followed hierarchical and well controlled steps for high reproducibility and function of all moieties (20,21). The complete NBC based on PMLA scaffold contained AONs to inhibit cathepsin F and miR-409–3p or MMP-10, polyethylene glycol copolymer (mPEG5000) that protects against enzymatic degradation, targeting mAb to TfR, endosome escape unit (LLL) for pH-dependent drug release inside the cell (20,21), and an optional fluorescent dye Alexa Fluor 488 for construct localization (Figure 1B). Free or NBC-conjugated AONs were verified for efficacy against their targets (Figure 3).

Receptor-mediated NBC uptake by cultured LEC

Previous studies indicated that TfR/CD71 was enriched in the stem cell-harboring limbal basal epithelial cells (36). To evaluate TfR mAb usability for delivering NBCs inside the diabetic LEC, we determined their TfR levels. Western analysis showed substantial expression of TfR in LEC cultures from four diabetic donors (Supplementary Figure S4A). This was corroborated by flow cytometry. Low signal was seen in unstained diabetic LEC and mouse breast cancer cells. After anti-TfR staining, diabetic LEC showed strong signal, comparable to brain endothelial cells, human breast cancer and glioma cells (Supplementary Figure S4B). About 95% of diabetic LEC expressed TfR.

A 2 h incubation of diabetic LEC with Alexa Fluor 488-labeled NBC (PMLA/mPEG/LLL/AON MMP-10/Alexa Fluor 488) resulted in its significant uptake by most cells (Supplementary Figure S4C, left). Preincubation of the cells with unlabeled anti-TfR antibody for 2 h followed by a 2 h incubation with the NBC, resulted only in background uptake, consistent with receptor-mediated endocytosis (Supplementary Figure S4C, middle). A strong NBC uptake was observed after 24 h incubation with diabetic cells (Supplementary Figure S4C, right). Previous studies have confirmed the accumulation of the anti-TfR containing NBC in endosomes of cultured cells (31).

Modulation of therapeutic target expression in cultured diabetic LEC by NBCs

By western blot analysis, free AON to miR-409 delivered using Endo-Porter reagent increased c-Met expression compared to negative control or untreated LEC. Free AON to either MMP-10 or cathepsin F downregulated their respective targets (Figure 3A), indicating the efficacy of selected AONs. Similar effects were observed after direct treatment of diabetic LEC with complete NBCs carrying corresponding AONs (Figure 3B).

NBC effects on protein marker expression and wound healing in diabetic LEC

We next examined whether NBC treatment could normalize decreased expression of putative stem cell markers and impaired wound healing in cultured diabetic LEC. By western analysis, lead NBC treatment led to a significant increase of the expression of putative stem cell markers keratin 15 (K15), K17, ΔNp63, and ATP-binding cassette sub-family G member 2 (ABCG2) in unwounded diabetic LEC (Figure 4). Comparative immunostaining for K15 confirmed that its expression became similar to normal LEC (Figure 5A).

Wound healing in diabetic LEC treated with lead NBC was significantly accelerated as compared to control NBC (by 32.1%; Figures 5B, C) and became similar to normal LEC [the difference in healing between diabetic and normal LEC was previously found to be about 26% (14)]. A combination of lead NBC modulating c-Met and cathepsin F with NBC inhibiting MMP-10 was more efficient, however, the difference between the two treatments did not reach significance (Figure 5C).

In organ-cultured diabetic corneas treated with NBC bearing AON CF+miR409, significant increase of c-Met (Figure 6A) and a decrease in cathepsin F (Figure 6B) as compared to control AON were observed by western analysis. By immunostaining, the treatment with lead NBC compared to control AON markedly increased central corneal and limbal

expression of diabetes-downregulated (13) integrin $\alpha_3\beta_1$ and nidogen-1 (Figure 7A). Similar to cultured LEC, this treatment in most cases (Table 4) also increased the expression of putative stem cell markers ΔNp63, K15 and K17 (Figure 7A), as well as ABCG2 (Figure 7B). The treatment also significantly accelerated healing of 5-mm epithelial wounds in diabetic corneas *vs*. control NBC (Figure 7C).

We have previously found that downregulation of cathepsin F and MMP-10 and upregulation of c-Met increased the expression of activated/phosphorylated signaling mediators EGFR-Akt and p38, respectively, in diabetic corneas (37, 38). In line with this observation, NBC treatment of diabetic organ-cultured corneas also caused the activation/phosphorylation of these wound healing-mediating signaling molecules including p-Akt, p-p38 (Figure 8) and p-EGFR (Supplementary Figure S5). This was revealed by both western analysis and immunostaining of sections of wounded corneas. The observed effects were similar in magnitude to those documented previously with AV-based gene therapy (37, 38).

DISCUSSION

Clinical and experimental studies of diabetes have established distinct pathogenic traits of this systemic and relentless disease. In the cornea, diabetic keratopathy and neuropathy are considered the main complications leading to severe impairment of wound healing and epithelial stem cell dysfunction, as well as to progressive loss of corneal nerves (2, 8, 39). The major clinical issue with diabetic corneal disease is that it is a widespread complication, but is often underdiagnosed and has an urgent need for a specific treatment (2, 5). In recent years, distinct molecular markers of diabetic cornea have been described that can be targeted by specific therapy to ameliorate corneal condition in diabetics (2, 5, 12, 39, 40–42).

In our previous work, we have identified markers that were over- or underexpressed in human diabetic corneas including MMP-10, cathepsin F and c-Met (9, 10). To revert the expression of these markers to normal we used viral gene therapy that is an efficient tool to correct abnormal protein expression in different systems including cornea (43–45). Adenoviral gene therapy allowed us to normalize levels of these proteins in organ-cultured diabetic corneas with concomitant significant amelioration of wound healing, various markers and signaling intermediates (2,12).

In cases of diabetic keratopathy with impaired healing following vitrectomy or refractive surgeries, or with persistent epithelial defects, the dysfunctional limbal epithelium (11) may need to be replaced. However, allogeneic LEC transplant will entail continuous immunosuppression (46). For autologous transplantation, marker expression and wound healing ability of cultured LEC will need to be first normalized by gene therapy. However, when adenoviral therapy standardized for whole corneas was attempted on stem cell-enriched cultured LEC, marked toxicity was observed (Figure 2A).

In recent years, nano constructs elicited considerable interest for drug delivery because of frequent lack of toxicity, biodegradability, ability to carry a number of therapeutic and targeting modules and cell penetrating moieties, and increased retention time (20, 47, 48).

Nano constructs have been successfully used in preclinical studies in rodents to deliver drugs to various parts of the eye (49, 50).

Our lead NBC was readily accumulated in LEC by receptor-mediated endocytosis through the conjugated antibody to TfR expressed on the basal corneal epithelial cells both *in vitro* (Supplementary Figure S4) and *ex vivo* without the need of transfection enhancers unlike free AON (36). It was shown to be non-toxic in cultures of diabetic LEC unlike adenoviral constructs used previously. Treatment with lead NBC led to significantly faster wound healing and increased levels of several stem cell markers in diabetic LEC. Importantly, the lead NBC was beneficial for the organ-cultured diabetic corneas similarly to the AV gene therapy in terms of stem cell marker expression and wound healing. Also, the lead NBC was able to normalize the levels of proteins decreased in diabetic cornea, integrin $\alpha_3\beta_1$ and nidogen-1 (Figure 7A).

Like AV treatment, the NBC that increased the expression of c-Met and decreased cathepsin F (and another one that decreased MMP-10) normalized the levels of activated signaling mediators p-p38, and p-Akt – p-EGFR that are important for corneal wound healing and cell proliferation (2,17,18). A similar influence of c-Met on p38 activation and of cathepsin F or MMP-10 on Akt activation was also found in other cell types. In the cancer-associated human pancreatic stellate cells, c-Met inhibition significantly reduced phosphorylation of p38 but not of phosphatidylinositol-3 kinase (PI-3K, upstream of Akt) (51). In gastric cancer cells, cathepsin F knockdown by shRNA induced cell proliferation presumably through PI-3K/Akt pathway, and increased RalB that promotes cell migration (52). In skin wounds of transgenic mice, a constitutively active MMP-10 in keratinocytes reduced Akt phosphorylation at the tip of migrating epithelium (53). These results are in line with our data showing that increase of c-Met and decrease of cathepsin F or MMP-10 achieved by the NBC are associated with activation of p38 and Akt-EGFR. Activation of these signaling pathways in the diabetic cornea by modulating levels of diabetic markers appears to be the molecular mechanism of NBC influence on stem cells and wound healing.

Overall, the used NBCs were able to effectively deliver AON to both diabetic human LEC and organ-cultured corneas and restore impaired wound healing and expression of various markers suppressed by the disease.

Previous corneal studies in preclinical models have already shown promise of nano drug carriers (54,55). The advantage and novelty of the present work is the use for the first time of non-toxic and biodegradable cell-targeted nanoconstruct that was able to correct both protein expression levels and functional defects in a common human corneal disease with stable, gene-specific and long-lasting AONs that show surge in recent FDA approvals (20, 34, 56). Non-toxic and efficient NBCs may constitute a new alternative to viral gene therapy in normalizing diabetic corneal stem cells and wound healing. They should be also suitable for preparing cultured diabetic cells for potential transplantation to diabetic corneas in cases with diabetic LESC deficiency. The described NBCs are fairly versatile and can carry inhibitors or boosters of various targets (19–23). Additional to diabetic corneal cell normalization, they could potentially inhibit keratocyte apoptosis following epithelial wounding if they have an attached antibody to interleukin-1 or its receptor antagonist (18).

Alternatively, an NBC with conjugated TGF-β inhibitor could be used as a therapeutic against stromal fibrosis or haze caused by various reasons including refractive surgery. Optimized and highly reproducible NBC synthesis and relatively long-lasting effect of AONs would ensure feasibility of such treatments.

The used NBCs not only normalized cultured diabetic LEC but also organ-cultured corneas, which may be useful for the future translation to the clinic in cases not requiring cell replenishment. In this respect, it would be advantageous to use topically applied NBCs, e.g., as eye drops. However, the multilayered corneal epithelium presents a serious barrier for the penetration of drugs. *Ex vivo* studies have shown that the intact epithelium allows only 0.5% of the applied nanoparticles to penetrate into the bovine cornea as opposed to 22% after cell removal (57). It should be noted that many studies of topical nano drug application were performed in mice and rabbits with significantly thinner corneas than humans (58, 59). Additionally, the stem cell harboring limbal part is thicker than central part in humans but thinner in rabbits or mice. Therefore, drug delivery to the human corneal cells especially, epithelial stem cells may present a bigger obstacle than in rodents. In our experiments with whole human diabetic corneas, an enhancer was needed for the NBC to efficiently penetrate through the epithelial layer and influence basal LEC harboring stem cells. Currently, no approved lipophilic drug delivery enhancers are available. However, many enhancers are being studied for ocular drug delivery giving hope for the development of safe and efficient agents for enhancing corneal penetration of promising new nano drugs (60).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- New nanopolymers with covalently attached antisense RNA therapeutics and cell-targeting antibody were synthesized and characterized
- Nanopolymers inhibited diabetic corneal targets cathepsin F and MMP-10, and boosted c-met expression in human cultured progenitor cells and corneas
- Nanopolymer treatment of cultured progenitor cells and corneal organ cultures accelerated wound healing and stem cell marker expression
- Non-toxic and biodegradable nano RNA therapeutics may be useful for alleviating signs of diabetic eye disease

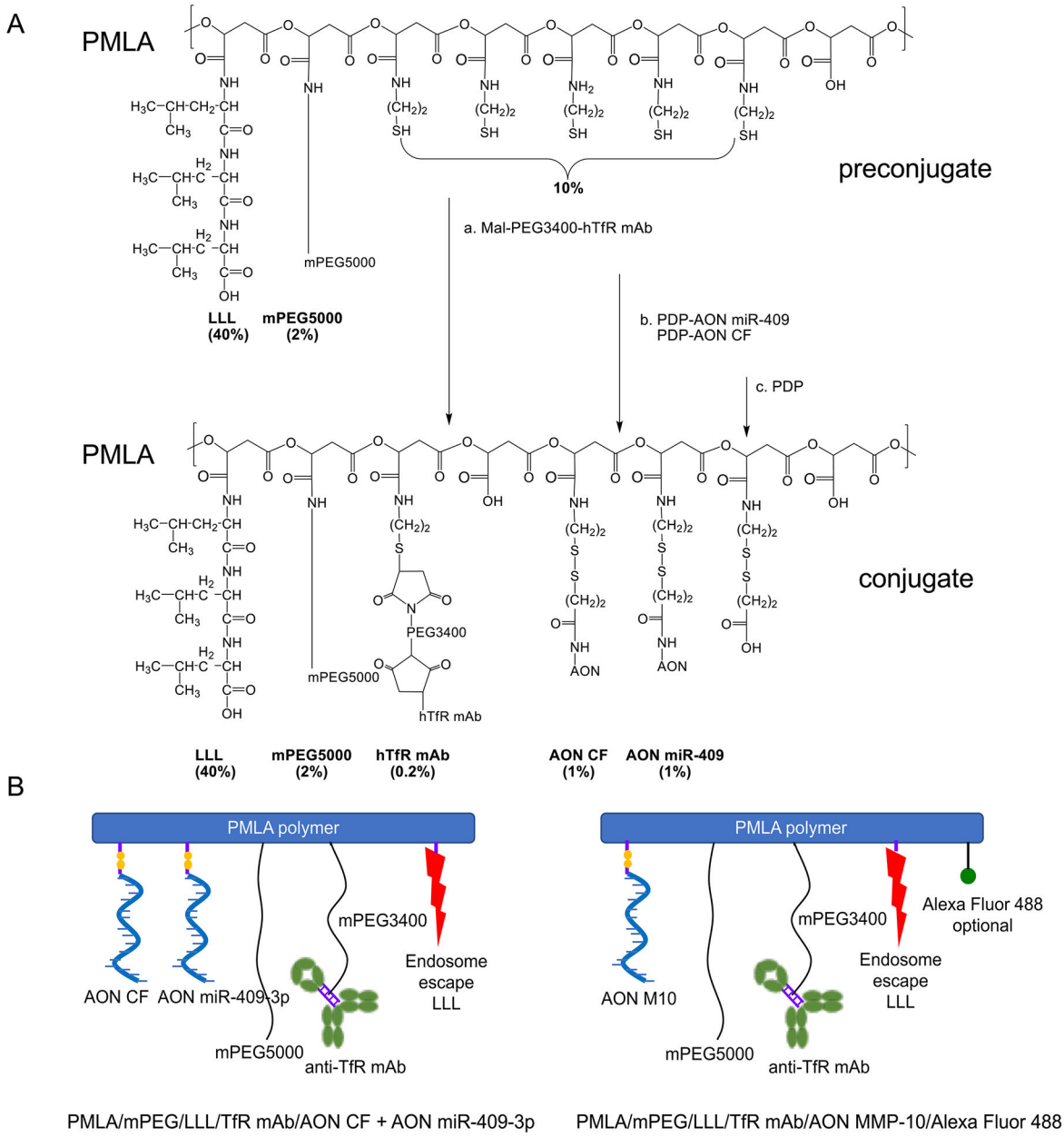

Figure 1.

NBC synthesis. A, first, a pre-conjugate is synthesized with 40% LLL, 2% mPEG5000 and 10% MEA (top). It is sequentially conjugated with (a) mixture of Mal-PEG3400-TfR mAb, (b) mixture of PDP-AON miR-409-3p, PDP-AON CF, and (c) PDP to block remaining free thiol groups (bottom). AON MMP-10 is conjugated separately to the same platform. Alexa Fluor 488 fluorescent dye is optionally conjugated to follow NBC distribution and transfection efficiency. B, PMLA-based NBC structure and variants for gene therapy of diabetic corneal cells. AONs inhibit CF and miR-409-3p or M10, PEG copolymer (mPEG5000) protects against enzymatic degradation, TfR mAb targets cells, and trileucine (LLL) ensures endosomal escape and drug release inside the cell. Abbreviations: P, polymalic acid; LLL, trileucine; CF, cathepsin F; TfR, transferrin receptor; AON,

Morpholino antisense oligonucleotide; miR-409-3p, miRNA inhibiting c-Met expression, M10, MMP-10.

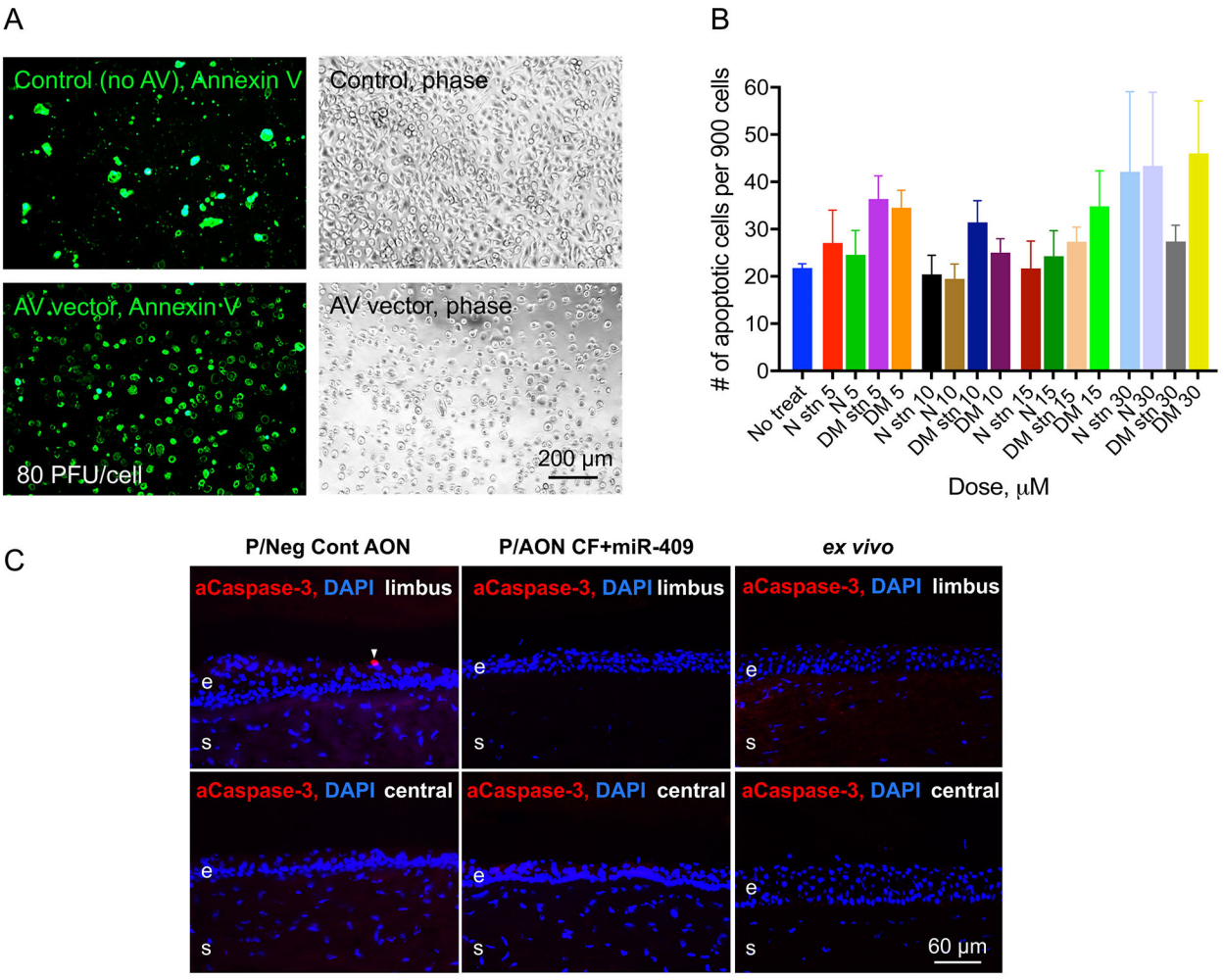

Figure 2.

Effects of treatments on LEC and corneal cell viability. A, AV-GFP treatment significantly increased LEC apoptosis *vs.* untreated LEC (Annexin V staining) even at 80 pfu/cell. Positive cells in untreated cultures are mostly dying cells on top of the monolayer. Initial cell density in treated and untreated cultures was the same. Bar = 200 µm. B, Cell death (Annexin V) assay of various NBC doses in LEC. At 5-30 µM AON in NBC, the treatment did not significantly increase the number of apoptotic cells in non-diabetic (N) or diabetic (DM) LEC cultures over no treatment control or NBC with standard AON (stn). At all doses, the apoptotic cell fraction did not exceed 5%. C, activated caspase-3 staining in control or lead NBC-treated corneas (n=5). Only occasional epithelial cells (arrowhead) are positive, mostly in the limbus. This is similar to *ex vivo* corneas (n=3). e, epithelium; s, stroma; P, PMLA. Bar = 60 µm.

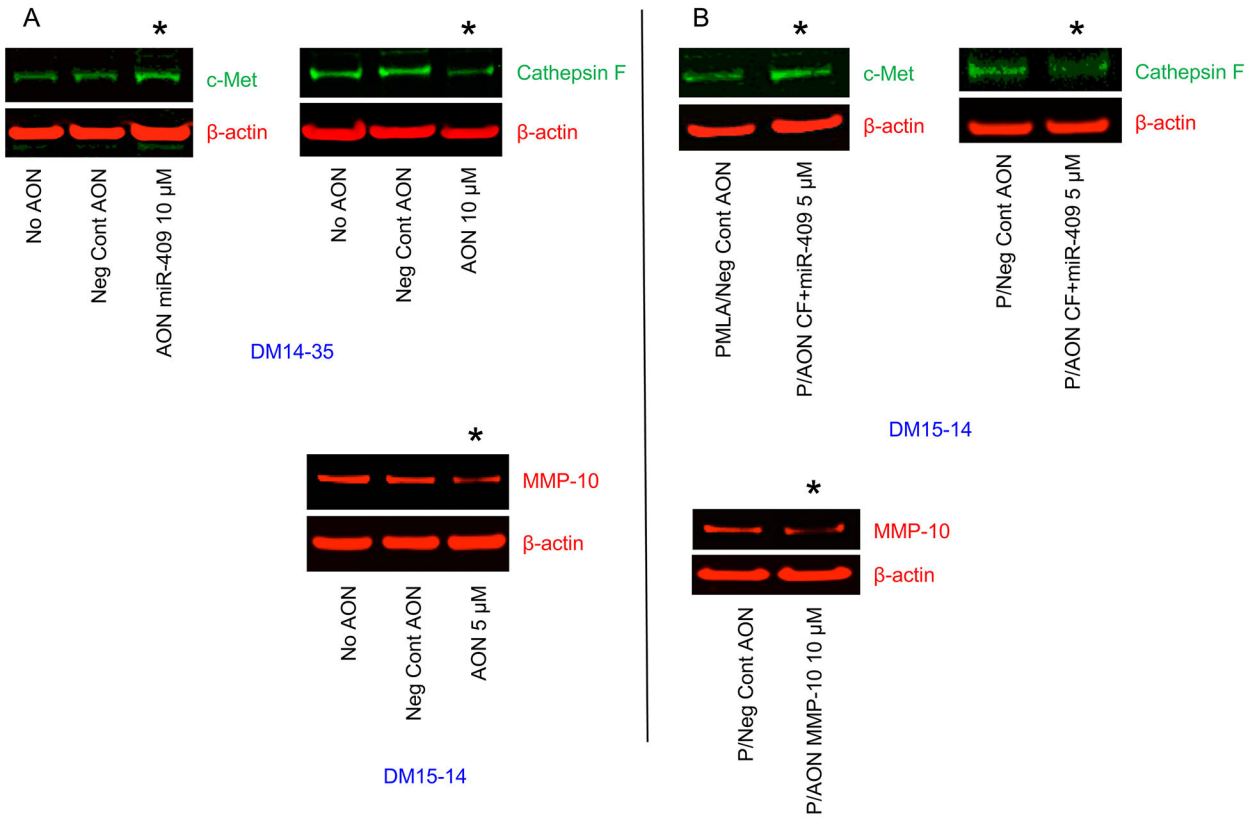

Figure 3.

Effects of free AONs (A) or AONs conjugated to NBCs (B) on target protein expression in diabetic LEC. In both cases, treatment led to increased c-Met and decreased cathepsin F (CF) and MMP-10. Representative western blots of cell lysates normalized to β-actin from different diabetic cases (DM14-35 and DM15-14) are shown. Asterisk denotes a band of interest after NBC treatment. Neg Cont, negative control with scrambled AON; P, PMLA. Experiments were performed by Dr. P.R. Gangalum (UCLA School of Medicine, Los Angeles, CA, USA).

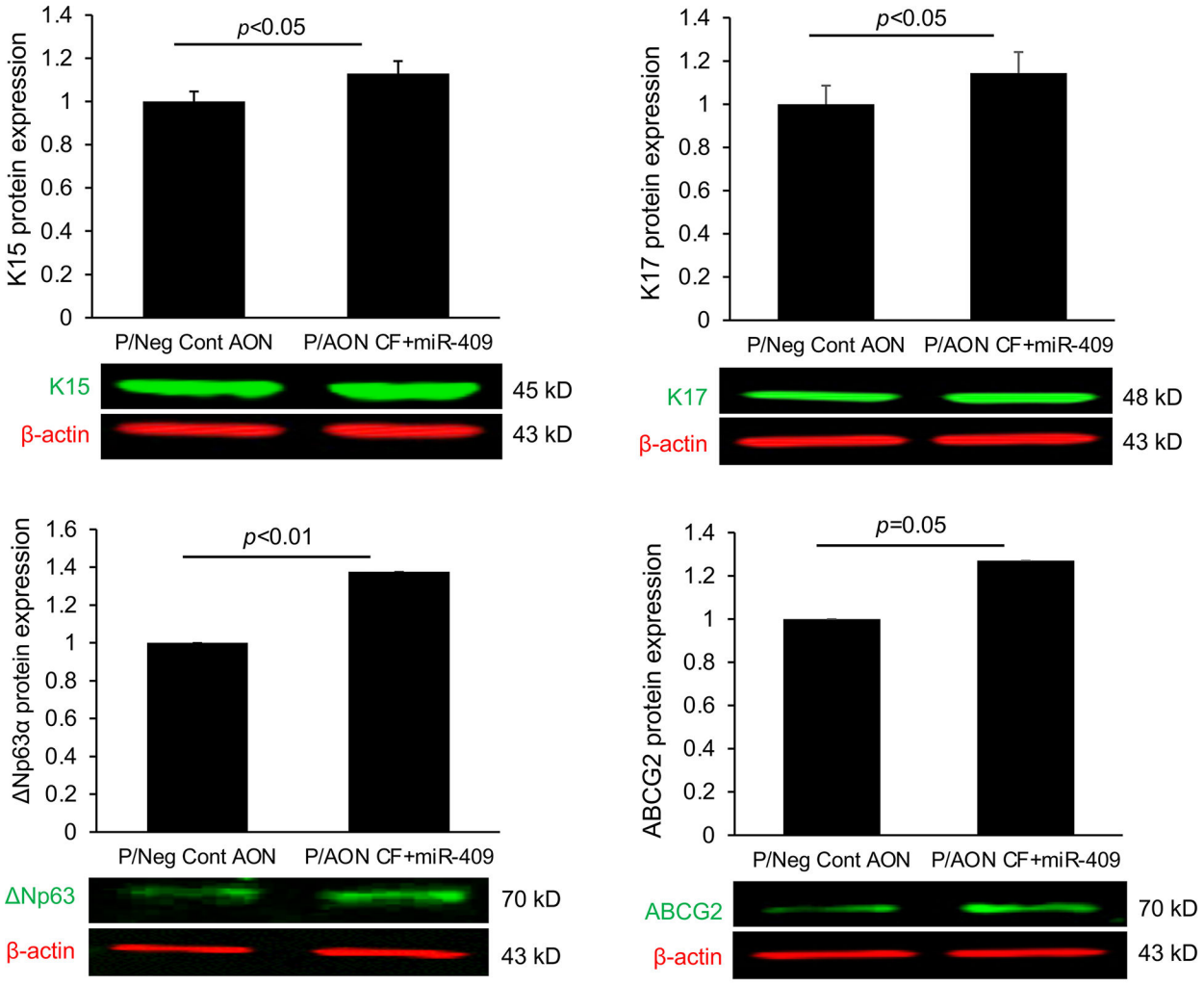

Figure 4.

Increased expression of putative LESC markers in cultures of unwounded diabetic LEC after lead NBC treatment. By western analysis, NBC treatment of diabetic LEC led to enhanced expression of LESC markers keratin 15 (K15, number of independent diabetic cases n=3, with at least two independent experiments with each case), keratin 17 (K17, n=3), ΔNp63 (n=5), and ABCG2 (n=5). The increase after treatment vs. negative control NBC was statistically significant except for ABCG2 that still showed a clear trend with p=0.05. Data are relative to β-actin. Here and below, the relative expression after negative control treatment was taken as 1. Here and below, data are mean ± SEM.

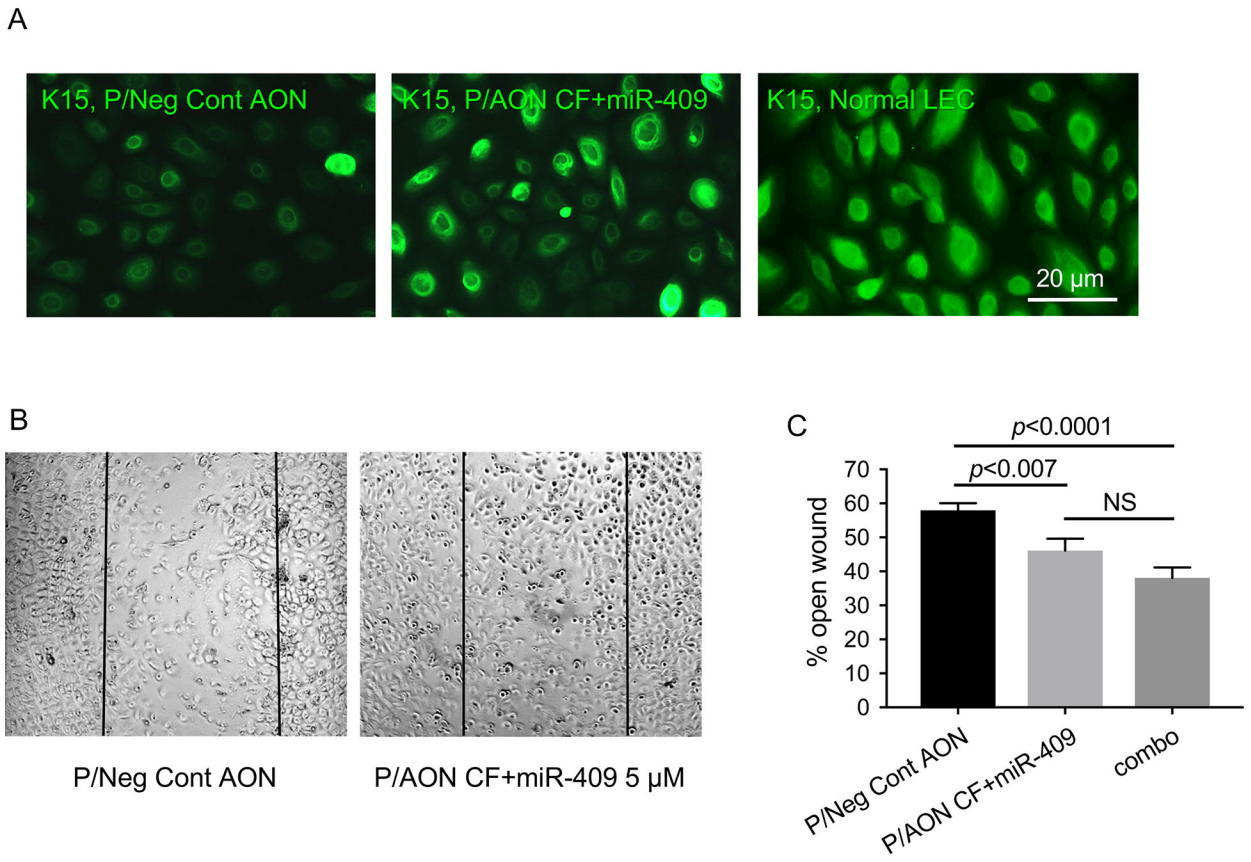

Figure 5.

Normalizing effects of NBCs in diabetic LEC cultures. A, By immunostaining, control LEC showed rare K15-positive cells. After lead NBC treatment, the number of positive cells markedly increased and became similar to normal LEC. B, NBC accelerated scratch wound healing in diabetic LEC (phase contrast). C, lead NBC significantly reduced open scratch wound area compared to NBC with scrambled AON. The addition of a second NBC inhibiting MMP-10 (combo) further accelerated wound healing; however, the difference from lead NBC was not significant. The graph comprises data from four to five independent experiments using LEC from three independent cases. Bar = 20 μm.

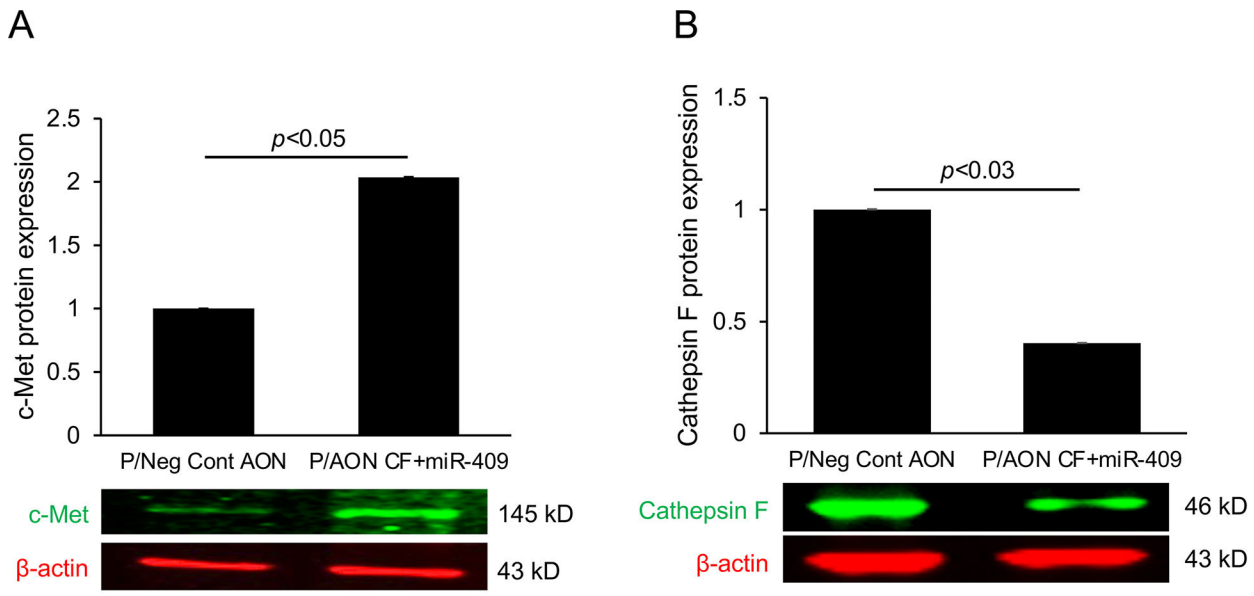

Figure 6.

NBC effects on treatment targets in organ-cultured diabetic corneas. By western analysis, the lead NBC significantly increased c-Met (A, number of independent cases n=3) and decreased cathepsin F (B, n=4) *vs.* negative control NBC. Data are relative to β-actin.

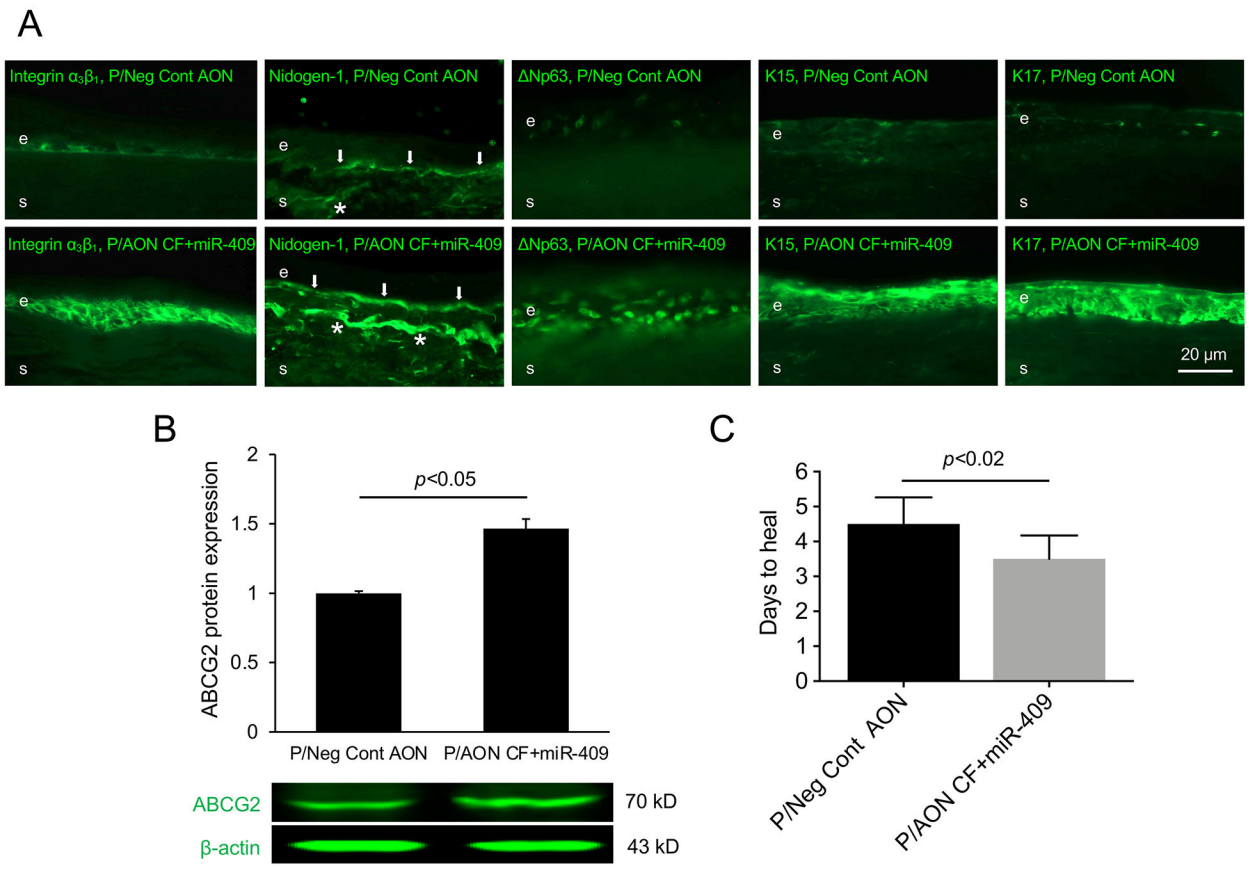

Figure 7.

Effects of lead NBC on marker expression and wound healing in organ-cultured diabetic corneas. A, immunostaining of the limbus harboring stem cells for diabetic (integrin $\alpha_3\beta_1$ and nidogen-1) and LESC (ΔNp63, K15, and K17) markers. Note substantial increase of all markers after NBC treatment (n=5). Asterisks in nidogen-1 panels denote positive limbal blood vessels; arrows denote the epithelial basement membrane. B, western analysis for LESC marker ABCG2 also shows significant increase after treatment (n=5). C, lead NBC significantly decreases corneal epithelial healing time vs. control NBC (n=6). e, epithelium; s, stroma. Bar = 20 μm.

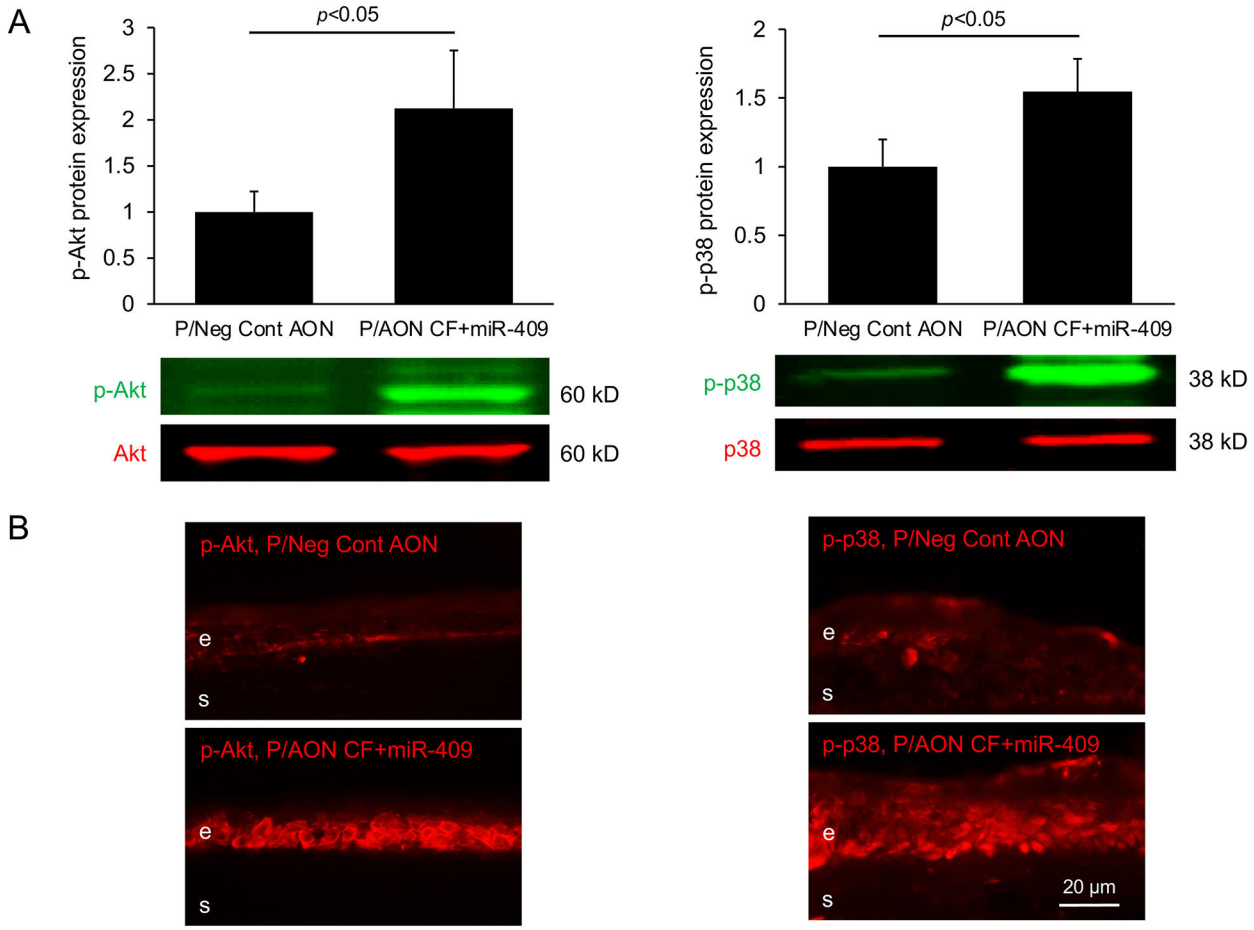

Figure 8.

Lead NBC activates wound healing-related signaling. A, by western analysis, NBC treatment significantly increased phosphorylated/activated signaling intermediates p-Akt (n=4) and p-p38 (n=5) vs. total Akt and total p38, respectively, in wounded and healed diabetic organ-cultured corneas. B, immunostaining showed a similar increase of signaling intermediates *vs.* negative control NBC. e, epithelium; s, stroma. Bar = 20 µm.

Table 1.

Donor characteristics

Case #	Age (years)	Sex	DM Type	Duration (years)	Cause of death	Culture conditions
DR10-16	63	M	NIDDM	10	Aspiration pneumonia	*ex vivo*
DM18-17	79	F	NIDDM	10	Cardiac arrest	*ex vivo*
DM19-28	79	M	NIDDM	15	Cardiac arrest	*ex vivo*
DM13-02	91	M	NIDDM	6	Pneumonia	LEC culture
DM13-15	54	M	IDDM	46	Acute renal failure	LEC culture
DM14-35	62	F	NIDDM	30	Multisystem organ failure	LEC culture
DM14-41	67	M	NIDDM	21	Myocardial infarction	LEC culture
DM15-04	81	M	NIDDM	20	Congestive heart failure	LEC culture
DM15-14	60	M	NIDDM	25	Pancreatic cancer	LEC culture
DM15-15	71	F	NIDDM	30	Cardiac arrest	LEC culture
DM15-25	88	M	NIDDM	8	Cardiac arrest	LEC culture
DM16-28	88	F	NIDDM	40	Cardiac arrest	LEC culture
DM17-02	71	M	NIDDM	9	Pneumonia	Organ culture
DM17-09	75	M	NIDDM	26	Brain cancer	Organ culture
DM17-13	52	F	IDDM	37	Myocardial infarction	Organ culture
DM17-54	61	M	NIDDM	15	Heart failure	Organ culture
DM18-13	58	M	NIDDM	4	Myocardial infarction	Organ culture
DM18-16	53	F	NIDDM	20	Arrhythmia-induced cardiac arrest	Organ culture
DM18-18	69	F	NIDDM	8	Cardiac arrest	Organ culture
DM19-28	79	M	NIDDM	15	Cardiac arrest	LEC culture
N13-39	59	F	N/A	N/A	Intracranial hemorrhage	LEC culture
N14-40	27	M	N/A	N/A	Multi-trauma	LEC culture
N16-12	86	F	N/A	N/A	Stroke	LEC culture
N16-18	64	M	N/A	N/A	Lung cancer	LEC culture
N16-29	69	M	N/A	N/A	Metastatic colon cancer	LEC culture
N17-44	38	F	N/A	N/A	Trauma	LEC culture

M, male; F, female; N/A, not applicable; IDDM, type 1 diabetes; NIDDM, type 2 diabetes.

Table 2.

Characterization of used nanoconjugates

Parameter	**PMLA/LLL/OKT-9/Control AON/Alexa Fluor 488**	**PMLA/LLL/OKT-9/AON MMP-10/Alexa Fluor 488**	**PMLA/LLL/OKT-9/AON CF/AON miR-409/Alexa Fluor 488**
	(n=8)	**(n=8)**	**(n=8)**
Polymer MW	412 kD	412 kD	412 kD
Alexa Fluor 488 (μM)	20	18.3	30
OKT-9 TfR antibody (%)	0.2%	0.2%	0.2%
AON Conc (μM)/each	200	200	200
Conjugate size (nm)	19.9	22.7	23.8
ζ-potential (mV)	−11.2	−11.5	−10.6

Table 3.

Primary antibody list

Antigen	Antibody	Catalog number and source	Dilution	Application
β-Actin	Mouse mAb	A5316, Sigma-Aldrich (St. Louis, MO)	1:1000	WB
β-Actin	Rabbit mAb	8457, Cell Signaling Technology (Danvers, MA)	1:1000	WB
c-Met	Rabbit mAb	8198, Cell Signaling Technology	1:1000	WB
Cathepsin F	Rabbit pAb	sc-13987, Santa Cruz Biotechnology (Dallas, TX)	1:1000	WB
ABCG2	Mouse mAb	MAB4155, EMD Millipore (Billerica, MA)	1:1000	WB
Keratin 15	Mouse mAb	sc-47697, Santa Cruz Biotechnology	1:10-1:50	IHC
Keratin 15	Rabbit mAb	Ab52816, Abcam (Cambridge, MA)	1:2000	WB
Keratin 17	Mouse mAb	sc-58726, Santa Cruz Biotechnology	1:1	IHC
Keratin 17	Rabbit mAb	4543S, Cell Signaling Technology	1:2000	WB
ΔNp63/p40	Mouse mAb	ACI3066, Biocare Medical (Pacheco, CA)	1:5	IHC
ΔNp63/p40	Rabbit mAb	ab167612 Abcam (Cambridge, MA)	1:1000	WB
Nidogen-1	Mouse mAb	MAB2570, R&D Systems (Minneapolis, MN)	1:50	IHC
Integrin $\alpha_3\beta_1$	Mouse mAb	MAB1992, EMD Millipore	1:100	IHC
p-Akt (S473)	Rabbit pAb	9271, Cell Signaling Technology	1:1000; 1:50	WB, IHC
Akt	Mouse mAb	2920, Cell Signaling Technology	1:1000	WB
p-p38 (T180/Y182)	Rabbit mAb	05-1059, EMD Millipore	1:5	IHC
p-p38 (T180/Y182)	Rabbit pAb	4511, Cell Signaling Technology	1:1000	WB
p38	Mouse mAb	ab31828, Abcam	1:1000	WB
p-EGFR	Rabbit pAb	44784-G, Thermo Fisher Scientific (Waltham, MA)	1:50	IHC
Activated caspase-3	Rabbit mAb	559565, BD Pharmingen (San Jose, CA)	1:100	IHC

Table 4.

Marker changes after NBC treatment of organ-cultured diabetic corneas

Marker	DM17-09	DM17-13	DM17-54	DM18-13	DM18-16	% with increase (+)
K15	+	+	+	+	+	100 (5/5)
K17	+	−	+	+	+	80 (4/5)
ΔNp63	+	−	+	+		75 (3/4)
Integrin $\alpha_3\beta_1$	−	+	+	+	+	80 (4/5)
Nidogen-1			+	−	+	67 (2/3)
p-Akt	−	−	+	+	+	60 (3/5)
p-p38	−	+	+	+		75 (3/4)

Arbitrary increase *vs.* treatment by control NBC with scrambled AON.

Numbers of independent cases are in parentheses