LETTER TO THE EDITOR

Polynucleotide biogel enhances tissue repair, matrix deposition and organization

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To the Editor,

Wound healing is a complex phenomenon that requires the interplay of different signal cascades and cell phenotypes (1), which eventually deposit a provisional matrix that progressively matures to normal healthy tissue (2). New cell progenitors from the neighboring healthy tissues grow into the provisional matrix and create sound tissue; in the relatively common case of skin wounds, these cells are both epithelial cells, which cover the defect and its provisional matrix, thus isolating and protecting it from the outside environment (3), and fibroblasts, which deposit the underlying connective tissue, mostly composed of collagen, in its diverse isoforms, and proteoglycans (4). If the deposition of extracellular matrix is insufficient, the final tissue will not possess adequate mechanical, functional or even esthetical characteristics (5). As fibroblasts are key to wound healing, it is unsurprising that strategies aiming at improving healing in clinical situations where this is impaired often rely on stimulating or improving fibroblast functionality (6). One such strategy is the use of polynucleotides (PN), polymers consisting of a mixture of double stranded deoxyribonucleotides. They have been used since 2004 in different class III medical devices, e.g. as wound healing accelerators, for intra articular supplementation (7), thanks to their lubricating and visco-elastic properties, and as filler to improve skin turgor and hydration, both over the face and other body areas (8), in skin rejuvenation medical devices. The present study investigated the effects of PN in stimulating the viability of human primary fibroblasts *in vitro* and the regulation of some important matrix components. We further used a mouse model of subcutaneous pouch to assess whether PN improved wound healing and how matrix deposition was affected *in vivo* by comparing its effect with hyaluronic acid, the most popular type of dermal filler.

MATERIALS AND METHODS

PN (Mastelli s.r.l., Sanremo, Italy) are a compound that contains a mixture of deoxyribonucleotide polymers of different lengths. PN are obtained from salmon trout gonads through purification and high-temperature sterilization, to ensure a very high percentage of DNA.

Cell culture

Cell assays were performed with human primary fibroblasts obtained from American Type Culture Collection (LGC Standards S.R.L., Milan, Italy). Cells were routinely maintained in complete Dulbecco modified MEM (DMEM, Life Technologies, Carlsbad,

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CA, USA) supplemented with 10% Fetal Bovine Serum (FBS, LifeTechnologies, Carlsbad, CA, USA), 4 mM L-glutamine (Merck KGaA), 100 IU/ml penicillin and 100 µg/ml streptomycin (PenStrep, Merck KGaA) in a humidified atmosphere with 5% CO2 at 37°C. Culture medium was replaced every 3 days. Cells were monitored by Nikon TMS inverted optical microscope (Nikon, Tokyo, Japan) in phase contrast for morphological characterization. The microscope was equipped with Nikon Digital Sight DS-2Mv acquisition system (Nikon) and images were acquired with NIS Elements F software (Nikon). After reaching 80% confluence, cells were washed with Dulbecco's phosphate buffered saline (PBS w/o Ca^{2+}, w/o Mg^{2+}, Euroclone, Pero, MI, Italy), harvested using trypsin-EDTA (Merck KGaA), resuspended in 10 ml of NaCl cold solution to be counted and seeded onto new plates. Two experimental groups were used in this *in vitro* study, a Control group and a PN group. In each experiment, 24 h after seeding, cells were stimulated with either culture medium (Control) or 100 µg/ml PN to evaluate its effects.

Cell count and viability

Cells were seeded in a 24-well plate at a density of $2x10^4$ cells/well to be stimulated 24 h after seeding. The experiment was performed in triplicate. The number of cells was evaluated for the Control group and PN group after 96 h and 1 week of stimulation by detaching and direct counting with a Coulter Counter (Coulter Electronics Limited, England). Briefly, after collecting the supernatant for the ELISA assays, each sample was washed with PBS (Ca^{2+} and Mg^{2+} free), detached with 300 µl of trypsin-EDTA and resuspended in 10 ml of NaCl cold solution for counting.

For cell viability evaluation, cells were seeded in a 96-well plate at a density of $8x10^3$ cells/well in a final volume of 100 µl of culture medium and stimulated 24 h after seeding. The Control group and PN group were evaluated after 96 h and 1 week from stimulation by MTT (Roche Applied Science, Monza, Italy) and Cell Titer-Glo® (Promega, Madison, WI, USA) assays.

The MTT assay was used according to the manufacturer's indications and eight wells were assigned for each experimental condition. Briefly, 10 µl of MTT labeling reagent was added at final concentration 0.5 mg/ml and the plate was placed into a thermostatically controlled incubator for 3 h. Once the incubation was completed, 110 µl of solubilization solution (anhydrous isopropanol containing 0.1 N HCl and 10% Triton X-100) was added, the contents were mixed for 5 min on an orbital shaker and the absorbance was read at 570 nm by a microplate reader (Infinite F200 TECAN, Switzerland).

The bioluminescence assay was performed according to the recommendations of the manufacturer and four wells were assigned for each experimental condition. Briefly, the plate was equilibrated to room temperature for 30 min and a volume of Cell Titer-Glo® Reagent equal to the volume of cell culture medium was then added. The content was mixed for 2 min on an orbital shaker to induce cell lysis and intracellular adenosine 5'-triphosphate (ATP) release into the culture medium and transferred from each well to a new opaque-walled multi-well plate. The plate was incubated at room temperature for 10 min to stabilize luminescence signal and finally the samples were read with a microplate reader (Infinite F200 TECAN, Switzerland). Control wells containing medium without cells were used to obtain a value for background luminescence.

Protein content and ELISA assay

To quantitate the protein content of Control and PN groups, cells were seeded in a 24-well plate at a density of $2x10^4$ cells/well, stimulated after 24 h and evaluated 96 h and 1 week after stimulation. The experiment was performed in triplicate. Briefly, samples were gently rinsed with PBS, left to dry for 2 h to be then solubilized with 200 µL of 5% NaOH DOC (Sigma-Aldrich) and left to stir for 1 h. After adding 2 ml of Lowry Reagent Solution containing $Na_2CuEDTA$, Na_2CO_3 and NaOH, samples were left to stir for 10 min and transferred to a sonicator, 200 µL of Folin & Ciocalteu's Phenol Reagent Working Solution (Sigma-Aldrich) were added and after 2 min the content was mixed for 1 h on an orbital shaker. The amount of proteins was estimated by reading the absorbance at 750 nm by a microplate reader (Infinite F200 TECAN, Switzerland) and the amount of proteins in the given unknown solution was calculated from the standard curve.

Collagen 1 and Collagen 3 expressions were measured by quantitating the protein in the supernatant collected in the Control group and PN group as described above. Cell culture media was centrifuged at 1000 rpm for 20 min

to eliminate cell remnants and all reagents and working standards were prepared following the manufacturer's recommendations. Samples were then assayed using Human Pro-Collagen I alpha 1 SimpleStep ELISA Kit (Abcam, Cambridge, UK) and Human Collagen III Alpha (COL3A1) ELISA Kit (Abbkine, Wuhan, China). The experiments were performed in triplicate.

For Collagen I secretion, supernatants were diluted into Sample Diluent NS and assayed. Fifty µL of all sample and standard were added to appropriate wells. After adding (50 µL) of the Antibody Cocktail to each well, the plate was sealed and incubated for 1 h at room temperature on a plate shaker set to 400 rpm. Each well was then washed with 350 µL 1X Wash Buffer PT for 3 times and, after complete removal of the liquid, 100 µL of TMB Development Solution were added to each well. The plate was incubated for 10 min in the dark on the plate shaker, 100 µL of stop solution were then added to each well, the plate was shaken for 1 min and the optical density (O.D.) recorded at 450 nm by a microplate reader.

For Collagen III secretion, after adding diluted standard (50 µL) to standard well, sample diluent (40 µL) and sample (10 µL) were added to testing sample well. The plate was covered and incubated for 45 min at 37°C and each well was then washed with Wash buffer (250 µL) for 5 times. After removing any remaining Wash buffer, HRP-Conjugated detection antibody (50 µL) was added and the plate was covered and incubated for 30 min at 37°C. The washing process was repeated for five times as described above and then chromogen solution A (50 µL) and chromogen solution B (50 µl) were added to each well. The contents were gently mixed and the plate was incubated for 15 min at 37°C while protecting it from light. (50 µl) stop solution was then added to each well and the O.D. was recorded at 450 nm by a microplate reader.

In vitro scratch assay

To determine the polydeoxyribonucleotide effect on cell spreading and migration capabilities, cells were seeded into a 12-well plate at a density of $20x10^4$ cells/well and grown to full confluence. The assay was performed in triplicate. After aspirating the culture medium from all wells, a linear wound was generated in the monolayer of cells with a 200 µL plastic pipette tip by gliding the tip across cell surface from a 12-o'clock to 6-o'clock location. Any cellular debris was removed by washing with PBS and cells were stimulated by adding culture medium (control group) or PN. After 48 and 96 h of incubation, images of migrated cells were taken using a digital camera connected to an inverted optical microscope in phase contrast to observe the closure of the wound area.

After 96 h, samples were fixed to be stained with Giemsa (Merck KGaA). Briefly, pure methanol was added to each well to then be replaced with 1ml of Giemsa solution. After 2 min, 3 ml of H_2O were added and the components were mixed by shaking the plate for 2 min. After removing the staining solution, the samples were rinsed with tap water and allowed to dry. Samples were observed with a Nikon Eclipse 80i optical microscope (Nikon), equipped with a Nikon Digital Sight DS-2Mv camera and imaged with NIS Elements F control software (Nikon).

In vivo experiment and histology

The test was conducted at the University of Messina after ethics committee approval and in accordance with the National Ethical Guidelines (Italian Ministry of Health; D.L.vo 116, January 27, 1992) and the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85–23, revised 1996). For this protocol 6 female C57BL mice, 14 weeks old, weighting 25-32 g were used. Animals were divided into 3 groups of 2 animals each. The first group was injected with polynucleotide gel (20mg/ml), the second group was injected with hyaluronic acid and the third group (control) was injected with NaCI 0.9% saline solution. The injection technique (microwheals technique) consisted of serial superficial intradermal injections of 0.3 ml of product.

Four injections were performed in sites with 1 cm spacing for each animal. All the animals were observed for 24, 48 and 72 h, 1 week and 2 weeks, to monitor the appearance of any toxic effects. Injection sites were examined for any signs of cutaneous irritation (erythema, edema and eschar). Moreover, the mice were anaesthetized after 2 weeks and received cutaneous punch biopsy (diameter 4 mm) for histological analysis in the treated areas.

The tissues obtained from the sacrificed animals were fixed in 4% neutral formaldehyde diluted in phosphate-buffered saline, were dehydrated in graded ethanol, cleared in xylene and embedded in paraffin after 24 h.

Samples were then sliced with a microtome at a 6 μm thickness and mounted on glass slides. Paraffin was then removed from the slides using xylene and the slides were subsequently dehydrated in alcohol. After washing with demineralized water, in order to morphologically assess tissue composition, some slides were dipped into Harris hematoxylin (Hematoxylin BCS certified-C.I. 75290 by Bio-Optica Milano S.p.A.) for 60 s and rinsed with tap water until clear, dipped in yellowish eosin in 25% solution (Eosin Y BSC certified-C.I. 45380 by Bio-Optica Milano S.p.A.) for 3 min and rinsed again with water. To highlight the presence of collagen in the EMC, other slides were incubated with a 0.1% Sirius Red solution (Sirius Red F3B 200, Mobay Chemical Co., Union, New Jersey, USA), dissolved in aqueous saturated picric acid for 1 h and washed in acidified water (0.5% hydrogen chloride). All samples where then washed with demineralized water, dehydrated in ethanol, cleared in xylene and mounted in a resinous medium (Fisher Chemical Permount Mounting Medium). Microphotographs of experimental H&E and picro-Sirius Red stained tissues were taken using an optical microscope Nikon Eclipse 80i (Nikon Instruments, Italy) equipped with a camera, Nikon Digital Sight DS-2Mv, connected to the control software, NIS-Elements D version 3.06 (Nikon Instruments). The samples stained with picro-Sirius Red were observed also with a polarized light microscope (the same instrument as above with the addition of polarizing filter).

Statistical analysis

Data were analyzed using Prism X (GraphPad, La Jolla, CA, USA). All values were reported as the mean ± Standard Deviation of repeated experiments performed in triplicate. ANOVA test was used to determine the statistical significance of the differences observed between groups. P values < 0.05 were considered significant.

RESULTS

PN improved the cell number in human fibroblasts cultures

We first investigated whether PN improved the cell growth of primary human fibroblasts. To this purpose, fibroblasts were cultured in the absence or in the presence of 100 μg/ml PN. Transmission light microscopy revealed that cells in both groups appeared healthy, elongated and, mostly, spindle shaped, but cells in the PN group were considerably more tightly packed than in control samples (Fig. 1 A-B). We then detached the cells at 96 h and 1 week of culture and counted them: an average of more than $4x10^4$ cells were counted in the treated wells, versus fewer than half that number for the control group at both time points (Fig. 1E, $p<0.001$).

These results were validated, with established viability assays. We decided to first use MTT, a well-established assay based on the reduction of this compound to insoluble and colored Formazan. Once the MTT was added to the wells, blue precipitates formed in the cell cytoplasm, as a consequence of their respiratory activity (Fig. 1 C, D). These precipitates revealed a higher number of cells in the treated wells. We then proceeded to solubilize Formazan and quantitate it by reading its absorbance at 570 nm. Absorbance was slightly but significantly higher in the PN-treated group after 96 h of culture (Fig. 1F, $p<0.01$), suggesting increased cell viability following PN treatment, although no significant difference was detected after 1 week of culture. To confirm these data, cell viability was also assessed by chemiluminescence, using the Cell titer GLO assay. The assay revealed that viability levels for cells cultured in medium enriched with 100 μg/ml PN were significantly higher than in the control group both after 96 h and after 1 week of treatment (Fig. 1G, $p<0.001$).

PN enhanced protein synthesis and the expression of collagen I and III

We measured the amount of proteins in the wells by Lowry's assay, a well-known method for protein quantification. Consistently with the tests above, the amount of proteins in the lysates from PN-stimulated samples was significantly higher than in the control group at both 96 h and 1 week of culture (Fig. 1H, $p<0.01$).

We then proceeded to quantitate the levels of Collagen protein in the supernatants of cell cultures grown in the presence or in the absence of 100 μg/ml PN for 96 h or 1 week. The expression levels of Collagen 1a1 and 3a1 increased over time in both groups, as did cell number, as shown in Figs. 1 and 2.

However, when normalizing the increase in Collagen production by the increase in cell number, it was still possible to observe that Collagen 1a1 and 3a1 production in the PN-stimulated group significantly exceeded that of the control group (Fig. 2 A, B, $p<0.01$), pointing to a specific regulation by PN.

PN enhanced wound healing in vitro and in vivo

We next assessed the reparative potential of PN by performing a scratch assay *in vitro*. After the well surface was scratched with a pipette tip, thus freeing cells from contact inhibition, the cells were followed up as they bridged the gap (Fig. 2 C, D). Although, as expected, cells in both groups started to proliferate and fill the scratched area, by 96 h of culture from stimulation, cells in the PN-treated group had occupied significantly more well surface than the control cells (Fig. 2E), prompting us to further investigate the regenerative potential of PN *in vivo*.

When the *in vivo* experiment was performed, during the period of clinical observation, it was impossible to detect any phenomenon of erythema, edema and eschar in the experimental mice cohorts at any experimental time (24, 48 and 72 h, 1 week and 2 weeks, data not shown).

Histological analysis of the skin samples 2 weeks after the injection procedure revealed that the wounds had healed uneventfully in all groups. However, abundant granulation tissue was observable in the control group (Fig. 3 A, D). The edges of the lesion were still visible and the newly formed tissue was occupied by a large inflammatory infiltrate (Fig. 3D). The healing area was markedly narrower in the hyaluronic acid group (Fig. 3 B, E), especially in the mid-portion, where tissue contraction had reduced granulation tissue to a thin band, constellated by a reduced but visible inflammatory infiltrate (Fig. 3E). Tissue healing was at an even more advanced stage in the PN-treated animals (Fig. 3 C, F), where the wound had almost completely disappeared and the two healing margins were tightly juxtaposed, with scarce inflammatory cells to mark the former

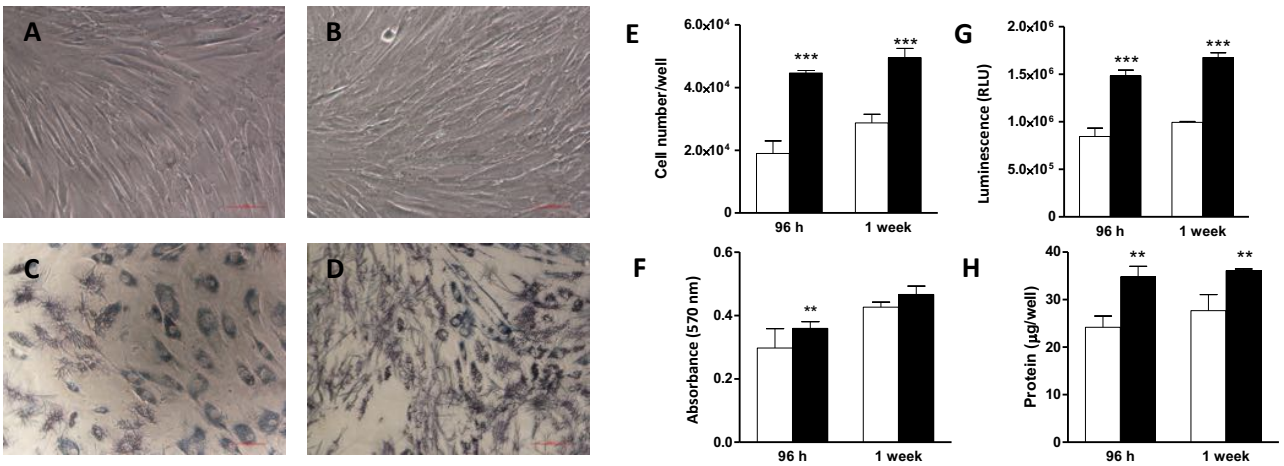

Fig. 1. ***A, B)*** *Microphotographs of primary human fibroblasts after 1 week of culture in the absence (A) or in the presence (B) of Polynucleotides (PN), observed at phase contrast. Magnification= 10 x.* ***C, D)*** *Microphotographs of primary human fibroblasts after 1 week of culture in the absence (C) or in the presence (D) of PN, after addition of MTT reagents. MTT creates blue needle-shape precipitates when it gets reduced to Formazan. Cells appeared more numerous in the presence of PN (B, D).* ***E)*** *Cells were also counted with a Coulter Counter at 96 h and 1 week of culture from stimulation. Cell number was significantly higher in the presence of PN. ***=$p<0.001$.* ***F)*** *Formazan deposits were solubilized and quantitated by absorptiometry at 96 h and 1 week of culture from stimulation. Viability levels were significantly higher in the presence of PN at 96 h of culture from stimulation. **=$p<0.01$.* ***G)*** *Cell viability was quantitated by Cell Titer GLO assay in the absence or in the presence of PN at 96 h and 1 week of culture from stimulation. Viability levels were significantly higher in the presence of PN at both time-points. ***=$p<0.001$. H) Protein content was also quantitated by Lowry assay in the absence or in the presence of PN after 96 h and 1 week of culture from stimulation. Protein levels were significantly higher in the presence of PN at 96 h and 1 week of culture from stimulation. **=$p<0.01$.*

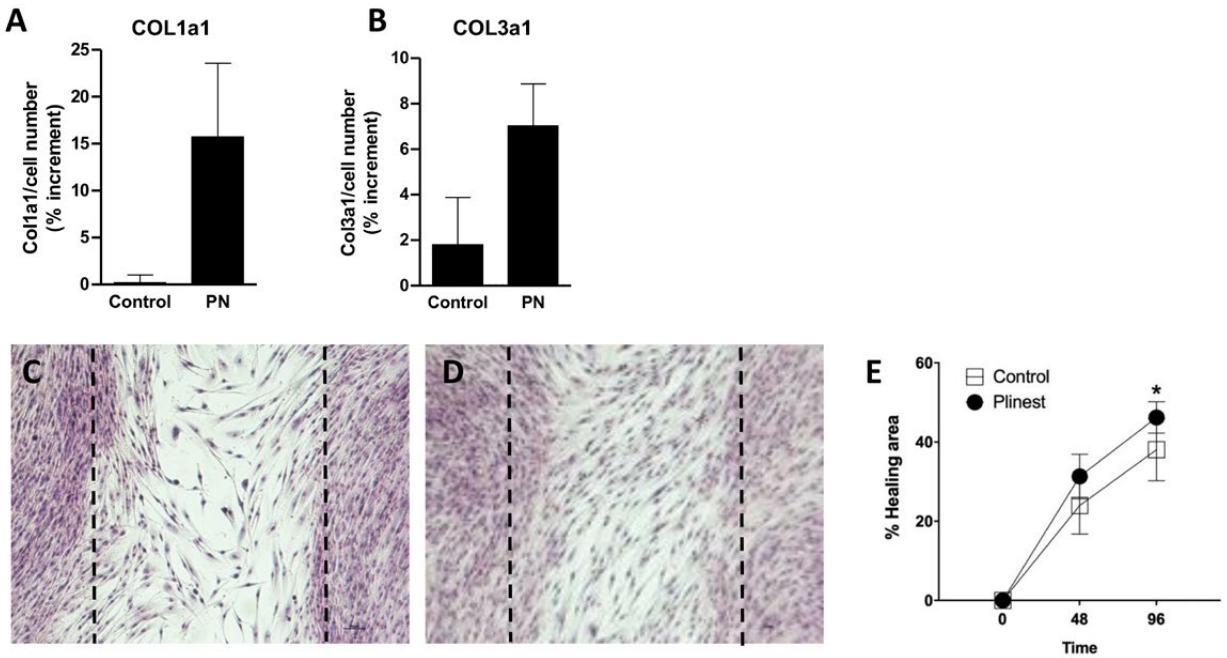

Fig. 2. *Histograms representing the expression of Collagen 1a1 (**A**) and Collagen 3a1 (**B**) in the culture supernatants of primary fibroblasts grown in the absence or in the presence of PN, as the ratio between the percent increase in Collagen production from 96 h to 1 week of culture from stimulation and the percent increase in cell number during the same time span. PN stimulated a higher expression of Collagen 1a1 and Collagen 3a1 compared to cell growth (A, B). **p<0.01. Transmitted light microphotographs of scratch test assay of (**C**) control cells and (**D**) cells stimulated with PN for 96 h of culture, stained with Giemsa protocol (Magnification 10 x). A visibly higher number of cells is bridging the scratch area in the presence of PN, as confirmed by (**E**) quantification of occupied area by a common image processing software at 3 time points.*

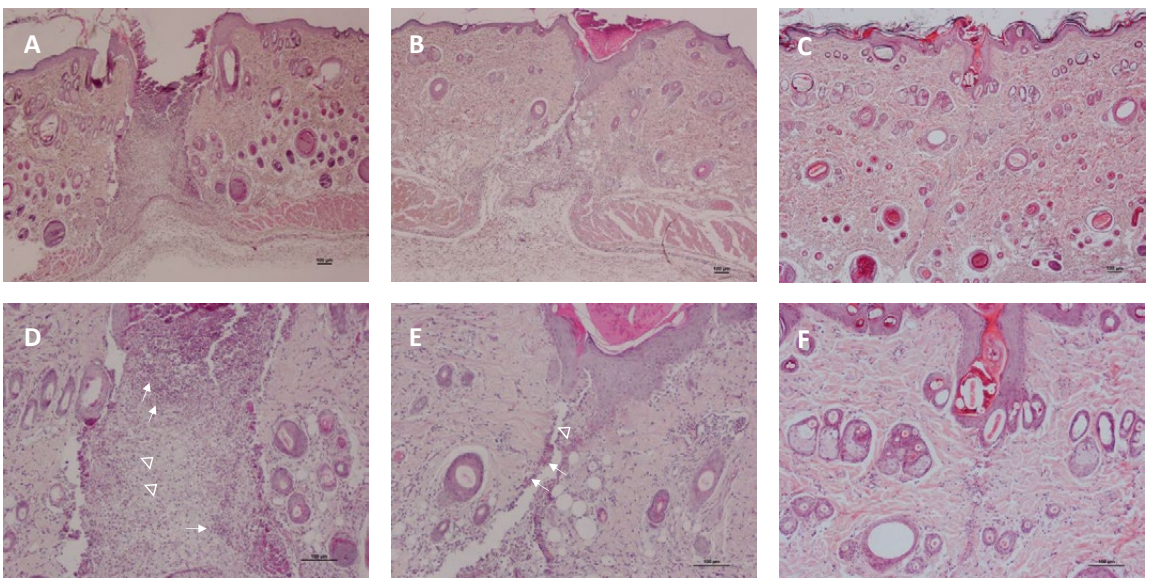

Fig. 3. *Microphotographs of healing wounds in the control (**A, D**), HA (**B, E**), and PN (**C, F**) group after Hematoxylin & Eosin staining. After incision in the dorsal skin of mice, wounds were sutured (control group) or HA or PN were inserted in the wound prior to suturing. The healing area is still visible in the control group, it is strongly reduced in the HA-treated animals and it almost completely disappeared in the PN-treated group 2 weeks after surgery. White arrows indicate the inflammatory infiltrate; white void arrowheads indicate the granulation tissue. Magnification = 4x (A, B, C) or 10 x (D, E, F).*

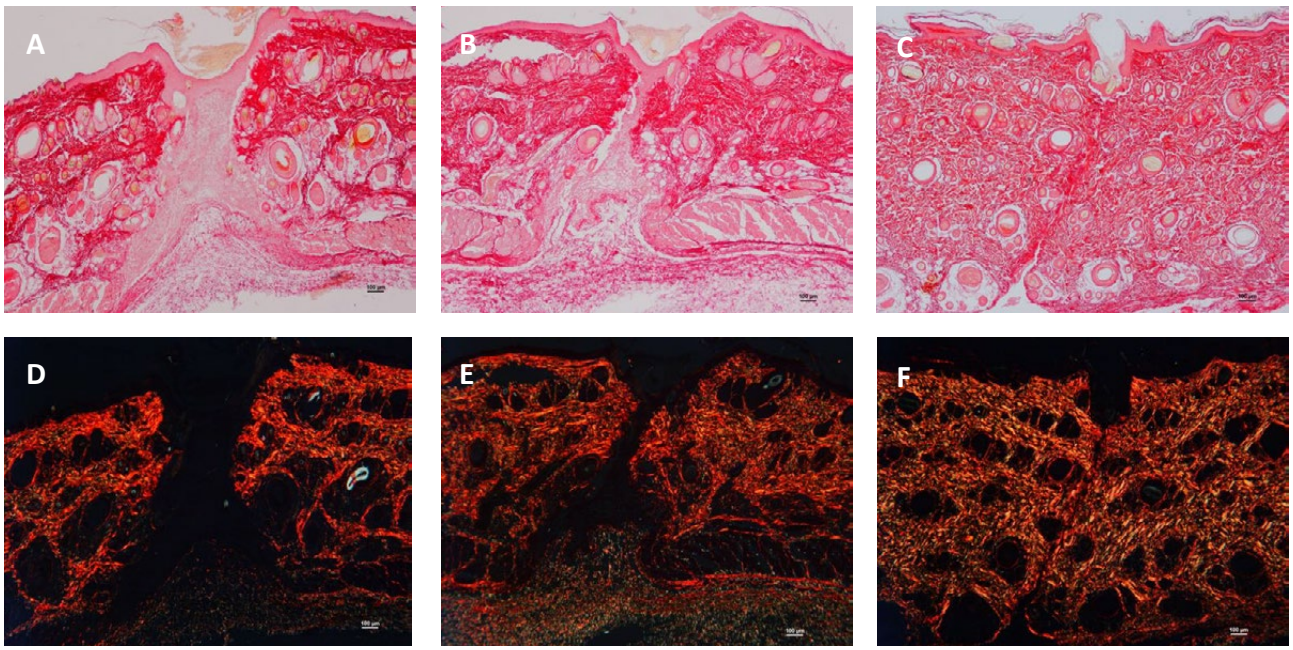

Fig. 4. *Microphotographs of healing wounds in the control (**A, D**), HA (**B, E**), and PN (**C, F**) group after Sirius Red staining for collagen at transmitted (A, B, C) or polarized light (D, E, F). The wounds are almost completely healed in the PN-treated group (C, F), and collagen fibers appear mature and well organized. This stands in stark contrast to the control group (A, D) where the defect is still devoid of collagen fibers or the HA-treated wounds (B, E), where the collagen fibers only partially occupy the healing tissue area. Magnification= 4x*

location of the wound. Histology failed to detect the persistence of polynucleotide gel inside the skin, due to its complete absorption. An abundant fibroblast population of normal appearance was also visible in the wound (Fig. 3F). Sirius Red staining was then used to disclose the deposition of extracellular matrix in and around the wound (Fig. 4). Polarized light observation was used to further enhance the appearance of the woven fibrils of collagen in the tissue (Fig. 4 D-F). No assembled mature fibrils were visible in the control wound tissue (Fig. 4 A, D), which appeared as a black gap separating the wound margins at polarized light (Fig. 3D). Similarly, although a signal reinforcement was observed in the margins of wounds treated with hyaluronic acid, where new matrix was being deposited, and although the gap in this group was significantly narrower than in the control group (Fig. 4E), a collagen-void area was still visible. In contrast, the wound margins appeared in contact in the PN-treated wounds and the continuity of the extracellular matrix was apparently restored. Only a band of signal reinforcement was visible along the wound line (Fig. 4C), and polarized light confirmed the still relative immaturity of the tissue in that area, where fibrils were still only partially organized (Fig. 4F).

DISCUSSION

Polynucleotides (PN) are a medical device gel made up of salified chains of deoxyribonucleic acids. Thanks to the abundance of polar functional groups (9), PN can attract and permanently bind water molecules. Polynucleotides are subject to physiological degradation by specific enzymes (endogenous DNases naturally present in tissues), which thus leads to the slow and progressive release of water molecules *in situ*. This process produces also oligonucleotides and free nucleotides, which are known to be physiologically present in the cell interstitium as degradation products (9). They can therefore be gradually and completely eliminated through a physiological cellular mechanism, which thus ensures the biocompatibility of the product.

Our results show that PN enhance fibroblast growth and viability *in vitro*. We observed a significant increase in cellularity in the treated cultures, using a selection of different methods, and promoted wound healing *in vitro*, as measured by scratch assay. Interestingly, our results also show that PN significantly stimulated the *in vitro* production of Collagen 1a1 and Collagen 3a1, two important isoforms of Collagen. The former, and more abundant form, Collagen 1a1, is the predominant isoform in mature tissues, whereas Collagen 3a1 is highly expressed during wound healing, and is remodeled and replaced by Collagen 1a1 as healing proceeds (10). Though the increase in Collagen production could indicate promotion of natural tissue repair, which could prove clinically important, e.g. to fill contracted or depressed spaces, an important concern with increasing Collagen deposition is the possibility of actually promoting fibrosis, a clinical situation where the physiological tissue structure is subverted by an excess of Collagen, which leads to functional and morphological impairment (11). To address these concerns, we also conducted an *in vivo* experiment by comparing PN effect with that of HA, a dermal filler widely used in aesthetic medicine, in a well-established model of dorsal sub-cutaneous pouch in mice. The effects of PN on Collagen expression are indeed visible at the histological analysis, as the polarized light analysis of Sirius Red-stained samples revealed that PN-treated tissues present a mature matrix, with well-organized Collagen fibers that have almost completely bridged the surgically-created gap, unlike the controls and the hyaluronic acid-treated group. The animal study therefore confirmed that PN not only create an environment that favors collagen deposition, but also indirectly promote its prompt recovery, without showing signs of fibrosis (Fig. 4).

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