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LXA_4 Actions Direct Fibroblast Function and Wound Closure

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Abstract

Timely resolution of inflammation is crucial for normal wound healing. Resolution of inflammation is an active biological process regulated by specialized lipid mediators including the lipoxins and resolvins. Failure of resolution activity has a major negative impact on wound healing in chronic inflammatory diseases that is manifest as excess fibrosis and scarring. Lipoxins, including Lipoxin A_4 (LXA_4), have known anti-fibrotic and anti-scarring properties. The goal of this study was to elucidate the impact of LXA_4 on fibroblast function. Mouse fibroblasts (3T3 *Mus musculus* Swiss) were cultured for 72 hours in the presence of TGF-β1, to induce fibroblast activation. The impact of exogenous TGF-β1 (1 ng/mL) on LXA_4 receptor expression (ALX/FPR2) was determined by flow cytometry. Fibroblast proliferation was measured by bromodeoxyuridine (BrdU) labeling and migration in a "scratch" assay wound model. Expression of α-smooth muscle actin (α-SMA), and collagen types I and III were measured by Western blot. We observed that TGF-β1 up-regulates LXA_4 receptor expression, enhances fibroblast proliferation, migration and scratch wound closure. α-SMA levels and Collagen type I and III deposition were also enhanced. LXA_4 slowed fibroblast migration and scratch wound closure at early time points (24 hours), but wound closure was equal to TGF-β1 alone at 48 and 72 hours. LXA_4 tended to slow fibroblast proliferation at both concentrations, but had no impact on α-SMA or collagen production by TGF-β1 stimulated fibroblasts. The generalizability of the actions of resolution molecules was examined in experiments repeated with resolvin D2 (RvD2) as the agonist. The activity of RvD2 mimicked the actions of LXA_4 in all assays, through an as yet

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unidentified receptor. The results suggest that mediators of resolution of inflammation enhance wound healing and limit fibrosis in part by modulating fibroblast function.

Keywords

Docosahexaenoic Acids; Lipoxins; Resolvin D2; Inflammation; Wound Healing; Collagen

1. Introduction

Wound healing is a complex process involving three sequential, yet overlapping phases: inflammation, proliferation and remodeling [1]. Phagocytic cells release growth factors, and produce cytokines to regulate the subsequent proliferative phase [1, 2]. The proliferative phase involves the formation of granulation tissue and revascularization, regulated by fibroblasts and endothelial cells. During the final maturation stage, the extracellular matrix is remodeled, leading to the tissue repair. Aberrations in any step of the reparative process are likely to result in impairment, with the potential for development of chronic wounds and ulcers, especially inflammation [3]. Chronic inflammation delays epithelialization (wound closure) and interferes with the remodeling phase that results in poor wound outcome such as increased fibrosis and scarring. Myofibroblasts differentiated from fibroblasts regulate wound healing, secret extracellular matrix (ECM), and are responsible for the contractility of scar tissue [4]. Myofibroblasts express α-smooth muscle actin (α-SMA) in stress fibers for contractile activity [5]. Scar formation has been linked to the number of myofibroblasts and to the extracellular environment, including inflammation, which halts tissue remodeling causing fibrotic scars [6].

Differentiation of myofibroblasts involves both mechanical stimulus to the cells by tensile forces and chemical stimuli by growth factors, such as TGF-β [5]. TGF-β regulates fibrotic responses in wound healing; it is produced by inflammatory cells, such as macrophages, fibroblasts, myofibroblasts and epithelial cells [7]. TGF-β has pleiotropic actions that are temporal and concentration dependent, such as inflammation, angiogenesis, fibroblast proliferation, collagen synthesis and deposition, and remodeling of the new extracellular matrix [8, 9]. TGF-β has three isoforms. While TGF-β3 appears to reduce scarring, TGF-β1 and β2 are key factors promoting scar formation [7]. Connective tissue growth factor, a downstream mediator of TGF-β1, has been shown to be involved in fibrosis, scar contractility, and deposition of collagen types I and III in the ECM [10]. During the entire process of healing, orchestrated resolution of inflammation is crucial for restoration of homeostasis and tissue integrity. Uncontrolled inflammation results in chronic, non-healing wounds or excessive scarring [11].

Resolution of inflammation is an active process leading to coordinated, temporal clearance of pro-inflammatory cells facilitating healing [12]. The coordination between pro-inflammatory and pro-resolving processes actively prevents damage to self [13]. Damage to self occurs in non-resolving inflammation that is associated with chronic diseases, such as arthritis, periodontal disease, diabetes and cardiovascular diseases [14]. A deficiency in resolution of inflammation molecules likely plays a role in disease pathogenesis [15–19]. Active resolution of inflammation is mediated by the local biosynthesis of endogenous

specialized pro-resolving lipid mediators (SPMs), which include the lipoxins, resolvins, protectins and maresins [11]. SPMs are enzymatically synthesized and induce diverse actions on a variety of cells through specific receptors [11, 20]. Target cells for the SPMs are not confined to the immune system; they also include the cells of structural tissues, such as bone [21]. Resolvins (Rv) are derived from the omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), while lipoxins (LX) are from the omega-6 arachidonic acid. They effectively resolve inflammation in periodontal diseases [21, 22], asthma [23, 24], and colitis [25]. Lipoxins reduce inflammatory pain, block IL-1β transcription induced by TNF-α in microglial cells, and limit polymorphonuclear leukocyte infiltration into inflamed brain, skin, and peritoneum [26]. In an experimental model of periodontitis, RvE1 prevented chronic inflammation, tissue breakdown and resulted in wound healing with restoration (regeneration) of all lost tissues [22, 27] suggesting a major role in regulating wound healing and regeneration. These observations have been further supported using lipoxins in a large animal model of regeneration [28]. In diabetic wound healing, Tang et al. demonstrated that RvD1 enhances wound closure in mice with decreased accumulation of apoptotic cells, macrophages and the susceptibility to infection [29]. While the potential of SPM for promoting wound healing without scarring is promising, there is a critical need to understand the mechanism of action on fibroblasts.

Considering that SPMs have significant actions on regulation of wound healing in inflammatory diseases, we hypothesized that amplification of resolution of inflammation pathways during wound healing will regulate fibroblast function, modulate fibroblast migration and proliferation, and potentially extend actions to myofibroblast differentiation and the deposition of collagen type-I and III. The aim of this study was to elucidate the impact of LXA_4, on fibroblast function in an *in vitro* model of wound healing assessing migration, proliferation, differentiation and collagen deposition as surrogates for wound repair and scar reduction.

2. Materials and Methods

2.1. Cell Culture, Experimental Conditions, Scratch Wound Closure

Fibroblasts (3T3 *Mus musculus* Swiss; CCL92; ATCC, Manassas, VA, USA) were cultured in 1% D-MEM supplemented with sodium-glutamine (4.0 mM), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (GIBCO, Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO_2. Medium was changed every 3 days. Cells were passaged using 0.025% trypsin in phosphate buffered-saline (PBS) containing 0.02% EDTA (GIBCO, Invitrogen, Carlsbad, CA, USA). 3×10^4 cells were seeded in a 12-well plates using the same medium until they reached confluence, then differentiated into myofibroblasts within 72 hours with TGF-β1 (1 ng/mL, Millipore, Billerica, MA, USA). In order to test the impact of SPMs, fibroblasts were incubated with or without LXA_4 (10 and 100 nM; Cayman Chemical, Ann Arbor, MI, USA). In order to study cell migration and wound closure *in vitro*, a linear scratch wound (3mm wide) was created using a rubber policeman as previously described [30]. The cell monolayers were stained with 0.9% crystal violet after 24, 48 and 72 hours of culture to visualize the fibroblast migration. To measure wound closure, standardized computer images were taken. The distance between the wound margins was quantified using computer

software (Image J; NIH). The scratches were analyzed by two individuals blinded to the experimental conditions.

2.2. Receptor Expression on Fibroblasts

Fibroblasts from each culture were seeded in 75 cm^2 flasks at an initial density of 10^6 cells per flask. When the cells became 80% confluent, they were starved overnight and differentiated into myofibroblasts as described above. The medium was aspirated and the monolayer was washed twice with PBS. Cells were detached using trypsin/EDTA; spun at 500×g for 10 minutes, washed with PBS, and fixed with 4% paraformaldehyde. Two flasks were used for each experimental condition. Each flask yielded approximately 2×10^6 cells. In order to determine the expression of ALX (a.k.a. FPR2), cells (4×10^5) were treated with antibody buffer (5% normal donkey serum in PBS containing 0.03% sodium azide) and stained with phycoerythrin (PE)-conjugated mouse anti-human ALX/FPR2 antibody (1:200, Santa Cruz Biotechnology, Dallas, TX, USA) or isotype control (1:200), which is known to cross-react with mouse ALX. The cells were analyzed by fluorescence with FACScan (CellQuest software, eBiosciences, San Diego, CA, USA).

2.3. Myofibroblast Differentiation

α-Smooth Muscle Actin (SMA, Sigma Chemical, St. Louis, MO, USA) expression was analyzed by Western blot. Fibroblasts were solubilized in RIPA buffer with proteinase inhibitors (Sigma Chemical, St. Louis, MO, USA) and centrifuged at 10,000×g for 10 min. Protein content was measured by the Bradford method [31]. The samples were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred onto polyvinylidene difluoride membranes (100V; 60 min). The membranes were blocked with 5% BSA for 60 min, and incubated overnight at 4°C with a polyclonal antibody against SMA and horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Band density was measured using an imaging densitometer (ChemImager 5500 system, Alpha Innotech Corp., USA). The band densitometry in the TGF-β1 stimulated sample was used as the internal control.

2.4. Fibroblast Proliferation in Response to LXA_4

10^4 cells were seeded per well in 96-well plates and treated with TGF-β1 in the presence or absence of LXA_4 (10 and 100 nM). After 24 hours, 5-bromo-2′-deoxy-uridine (BrdU)-labeling (Calbiochem, EMD Millipore, Darmstadt, Germany) was quantified according to the manufacturer's instructions. The absorbance was measured at 370 nm.

2.5. Collagen Deposition in Response to LXA_4

Collagen deposition was measured as an indicator of extracellular matrix generation by fibroblasts by Western blotting, as described above. Membranes were incubated with polyclonal primary antibodies against collagen type-I (C18) or type-III (C15; Santa Cruz Biotechnology, Dallas, TX, USA).

2.6. Resolvin D2

Resolvin D2 is an SPM derived from DHA that exerts proresolving properties through an as yet unidentified receptor. In order to determine whether the properties of lipoxins mediated through ALX were generalizable to other SPM of different fatty acid origin; all experiments were repeated in an identical fashion using RvD2 as the SPM agonist at 10 and 100 nM.

2.7. Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) and corrected for multiple comparisons using Newman-Keuls correction. All values are reported as mean ± SEM. Statistical significance (p value) was set at 0.05.

3. Results

3.1. TGF-β1 Up-regulates Expression of ALX/FPR2 on Fibroblasts

In order to determine the impact of LXA_4 on normal fibroblasts, all assays were performed on resting fibroblasts in the absence of TGF-β1. Figure 1A shows that resting fibroblasts minimally expressed the ALX receptor. In functional assays (SMA up-regulation, Collagen I and III upregulation, migration), there is no response to SPM in the absence of TBF-β1 pretreatment (Supplemental Figure 1). TGF-β1 stimulates the upregulation of ALX 2-fold ($p<0.05$) and induces functional responses reported below.

3.2. Scratch Wound Closure in Response to LXA_4

Fibroblast migration in the “scratch” assay was used as a measure of fibroblast stimulation and function in response to TGF-β1. Fibroblast migration and scratch closure were significantly increased in response to TGF-β1 compared to the vehicle control at all time-points studied ($p<0.05$) (Figure 1B). In the presence of LXA_4 (10 and 100 nM) a bi-phasic response was seen inhibiting fibroblast migration induced by TGF-β1 in the first 24 hours ($p<0.05$), with restoration of the response recovered after 48 hours.

3.3. TGF-β1 induced fibroblast proliferation is reduced by LXA_4

TGF-β1 stimulated fibroblast proliferation was measured by BrdU incorporation ($p<0.05$). Stimulation was reduced significantly by LXA_4 at both concentrations in the first 24 hours (Figure 1C). Negative controls were performed using only LXA_4; no proliferation was observed in any group (data not shown).

3.4. TGF-β1 stimulates myofibroblast differentiation

Alpha-smooth muscle actin (α-SMA) expression was used to assess myofibroblast differentiation. TGF-β1 stimulated α-SMA deposition after 12, 24 and 72 hours. LXA_4 had no impact on myofibroblast differentiation at either concentration (Figure 2).

3.5. Collagen type-I and type-III expression is enhanced by TGFβ1

In order to determine the impact of LXA_4 on TGF-β1 induced collagen deposition, collagen types I and III were assessed. As expected, TGF-β1 stimulated the expression of collagen

types-I and III. LXA_4 had no impact on TGF-β1 stimulation of collagen Type I or Type III deposition ($p<0.05$, Figure 3).

3.6. Actions of Resolvin D2 (RVD2)

Experiments were repeated as above using RvD2 in order to determine whether the actions of SPM on fibroblasts were limited to lipoxins. RvD2 exhibited the same properties qualitatively and quantitatively as LXA_4 in the fibroblast proliferation, α-smooth muscle assays, the cell migration (scratch closure) and collagen production assays (Figure 4).

4. Discussion

Active resolution of inflammation is a process driven by novel endogenous lipid mediators such as lipoxins and resolvins that rescue excessive or prolonged inflammatory responses to promote wound healing. The actions of resolution agonists are receptor mediated; a feed forward system rather than inhibition of enzyme pathways or use of receptor antagonism [17]. In this study, we show that two SPMs, LXA_4 and RvD_2, counter-regulate fibroblast migration and proliferation in fibroblast cultures at early time points suggesting that SPMs play an active role in limiting fibrosis in part through the limitation of fibroblast activity. The activity of fibroblasts at later time points is not inhibited suggesting SPM will not have a negative impact on wound healing overall. RvD2 and LXA_4 do not impact myofibroblast differentiation or collagen deposition in the extracellular matrix induced by TGFβ1.

LXA_4 binds to the formyl-peptide receptor type 2 (ALX), a unique G-protein coupled receptor that conveys proresolving signals induced by proteins, peptides, and lipid ligands [32]. We observed that fibroblasts express the receptor ALX and that TGF-β1 increases its expression. Resolvins derived from docosahexaenoic acid, such as RvD_2, are synthesized by neutrophils during the resolution phase of inflammation and exhibit properties similar to lipoxins blocking the secretion of interleukin-1 beta (IL-1β) and tumor necrosis factor a (TNF-α), reducing neutrophil adhesion to the endothelium, and inhibiting neutrophil infiltration into the tissue [25]. The receptor for RvD_2 has yet to be determined.

The presence of ALX on fibroblasts suggests that the proresolving mediators play a regulatory role in fibroblast function. Our data clearly indicate that both RvD_2 and LXA_4 modulate fibroblast activity. Proliferation and collagen deposition by fibroblasts are integral to wound healing. Fibroblast proliferation contributes to the formation of granulation tissue in the wound bed to begin the repair process and collagen deposition is essential for increasing the strength of the wound and allows the cells involved in angiogenesis and connective tissue construction to attach, grow, and differentiate. Earlier studies demonstrated that inhibition of collagen synthesis was observed in fibroblasts in the presence of the lipoxygenase-derived inflammatory lipid mediators 12(S)-12S-hydroxy-(5Z, 8Z, 10E, 14Z)-eicosatetraenoic acid [12(S)-HETE] [33] suggesting that the eicosanoids influence the metabolism of fibroblasts, which could impact the skin repair process.

Collagen types I and III are some of the most abundant collagens found in healing wounds. They are important in maintaining arterial stiffness, for example. These collagens are formed via a complex biosynthetic pathway involving intracellular and extracellular

posttranslational modifications by various enzymes [34]. Some inflammatory cytokines, such as IL-1β and TNF-α down-regulate procollagen biosynthesis at the transcriptional level in various types of cells [35]. For example, Aoki *et al*. reported that IL-1β and NF-κB regulate collagen biosynthesis (types-I and III) in cerebral aneurysm walls. Pathologically, cerebral aneurysm is characterized by decreased collagen content resulting from a chronic inflammatory response in aneurysmal walls. The upregulation of IL-1β and NF-κB activation contributed to cerebral aneurysm progression [35] suggesting that unresolved inflammation has a negative impact on wound healing on two temporally distinct levels. First, early in healing, inflammation leads to excessive granulation tissue and fibroblast proliferation; late in healing, inflammation reduces collagen deposition and wound strength. The results reported here are in agreement with other studies; TGF-β1 induces both collagen type-I and III expression, but treatment with RvD_2 and LXA_4 does not interfere with deposition allowing the continued expression of collagens to take place by fibroblasts at later time points. At early time points, fibroblast migration and proliferation are slowed, which is consistent with limiting excess granulation tissue and subsequent fibrosis.

Previous studies have examined SPMs for their impact on wounds. Mustafa *et al*. showed that the treatment of human periodontal ligament cells (PDL; a fibroblast-like cell) with RvD1, reduces cytokine induced production of the pro-inflammatory mediator prostaglandin E_2, and up-regulates LXA_4 production. In addition, RvD1 significantly enhanced PDL proliferation, wound closure and fibroblast growth factor (FGF) release [36]. In another study, using a rat model of dorsal skin wound, Spite *et al*. showed that type-2 diabetes alters the resolution of inflammation; this alteration can be acutely corrected by stimulating resolution with RvD1, restoring diabetic defects in macrophage phagocytosis, which decreases the accumulation of apoptotic/necrotic cells and microbes present in chronically inflamed tissues [29]. These studies demonstrate the potential use of SPMs as therapeutics to improve wound outcomes.

In another *in vivo* study using two different mouse burn injury models involving significant partial thickness injuries, Bohr et al. [37] show that a systemically administered single dose of RvD2 (25 pg/g) effectively prevented thrombosis of the deep dermal vascular network, subsequently preventing dermal necrosis. In addition, RvD2 enhances neutrophil access to the dermis. RvD2 also inhibits TNF-α, IL-1β, and neutrophil platelet-endothelial cell adhesion molecule-1 [37].

Our data show that SPMs, in addition to known anti-inflammatory and proresolving actions, modulate fibroblast proliferation and migration directly to limit fibrosis at early time points without interfering with myofibroblast differentiation allowing wound healing and collagen deposition in the extracellular matrix to proceed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- TGF-β1 up-regulates LXA_4 receptor (ALX/FPR2) expression on fibroblast.
- LXA_4 regulates fibroblast migration and proliferation induced by TGF-β1.
- SPMs have no impact on α-SMA, collagen type-I and III expression by fibroblast.
- RvD2 regulates TGF-β1-induced fibroblast proliferation and scratch wound closure.

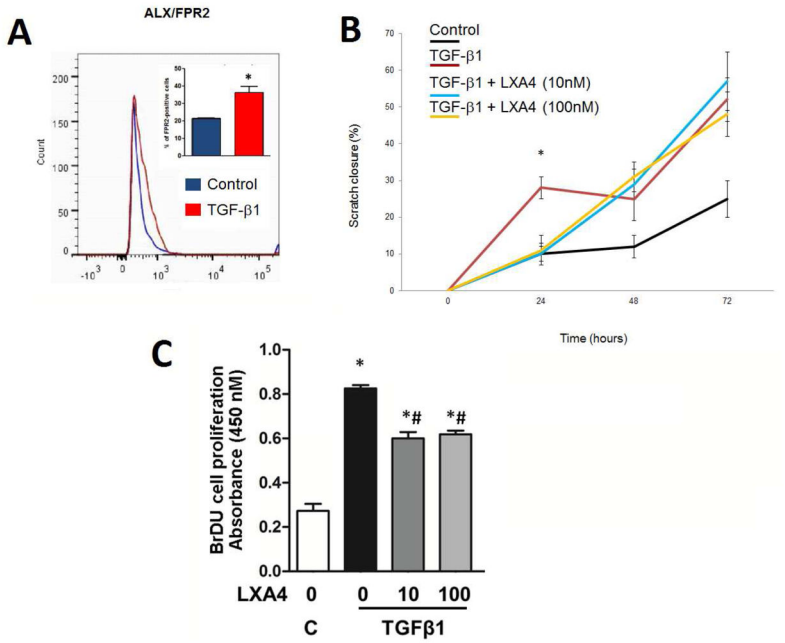

Figure 1.

Fibroblasts express the LXA_4 receptor, ALX/FPR2. LXA_4 regulates fibroblast migration and inhibits TGF-β1 induced fibroblast proliferation. Panel A: TGF-β1 upregulates ALX receptor expression analyzed by flow cytometry (PE-conjugated anti-ALX/FPR2 receptor antibody; $p<0.05$, n=4). Panel B: TGF-β1 increases fibroblast migration and scratch closure significantly at 24 hours ($p<0.05$). LXA_4 slows fibroblast migration at 24 hours at both doses, returning to TGF-β1 alone values at 48 and 72 hours ($p<0.05$). Panel C: TGF-β1 stimulated an increase in fibroblast proliferation, analyzed by BrdU incorporation, and it is reduced by LXA_4 (n=8; * = $p<0.05$ vs. control; # = $p<0.05$ vs. TGFβ1 alone).

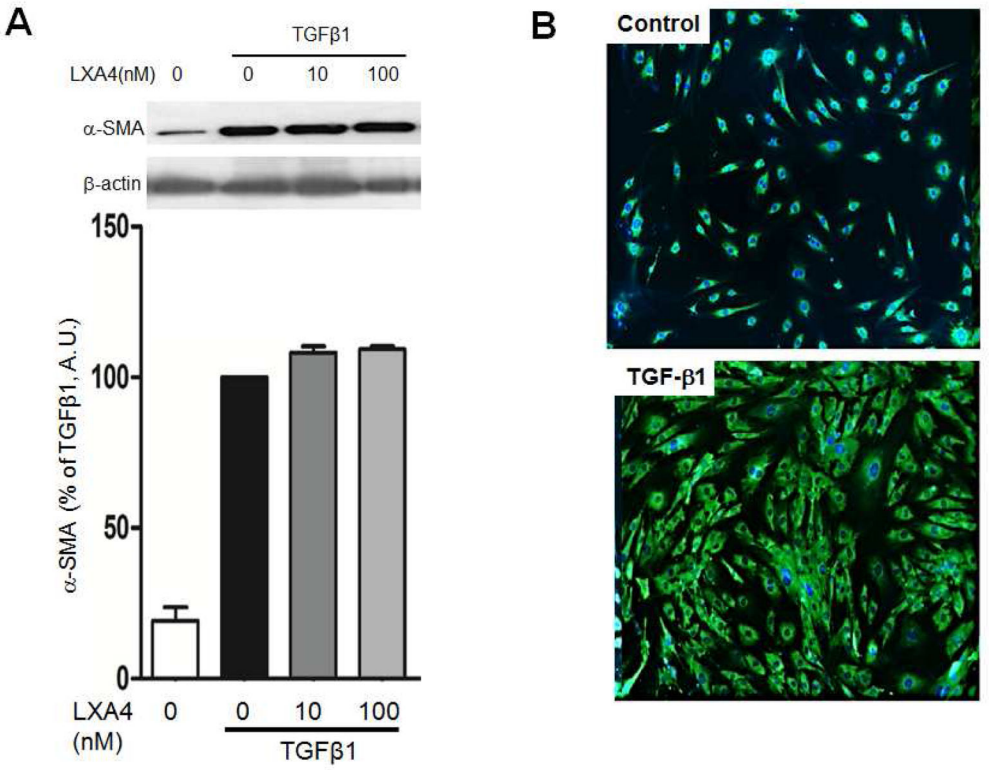

Figure 2.

Myofibroblast differentiation assessed by α-SMA protein expression. Panel A: TGF-β1 stimulates myofibroblast differentiation and α-SMA expression after 72 hours. LXA_4 has no impact on myofibroblast differentiation ($p<0.001$, $n=4$). Panel B: α-SMA stained with FITC (green) and the nuclei with Hoechst (blue).

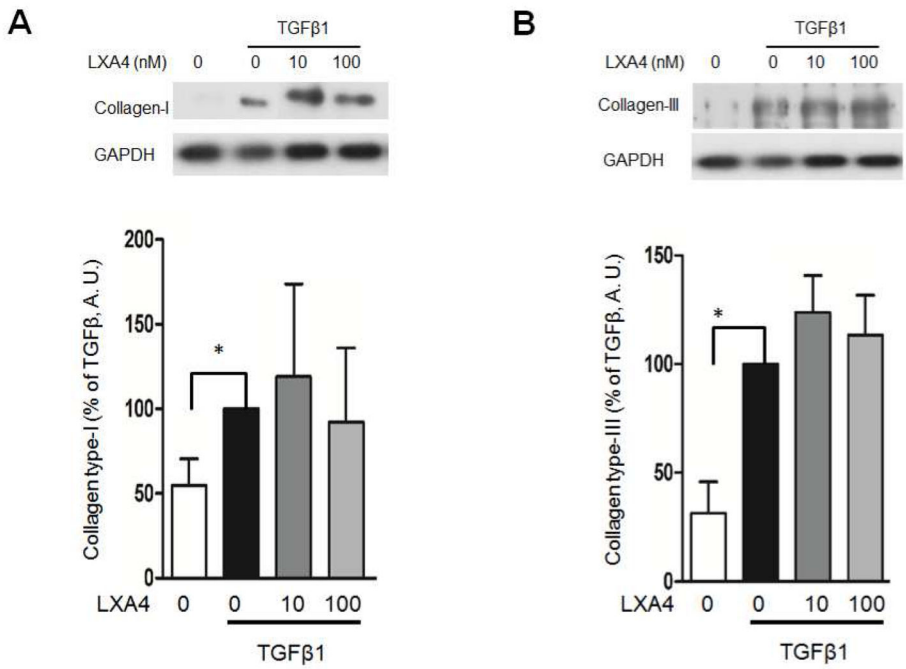

Figure 3.

TGF-β1 stimulates collagen type-I and III deposition by fibroblasts ($p<0.05$). LXA_4 has no impact on collagen expression (A.U. = Arbitrary Units, n=4).

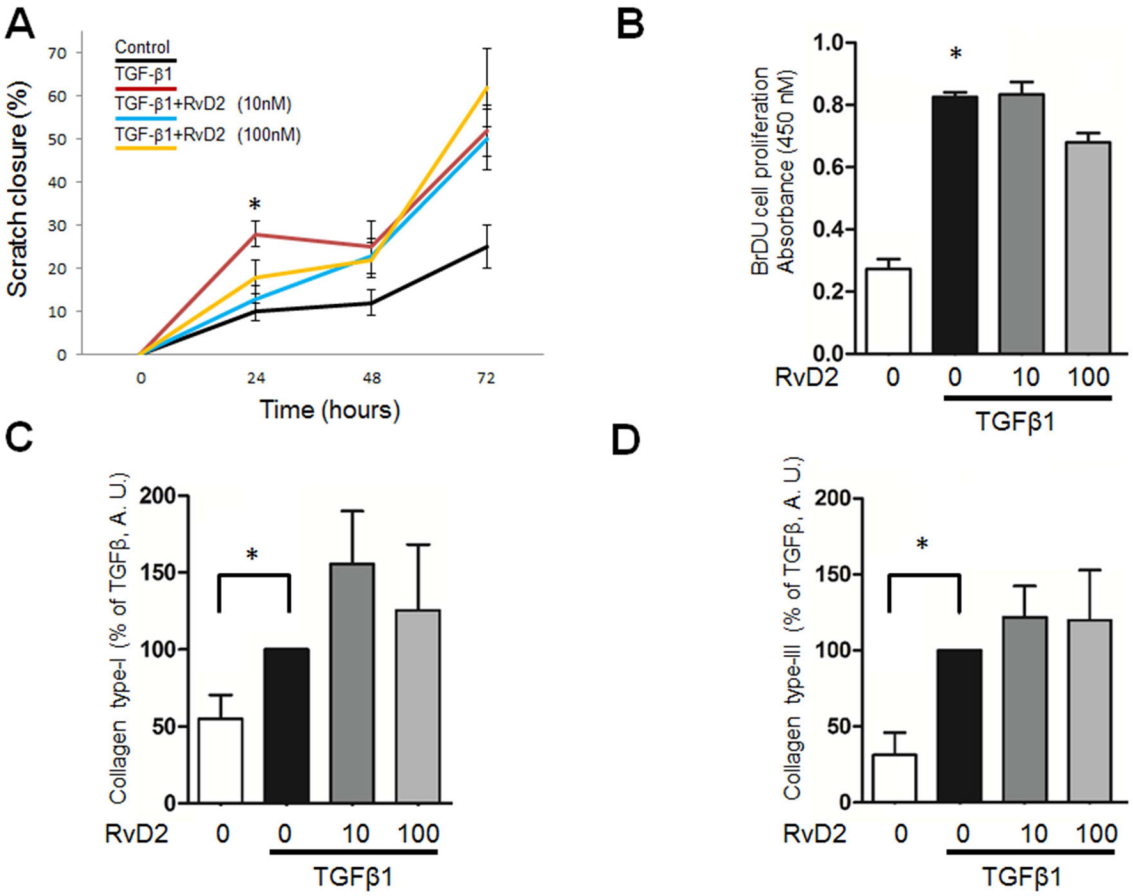

Figure 4.

RvD2 regulates fibroblast function *in vitro*. All experiments were repeated with RvD2; a resolution agonist derived from the n-3 fatty acid DHA. Panel A: RvD2 inhibits scratch wound closure induced by TGF-β1 at 24 hours ($p<0.05$); closure rate is restored at 48 and 72 hours. Panel B: RvD2 inhibits TGF-β1 induced cell proliferation ($p<0.05$; n=5). Panels C and D: RvD2 has no significant effect on Collagen expression (A.U. = Arbitrary Units, n=4).