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NO-dependent proliferation and migration induced by Vitamin D in HUVEC

Pamela Pittarella [a,1], Diletta F. Squarzanti [b,1], Claudio Molinari [b], Marco Invernizzi [c], Francesca Uberti [d], Filippo Renò [a,*]

[a] Innovative Research Laboratory for Wound Healing, Health Sciences Department, University of Eastern Piedmont "A. Avogadro", Via Solaroli 17, 28100 Novara, Italy
[b] Physiology Laboratory, Translational Medicine Department, University of Eastern Piedmont "A. Avogadro", Via Solaroli 17, 28100 Novara, Italy
[c] Physical and Rehabilitative Medicine, Health Sciences Department, University of Eastern Piedmont "A. Avogadro", Via Piazza d'Armi 1, 28100 Novara, Italy
[d] Department of Obstetrics and Gynecology, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Via Manfredo Fanti 6, 20122 Milan, Italy

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ABSTRACT

Recently, Vitamin D (Vit. D) has gained importance in cellular functions of a wide range of extraskeletal organs and target tissues, other than bone. In particular, Vit. D has displayed important beneficial effects in the cardiovascular system. Although little is known about the mechanism by which this response is exerted, a Vit. D-induced eNOS-dependent nitric oxide (NO) production in endothelial cells (EC) has been reported. The aim of this study was to evaluate whether Vit. D administration could affect human EC proliferation and/or migration through NO production. For this purpose, HUVEC (human umbilical vein endothelial cells) were used to evaluate Vit. D effects on cell proliferation and migration in a 3D matrix. Experiments were also performed in the presence of the specific VDR ligand ZK159222 and eNOS inhibitor L-NAME. This study demonstrated that Vit. D can promote both HUVEC proliferation and migration in a 3D matrix. These effects were NO dependent, since HUVEC proliferation and migration were abrogated along with Vit. D induced MMP-2 expression by inhibiting eNOS activity by L-NAME. These findings support the role of Vit. D in the angiogenic process, suggesting new applications for Vit. D in tissue repair and wound healing.

1. Introduction

Classically, the active form of vitamin D, $1\alpha,25(OH)_2D_3$ (Vit. D), is involved in the control of calcium and phosphate homeostasis, hormonal secretions, cell proliferation and differentiation. Moreover, Vit. D has been found to affect a wide range of extraskeletal organs and target tissues, including the heart and the vasculature, with a beneficial effect on cardiovascular function [1–4]. Indeed, Vit. D insufficiency/deficiency are associated with myocardial infarction, congestive heart failure and calcific aortic stenosis, which can lead to the massive vascular calcification seen in chronic kidney disease [2]. Endothelial cells (EC) are capable of synthesizing Vit. D due to the expression of the key biosynthetic enzyme 25 (OH)D3-1α-hydroxylase [5]. Moreover, EC also expressed the intracellular Vit. D Receptors (VDR), suggesting a possible autocrine/intracrine mechanism exerted by Vit. D as a modulator of endothelial functions [1,3,6]. Although the beneficial effects of Vit. D in the cardiovascular system are well established [7–11], little is known about the mechanisms by which this response is exerted. Nevertheless, our previous study demonstrated that Vit. D interaction with VDR induced nitric oxide (NO) production through endothelial nitric oxide synthase (eNOS) activation in cultured human endothelial cells [12]. NO plays a critical role in cardiovascular physiology and angiogenesis, enhancing EC survival, proliferation and migration [13,14]. Furthermore, it has been demonstrated that Vit. D is able to stimulate EC proliferation and to inhibit their apoptosis by increasing NO production in a murine model [15]. Because of the presence of both Vit. D and VDR in the EC and the key role of NO in the endothelial physiology, it is possible to hypothesize an interaction between Vit. D and NO capable of influencing EC proliferation and migration. In a previous work we have already demonstrated a Vit. D dependent proliferation and migration in a porcine endothelial cell model [6]. In order to confirm this mechanism also in human endothelial cells, Vit. D effects were evaluated in human umbilical vein endothelial cells (HUVEC), a well-known experimental model for the study of the regulation of endothelial cell functions and

* Corresponding author. Tel.: +39 321 660634; fax: +39 321 660634.
E-mail address: filippo.reno@med.unipmn.it (F. Renò).
[1] These authors contributed equally to the study.

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angiogenesis. In particular, proliferation and migration in a three-dimensional matrix have been investigated, along with the possible involvement of NO as a mediator of Vit. D effects.

2. Materials and methods

2.1. Endothelial cell culture

HUVEC, isolated from umbilical cord veins (ATCC), were cultured in 0.1% gelatin-coated flask with Endothelial Growth Medium-2 (EGM-2) containing 2% fetal bovine serum (FBS), 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% hEGF, 0.1% GA-1000, 0.1% heparin (all from Lonza, Walkersville, MD, USA) and maintained at 37 °C and 5% CO_2 as previously described [16]. For all experiments cells were used from passage 3 to 6.

2.2. Cell proliferation

In order to evaluate Vit. D influence on cell proliferation, 10^3 cells were seeded in gelatin-coated 96 well plates and allow to adhere; then complete medium was changed with low FBS (0.2%) DMEM medium without phenol red (starvation medium) and samples were treated with Vit. D (1α,25-dihydroxyvitamin D_3, Catalog Number D1530, SigmaAldrich, St. Louis, MO, USA; 1-10-100 nM, dissolved in ethanol), ethanol (maximum concentration 0.1%), or left untreated. To verify the effective involvement of VDR in the action mechanism of Vit. D, HUVEC were also treated with VDR ligand ZK159222 (Bayer Schering Pharma AG, Berlin, Germany). This Vit. D antagonist was used at the same concentration as Vit. D, alone or in co-stimulation. Moreover, in order to evaluate NO involvement in HUVEC proliferation following Vit. D treatment, some experiments were performed in the presence of the NOS inhibitor N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma–Aldrich, St. Louis, MO, USA). L-NAME was dissolved in serum free medium, used at a final concentration of 1 mM as reported in literature [17,18] and added 15 min before Vit. D treatment. For all the experiments, after 48 h incubation, cell culture medium was removed and cells were fixed in 3,7% formaldehyde-3% sucrose solution, stained with 1% toluidine blue solution and samples were photographed at 10× magnification using an optical microscope (Leica ICC50 HD). Cell proliferation was evaluated by counting cells in 10 random fields in 3 samples for each experimental condition from 3 different experiments. Results were expressed as cells/mm^2 ± standard deviation (S.D.). Moreover, some samples treated with Vit. D and L-NAME were fixed at 24 h, stained with crystal violet and photographed at 20× magnification using an optical microscope (Leica ICC50 HD) for morphological analysis.

2.3. Three-dimensional matrix migration assay

HUVEC were seeded in 12 well plates and grown in complete medium to reach a ~70 confluent monolayer. The three-dimensional hydrogel matrix (Epigel B, Epinova Biotech, Novara, Italy) were lean onto cell monolayers in 250 μl of complete medium containing different Vit. D concentrations (1-10-100 nM). In order to evaluate the involvement of NO synthesis in HUVEC migration following Vit. D treatment, some experiments were performed in the presence of L-NAME, alone or in combination with the most effective Vit. D concentration. To verify the effective involvement of VDR in migration, some experiments were also performed in the presence of ZK159222, used at the same concentration as Vit. D (100 nM), alone or in co-stimulation. After 3 days, cell culture medium was replaced with fresh medium. After 7 days, hydrogel samples were fixed in 3,7% formaldehyde-3% sucrose solution, stained with 2 μg/ml of Hoechst 33,342 solution (SigmaAldrich, St. Louis, MO, USA) to visualize cell nuclei and then

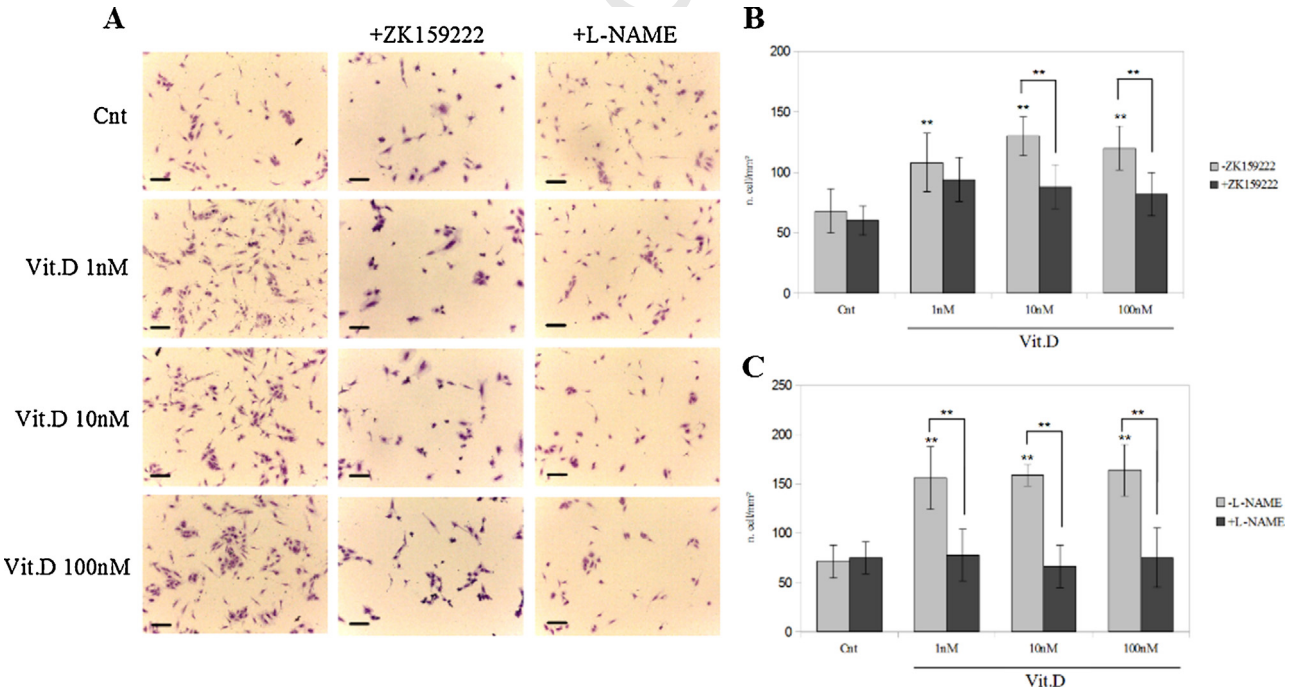

Fig. 1. HUVEC proliferation. (A) Optical microscopy images of control and Vit. D (1–100 nM) treated cells both in absence or presence of ZK159222 and 1 mM L-NAME after 48 h of incubation, stained with toluidine blue. Original magnification = 10×. Scale bar = 125 μm. (B) Determination of HUVEC proliferation in the presence of different Vit. D concentration (1–100 nM) and ZK159222 co-stimulation after 48 h of incubation. Results are expressed as n. cells/mm^2 ± S.D. Grey bars = cells without ZK159222 addition; black bars = cells + ZK159222. One-way ANOVA followed by Bonferroni post-test were used for statistical analysis. ZK = ZK159222. $^{**}P < 0.01$. (C) Determination of HUVEC proliferation in the presence of different Vit. D concentration (1–100 nM) and L-NAME after 48 h of incubation. Results are expressed as n. cells/mm^2 ± S.D. Grey bars = cells without L-NAME addition; black bars = cells + L-NAME. One-way ANOVA followed by Bonferroni post-test were used for statistical analysis. $^{**}P < 0.01$.

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transferred onto glass microscope slides and observed using Leica DM5500 fluorescence microscope. Cell migration was evaluated by counting migrated cells into the 3D matrix. For each experimental condition, 3 samples were analyzed at 10× magnification, selecting 10 random fields and results were expressed as n. cell/HPF (high power microscope field) ± standard deviation (S.D.).

2.4. Zymography

Conditioned media from HUVEC migrated into the 3D matrix for 7 days were separated by electrophoresis on SDS-polyacrylamide gels containing 0.2% gelatin. Samples were loaded onto zymograms without denaturation. After running, gels were washed at room temperature for 2 h in 2.5% Triton X-100 solution and incubated overnight at 37 °C in 0.5 M Tris–HCl, 0.2 M NaCl, 5 mM $CaCl_2$ buffer. Gels were then fixed in MetOH/acetic acid (50:10) solution and stained in 0.5% coomassie blue in MetOH/Acetic acid (40:10) solution. Images of stained gels were acquired after appropriate destaining. Gelatinolytic activity was detected as white bands on a dark blue background and quantified by densitometric analysis using ImageJ software.

2.5. Reverse transcription-PCR (RT-PCR)

To detect changes in MMP-2 gene expression after Vit. D stimulation, total RNA was extracted from control and treated samples (100 nM Vit. D, 1 mM L-NAME alone or in co-stimulation) using Ribozol (Amresco, Solon, OH, USA) according to the manufacturer's instructions. RNA integrity was assessed using nanodrop (Thermo Fisher Scientific, Wilmington, DE, USA). Single-strand cDNA was synthesized from 300 ng of total RNA using high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Aliquots of cDNA products were used as templates for PCR amplification in an automated thermal cycler (Technegene DNA Thermal Cycler, Techne, UK). The oligonucleotide primers used were: MMP-2: Forward 5'-TAC AAA GGG ATT GCC AGG AC-3' and Reverse 5'-GGC AGC CAT AGA AGG TGT TC-3'; β-actin: Forward 5'-ACA CTG TGC CCA TCT ACG AGG GG-3' and Reverse 5'-ATG ATG GAG TTG AAG GTA GTT TCG TGG AT-3'. RT-PCR runs were performed with an initial activation step of 4 min at 94 °C followed by 35 routine cycles. Each cycle included incubation at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 1 min. One additional cycle of 72 °C for 7 min was run to allow trimming of incomplete polymerization. Control reactions were performed in the absence of cDNA. Amplified PCR products were separated on 1.8% agarose gel containing ethidium bromide and visualized by UV illumination. Images of stained gels were acquired and the levels of MMP-2 and β-actin PCR products were quantified by densitometric analysis using ImageJ software. Data were expressed as MMP-2/β-actin ratio.

2.6. Statistical analysis

Results are expressed as means ± S.D. of at least 3 independent experiments for each protocol. One-way ANOVA followed by Bonferroni post-test were used for statistical analysis. Probability values of $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Vitamin D induced HUVEC proliferation through a NO dependent pathway

HUVEC proliferation has been evaluated after 48 h in starvation medium in the presence or absence of Vit. D (1-10-100 nM). As shown in Fig. 1, Vit. D induced a significant increase in cell growth at all the concentrations tested. In particular, the observed cellular densities of Vit. D-stimulated samples were at least doubled compared to untreated samples. Ethanol used as a vehicle for Vit. D administration did not affect cell proliferation (data not shown). The stimulation of HUVEC with the VDR ligand ZK159222 caused a significant decrease in Vit. D induced cell proliferation, confirming the role of VDR in the effects mediated by Vit. D (Fig. 1B). Furthermore, in order to evaluate NO involvement in the observed Vit. D effects, proliferation assays were performed in the presence of 1 mM L-NAME, an arginine analog that inhibits NO synthesis. Under these conditions, Vit. D was not able to induce cell proliferation. As shown in Fig. 1C, L-NAME presence did not alter control HUVEC proliferation, whereas completely reverted Vit. D-induced cell proliferation. Moreover, some samples treated as described above were fixed at 24 h and photographed for morphological analysis. Noteworthy, an increase in cell mitosis has been observed only in Vit. D treated specimens and not in samples with L-NAME (Fig. 2).

3.2. Vitamin D induced HUVEC migration in a 3D matrix through a NO dependent pathway

HUVEC migration has been evaluated in a three-dimensional model. A 3D matrix was lain on 70% confluent HUVEC monolayers. The 3D matrix used in these experiments was an anionic hydrogel made of gelatin and polyglutamic acid, which was previously described as a good substrate for cell growth [19,20]. As shown in

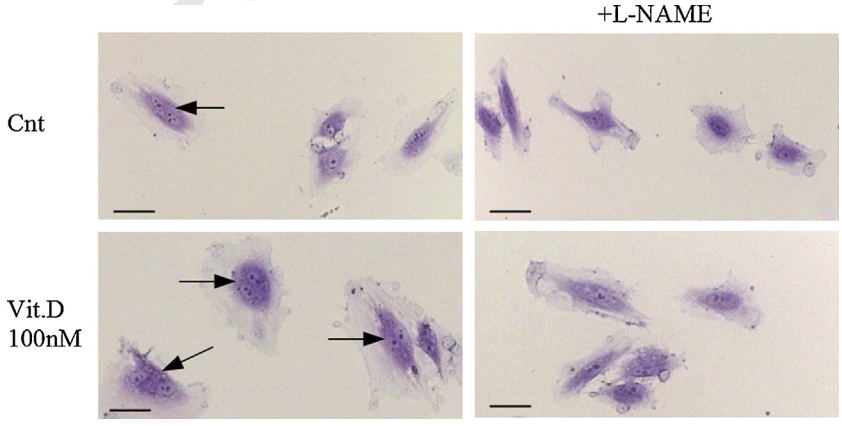

Fig. 2. Cell mitosis. Representative optical images of control and Vit. D-treated cells both in absence or presence of L-NAME after 24 h of incubation, stained with crystal violet. Original magnification = 20×. Scale bar = 15 μm.

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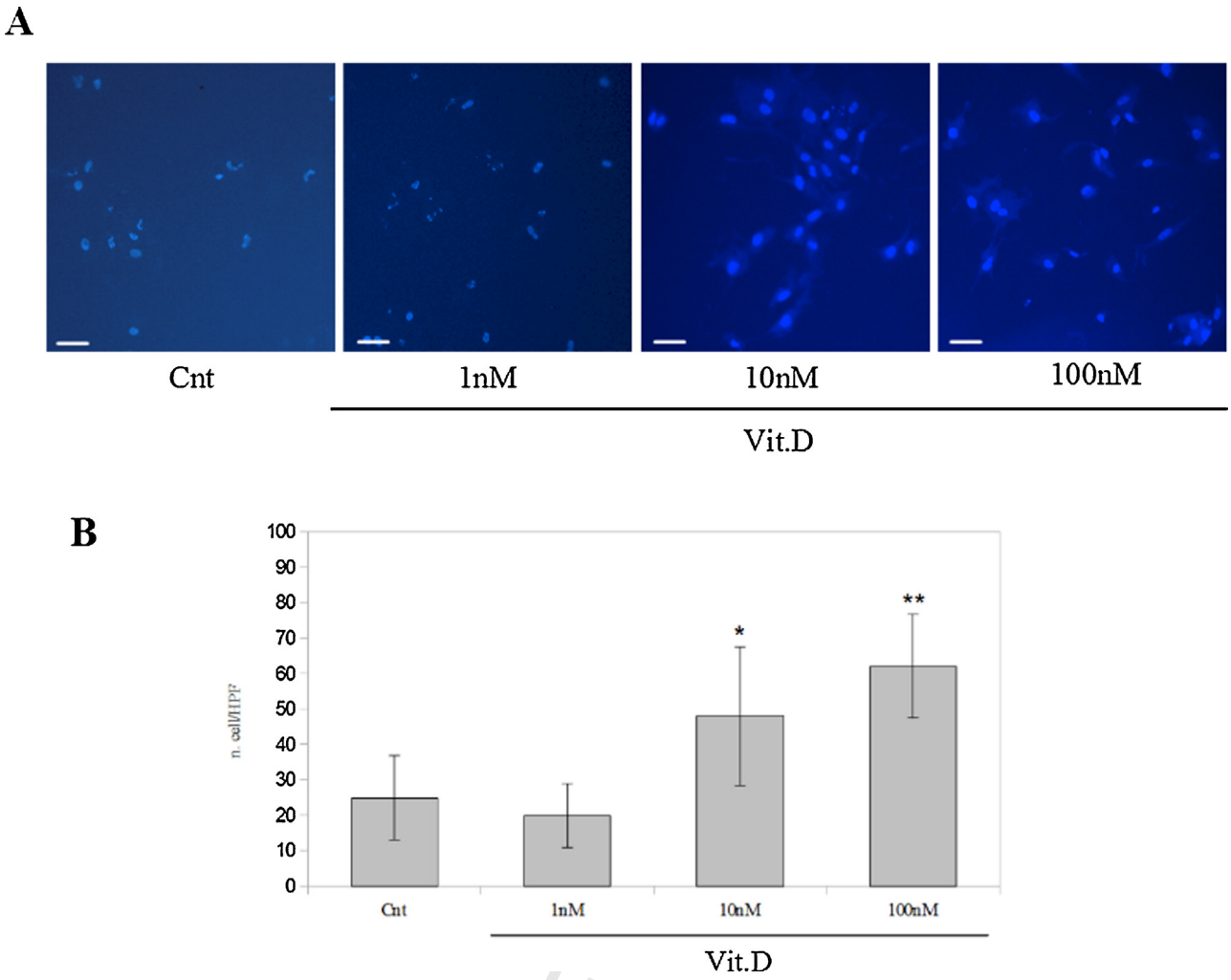

Fig. 3. Vit. D treated HUVEC migration into the 3D matrix. (A) Fluorescence microscopy images of control and Vit. D (1–100 nM) treated cells migrated in the 3D matrix after 7 days of incubation, stained with Hoechst 33,342. Original magnification = 10×. Scale bar = 60 μm. B) Determination of HUVEC migration after 7 days of incubation in the presence of different concentration of Vit. D (1–100 nM). Results are expressed as n. cell/HPF ± S.D. One-way ANOVA followed by Bonferroni post-test were used for statistical analysis. $^{*}P < 0.05$; $^{**}P < 0.01$.

Fig. 3A and B, HUVEC migration increased significantly only in the presence of 10 nM and 100 nM Vit. D ($P < 0.05$ and $P < 0.01$ respectively). In order to evaluate NO involvement in the observed phenomenon, the same experiments were also performed stimulating cells with 1 mM L-NAME in the presence or absence of the most effective Vit. D concentration (100 nM). As shown in Fig. 4A and B, L-NAME treatment did not affect control cells migration, whereas significantly reduced Vit. D-induced hydrogel invasion ($P < 0.01$ compared to Vit. D alone). To demonstrate the relevance of VDR on these effects, some experiments were also performed with Vit. D alone or in co-stimulation with ZK159222. This VDR ligand reduced Vit. D-induced migratory effect, as shown in Fig. 5.

3.3. Vitamin D induced MMP-2 mRNA expression and gelatinolytic activity via NO dependent pathway

Extracellular matrix (ECM) degradation is one of the main steps in cell migration. For this reason Vit. D effects on MMP-2 expression have been evaluated both in the conditioned medium of HUVEC migrating into the 3D hydrogel matrix and in RT-PCR. Gelatin zymography, performed on conditioned medium after 7 days of cell migration in the 3D matrix, demonstrated a statistically significant increase in MMP-2 activity in all the Vit. D treated samples. This effect was more evident in samples stimulated with 100 nM Vit. D ($P < 0.01$). The increase in MMP-2 expression appeared to be NO dependent, as L-NAME treatment totally abrogated Vit. D effects on MMP-2 expression (Fig. 6), according to the above described results for cell migration. In ZK159222-treated samples, MMP-2 activity reflected the data obtained for the migration assay (Fig. 7).

To evaluate whether the increased cell density into the 3D matrix was due to cell proliferation and/or migration, we investigated Vit. D effects on MMP-2 mRNA expression after 24 h of stimulation with the most effective Vit. D concentration (100 nM). As shown in Fig. 8, Vit. D was able to induce a statistically significant increase in MMP-2 mRNA expression, indicating that a Vit. D induced increase in cell migration is also involved in the observed process. As expected, L-NAME treatment decreased Vit. D-induced effects on MMP-2 expression (Fig. 8).

4. Discussion

Vit. D activity is currently of great interest, because its deficiency is causally associated with musculoskeletal diseases. Moreover, there has been an increasing interest in the relationship between Vit. D and extra-skeletal disorders, due to the identification of VDR in almost all human cells [10]. In particular, the

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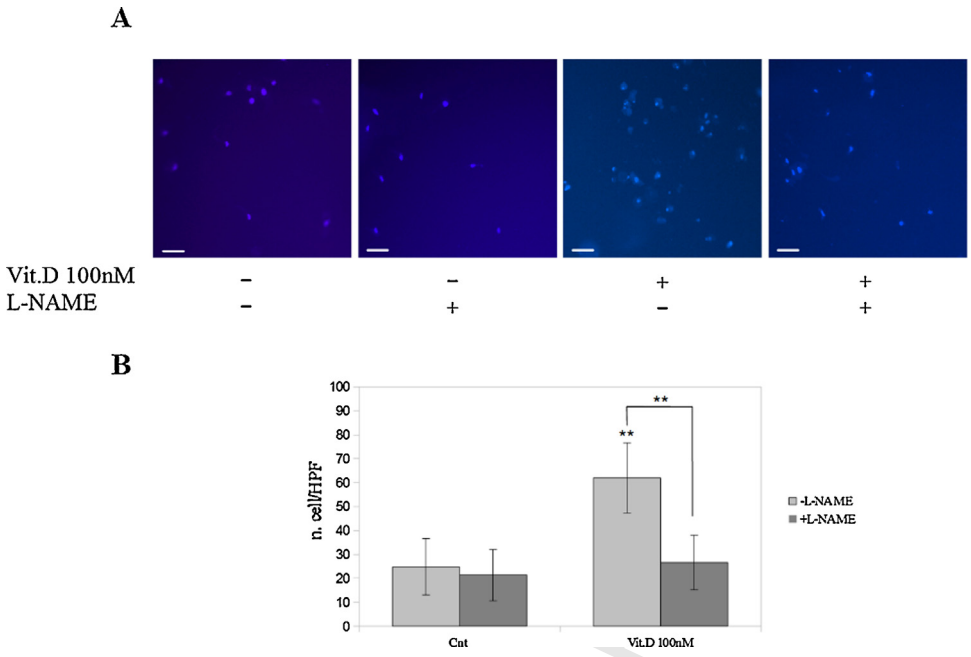

Fig. 4. Vit. D and L-NAME treated HUVEC migration into the 3D matrix. (A) Fluorescence microscopy images of control and Vit. D (100 nM) treated cells, both in the absence and presence of L-NAME, migrated in the 3D matrix after 7 days of incubation and stained with Hoechst 33,342. Original magnification = 10×. Scale bar = 60 μm. (B) Determination of control and Vit. D (100 nM) treated cells after 7 days migration both in the absence and presence of 1 mM L-NAME. Results are expressed as n. cell/HPF ± S.D. One-way ANOVA followed by Bonferroni post-test were used for statistical analysis. $^{**}P < 0.01$.

association between Vit. D deficiency and cardiovascular diseases has been deeply investigated [7,9] and recently Vit. D has been proposed as a novel and effective approach for vascular regeneration [21]. Concerning angiogenesis, therapeutic modulation of this process is a new promising field under investigation, since both macro- and micro-vascular ischaemic diseases remain a major concern. Indeed, dysregulated angiogenesis is involved in different pathologies, such as cardiovascular diseases, diabetes and chronic inflammation [14,22]. Angiogenesis is a multistep process including endothelial cell proliferation and migration into surrounding stroma/tissues. In the present study the effects of Vit. D administration in HUVEC have been investigated; a first set of experiments revealed a Vit. D-induced proliferation. Vit. D effects were actually mediated by VDR and its involvement was confirmed by the absence of increased cell proliferation in the presence of ZK159222, a VDR antagonist. In a second set of experiments focused on identifying Vit. D effects on HUVEC migration, a dose-dependent increase in cell migration in the 3D matrix was found. Angiogenesis requires the presence of proteases, such as the matrix metallo-proteinases (MMPs), which can degrade some of the components of the extracellular matrix (ECM) to let EC migrate into the surrounding environment. Moreover, MMPs also contribute to angiogenesis by detaching pericytes from vessels undergoing angiogenesis, by releasing ECM-bound angiogenic growth factors and by stimulating endothelial cell proliferation [23,24]. In particular, the main components of the vascular basal lamina (collagen IV, laminin and fibronectin) are degraded mainly by MMP-2 and by MMP-9, also known as gelatinases [25]. Considering the key role of MMP-2 in EC migration, MMP-2 expression has been investigated, both at transcriptional and translational level. For this purpose, the MMP-2 expression were evaluated by zymography and by RT-PCR in order to determine whether MMP-2 activation is involved in the mechanism underlying the 3D migratory responses induced by Vit. D. After 7 days of cell migration in the 3D matrix, MMP-2 activity was significantly increased in Vit. D-treated samples and the maximum effect was obtained in the presence

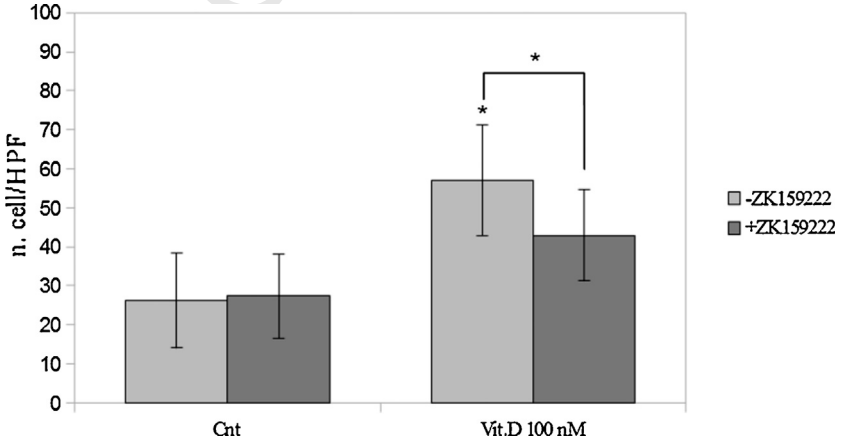

Fig. 5. Vit. D and ZK159222-treated HUVEC migration into the 3D matrix. Determination of control and Vit. D (100 nM) treated cells after 7 days migration both in the absence and presence of ZK159222. Results are expressed as n. cell/HPF ± S.D. One-way ANOVA followed by Bonferroni post-test were used for statistical analysis. $^{*}P < 0.05$.

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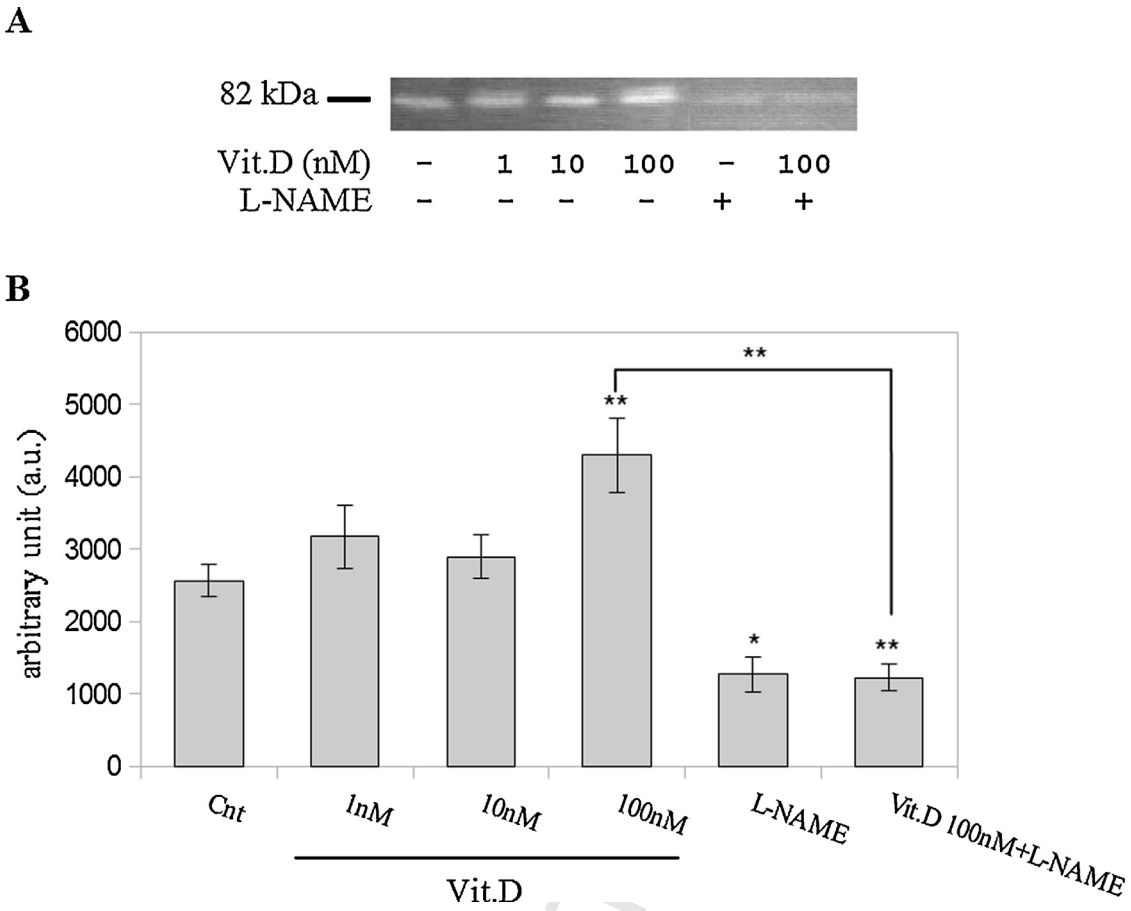

Fig. 6. MMP-2 activity in Vit. D and L-NAME-treated HUVEC. (A) Representative zymography of cell growth medium from Vit. D treated HUVEC migrated into the 3D matrix for 7 days in the absence or presence of L-NAME. (B) Densitometric quantification of MMP-2 expression. One-way ANOVA followed by Bonferroni post-test were used for statistical analysis. $^{*}P < 0.05$; $^{**}P < 0.01$.

of 100 nM Vit. D. To demonstrate VDR involvement on these effects, some samples were co-stimulated with Vit. D and ZK159222. This VDR ligand reduced Vit. D-induced migratory effect. To determine whether cells counted into the 3D matrix were all actually migrated or only a small population was migrated and subsequently proliferated, we investigated the MMP-2 mRNA expression after 24 h of stimulation with the most effective Vit. D concentration (i.e. 100 nM). Vit. D was able to induce an increase in MMP-2 expression regardless of cell proliferation, suggesting a combined and simultaneous action between enhanced proliferation and migration. Furthermore, L-NAME treatment did not affect control MMP-2 expression, whereas significantly reduced Vit. D-induced effects.

Endothelium derived NO is a bioregulatory molecule that controls the vascular tone and it is a critical mediator of angiogenic process [26,27]. NO exerts its pro-angiogenic effects by enhancing EC survival, proliferation and migration, and inducing the expressions of VEGF and FGF from vascular cells leading to an increase in angiogenic stimuli [14]. Since Vit. D was previously described as being able to induce NO production in HUVEC culture

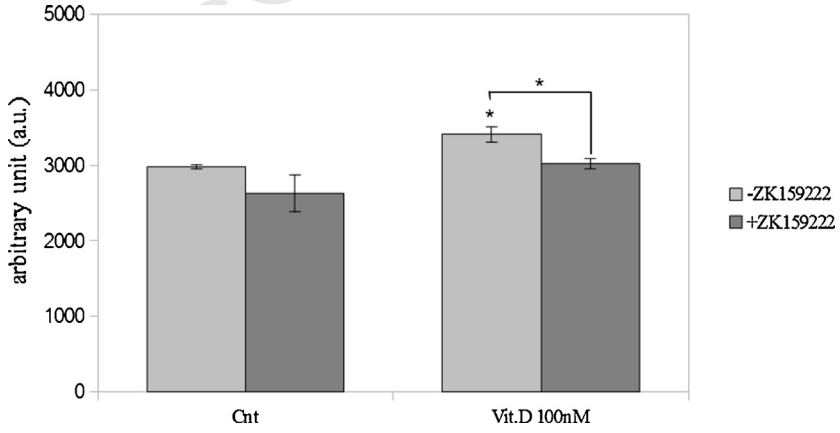

Fig. 7. MMP-2 activity in Vit. D and ZK159222-treated HUVEC. Densitometric quantification of MMP-2 expression. One-way ANOVA followed by Bonferroni post-test were used for statistical analysis. $^{*}P < 0.05$.

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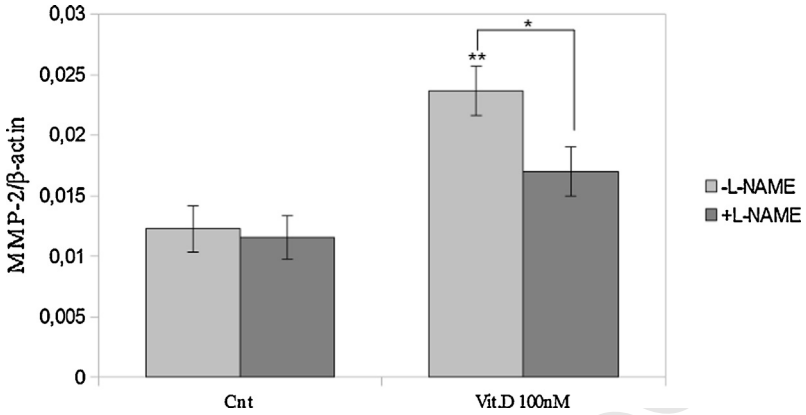

Fig. 8. MMP-2 mRNA expression. Densitometric quantification of MMP-2 mRNA expression of control and Vit. D 100 nM treated cells in the absence and presence of 1 mM L-NAME after 24 h of incubation. Results are expressed as MMP-2/β-actin ratio ± S.D. One-way ANOVA followed by Bonferroni post-test were used for statistical analysis. $^{*}P < 0.05$; $^{**}P < 0.01$.

through eNOS activation [12], a possible NO involvement in the proliferative and migratory effects induced by Vit. D can be hypothesized. For this purpose, further experiments were performed in the presence of L-NAME, an eNOS competitive inhibitor. As expected, L-NAME was able to prevent Vit. D-induced cell proliferation and migration, drastically reducing cell number. Moreover, L-NAME caused a dramatic reduction in MMP-2 activity confirming the involvement of NO as a mediator of Vit. D effects.

5. Conclusions

The results described herein highlight that Vit. D stimulated human EC proliferation and migration in a 3D matrix through NO-dependent mechanisms. These findings strongly support the role of Vit. D in human angiogenic process, suggesting new applications for Vit. D in tissue repair and wound healing.

Conflict of interest

None.

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