**Vitamin D Promotes Vascular Regeneration**

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Background—Vitamin D deficiency in humans is frequent and has been associated with inflammation. The role of the active hormone 1,25-dihydroxycholecalciferol (1,25-dihydroxy-vitamin D₃; 1,25-VitD₃) in the cardiovascular system is controversial. High doses induce vascular calcification; vitamin D₃ deficiency, however, has been linked to cardiovascular disease because the hormone has anti-inflammatory properties. We therefore hypothesized that 1,25-VitD₃ promotes regeneration after vascular injury.

Methods and Results—In healthy volunteers, supplementation of vitamin D₃ (4000 IU cholecalciferol per day) increased the number of circulating CD117+Flk1+Sca1+ angiogenic myeloid cells, which are thought to promote vascular regeneration. Similarly, in mice, 1,25-VitD₃ (100 ng/kg per day) increased the number of angiogenic myeloid cells and promoted reendothelialization in the carotid artery injury model. In streptozotocin-induced diabetic mice, 1,25-VitD₃ also promoted reendothelialization and restored the impaired angiogenesis in the femoral artery ligation model. Angiogenic myeloid cells home through the stromal cell–derived factor 1 (SDF1) receptor CXCR4. Inhibition of CXCR4 blocked 1,25-VitD₃–stimulated healing, pointing to a role of SDF1. The combination of injury and 1,25-VitD₃ increased SDF1 in vessels. Conditioned medium from injured, 1,25-VitD₃–treated arteries elicited a chemotactic effect on angiogenic myeloid cells, which was blocked by SDF1-neutralizing antibodies. Conditional knockout of the vitamin D receptor in myeloid cells but not the endothelium or smooth muscle cells blocked the effects of 1,25-VitD₃ on healing and prevented SDF1 formation. Mechanistically, 1,25-VitD₃ increased hypoxia-inducible factor 1-α through binding to its promoter. Increased hypoxia-inducible factor signaling subsequently promoted SDF1 expression, as revealed by reporter assays and knockout and inhibitory strategies of hypoxia-inducible factor 1-α.

Conclusions—By inducing SDF1, vitamin D₃ is a novel approach to promote vascular repair. (Circulation. 2014;130:976-986.)

**Key Words:** angiogenesis ■ endothelium ■ reendothelialization ■ regeneration ■ vitamin D

Vitamin D is a misnomer. The body produces this hormone through precursors with final hydroxylation to the most active metabolite, 1,25-dihydroxycholecalciferol (1,25-dihydroxy-vitamin D₃; 1,25-VitD₃). Vitamin D is ingested with food or synthesized from the precursor 7-dehydrocholesterol, which is then photochemically converted in the sunlight-exposed skin into cholecalciferol (vitamin D₃). Active 1,25-VitD₃ is generated by 2 hydroxylation steps: 25β-hydroxylation in the liver followed by 1α-hydroxylation in the kidney. The latter reaction is particularly rate limiting, and thus the effective concentration of 1,25-VitD₃ is much lower than that of vitamin D₃ or 25-hydroxy-vitamin D₃. By a positive feedback loop, 1,25-VitD₃ induces 24-hydroxylase expression. Because 1,24,25-trihydroxy-vitamin D₃ is water soluble and cleared by the kidney, this pathway limits the effects of the hormone, and 24-hydroxylase expression can be used as a marker for the biological activity of 1,25-VitD₃. Unless vitamin D is supplemented, intake with food is usually insufficient, and thus endogenous production contributes significantly to plasma levels. This leads to vitamin D deficiencies in up to 70% in some population groups in which sunlight exposure is low because of modern lifestyle, clothing, and few sunshine hours during winter.¹²

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The best-characterized vitamin D effects are enhanced intestinal calcium absorption and an increase in plasma calcium level. The biological effects of 1,25-VitD3, however, extend far beyond calcium metabolism. By controlling gene expression through the nuclear vitamin D receptor (VDR), 1,25-VitD3 has been suggested to limit inflammation and cancer, and thus lack of vitamin D has been associated with autoimmune disorders such as multiple sclerosis or neoplastic diseases. In the cardiovascular system, a low vitamin D status is inversely associated with blood pressure in blacks and increased renin-angiotensin II activity in mice. Vitamin D has also been suggested to delay the development of heart failure and potentially myocardial infarction. Furthermore, epidemiological evidence links low vitamin D status to an increased risk for cardiovascular disease. Although these observations are still controversial, they have attracted considerable attention in cardiovascular research to study the effects of this hormone.

Little is known about the role of vitamin D in endothelial cell biology. Initial data suggested that high doses of vitamin D inhibit endothelial cell migration, which could be used to treat tumor angiogenesis. Whether physiological concentrations of active vitamin D, however, have an effect on angiogenesis is elusive. Considering the anti-inflammatory and prodifferentiating effects of vitamin D, we hypothesized that the hormone promotes vascular regeneration. To address this, we subjected mice to the carotid artery injury model and femoral artery ligation. Furthermore, we investigated the effect of vitamin D supplementation on circulating angiogenic myeloid cells (AMCs) both in a mouse model of type 1 diabetes mellitus and in healthy volunteers.

Methods

An expanded methods section is available in the online-only Data Supplement.

Experimental Animals and Animal Procedures

All experimental procedures were approved by the local governmental authorities (approval Nos. F28/14 and F28/30) and were performed in accordance with animal protection guidelines exclusively in male mice. Hypoxia-inducible factor-1α (Hif1α)flox/flox, LysM-Creer×TG and Hif1αflox/flox-Hif2αflox/TG mice were provided by one of the coauthors (N.D.). Hif1αflox/flox- and Hif2αflox/flox mice were initially provided by R.S. Johnson (San Diego, CA). A previously reported lysosome M (LysM)-Cre deleter line was used for conditional gene knockout in mononuclear cells like macrophages and leukocytes. The generation and general characterization of VDRflox/flox and SMMC-CreERT2 mice have been described previously. Cdh5-CreERT2 mice were kindly provided by Ralf Adams, MPI Münster. Activation of CreERT2 was achieved previously with the biological activity of 1,25-VitD3, the expression of 24-hydroxylase was determined in myeloid cells obtained from the peripheral blood during the trial. The enzyme was almost undetectable before treatment but was massively induced by in vivo vitamin D3 (Figure 1D). After termination of the treatment, the level of active 1,25-VitD3 slowly decreased, as did the number of circulating AMCs and the 24-hydroxylase expression. This suggests that the effects observed during the study were related to vitamin D3 therapy.

Vitamin D3 Increases the Number of Circulating Angiogenic Myeloid Cells in Humans

To address whether 1,25-VitD3 could have a positive effect on factors promoting vascular repair in humans, healthy volunteers were treated with vitamin D3 (4000 IU orally once a day), and blood was drawn before, during, and after the 6-week treatment course. The treatment induced a significant increase in the levels of the vitamin D3 metabolite 25-hydroxy-vitamin D3 and importantly also doubled the plasma concentration of active 1,25-VitD3 (Figure 1A and 1B). Importantly, the number of CD45-CD117+Sca1+Flk1+ cells, denoted as AMCs, more than doubled during vitamin D3 treatment of humans (Figure 1C). To correlate this finding with the biological activity of 1,25-VitD3, the expression of 24-hydroxylase was determined in myeloid cells obtained from the peripheral blood during the trial. The enzyme was almost undetectable before treatment but was massively induced by in vivo vitamin D3 (Figure 1D). After termination of the treatment, the level of active 1,25-VitD3 slowly decreased, as did the number of circulating AMCs and the 24-hydroxylase expression. This suggests that the effects observed during the study were related to vitamin D3 therapy.

1,25-VitD3 Promotes Endothelial Regeneration in Mice

To determine whether 1,25-VitD3 indeed promotes endothelial regeneration, mice were treated with 1,25-VitD3 (100 ng/kg per day) for 5 days and were subsequently subjected to carotid artery electric injury. Although this approach only increased the plasma levels of 1,25-VitD3 by ≈2.5-fold, it elevated the number of CD45-CD117+Sca1+Flk1+ cells, denoted as AMCs, in blood and bone marrow and more than doubled the number of colony-forming units.
Diabetic Mice

1,25-VitD3 Restores Vascular Regeneration in Diabetic Mice

Diabetic patients are frequently affected by vitamin D deficiency, and the systemic inflammation in diabetes mellitus has been linked to functional vitamin D shortage. Because vascular dysfunction is the most important complication of diabetes mellitus, we hypothesized that diabetic vasculopathy will be alleviated by 1,25-VitD3 supplementation. Indeed, in streptozotocin-induced type 1 diabetic mice, carotid artery reendothelialization and colony-forming unit formation were greatly reduced (Figure 3A and 3B). Although vitamin D plasma levels, as expected, were normal in type 1 diabetic mice (Figure II in the online-only Data Supplement), 1,25-VitD3 supplementation had a beneficial effect so that colony-forming unit formation was increased by 1,25-VitD3 treatment and vascular healing was completely normalized to the level obtained in control animals (Figure 3A and 3B). Encouraged by this finding, vascular regeneration in the femoral artery ligation model was determined. In normal C57BL/6 mice, basal angiogenesis was very strong but was basically ablated by diabetes mellitus as determined by laser Doppler imaging and capillary density measurements. Remarkably, 1,25-VitD3 treatment completely restored the normal angiogenic response in these highly vascular dysfunctional animals (Figure 3C through 3F). These data indicate that 1,25-VitD3 can revert vascular dysfunction and that 1,25-VitD3 is a candidate to restore angiogenesis in diabetes mellitus. Although the benefit of 1,25-VitD3 in diabetic mice is greater than that in healthy mice, it can, however, not be inferred for the present data that diabetes mellitus is a situation of functional vitamin D deficiency.

**Figure 1.** Vitamin D increases angiogenic myeloid cells (AMCs) in human blood. Healthy subjects were studied before, during, and after a 6-week course of vitamin D (cholecalciferol, 4000 IU/d) supplementation. A and B, Plasma concentrations of 25-hydroxy-vitamin D3 (25-VitD3; A) and 1,25-dihydroxy-vitamin D3 (1,25-VitD3; B) as determined by enzyme-linked immunosorbent assay. *Significant differences from the mean of the values obtained before vitamin D supplementation. C, Percentage of CD45-CD117+Sca1+Flk1+ AMCs in the peripheral blood. D, 24-hydroxylase mRNA expression in myeloid cells isolated from the peripheral blood. n=6; *P<0.05. Ticks on the x axis of A and B indicate weeks.

CXC4-Positive Myeloid Cells Mediate the Regenerative Effect of 1,25-VitD3

The positive effect of 1,25-VitD3 on reendothelialization could be the consequence of a direct effect on endothelial cells or could be mediated by changes in the environment promoting endothelial migration in a paracrine manner. To distinguish between these possibilities, tissue-specific knockout mice of the VDR were generated. The regenerative effect of 1,25-VitD3 was lost in mice lacking the VDR in LysM-positive myeloid cells (Figure 4A). In contrast, deletion of VDR in endothelial or smooth muscle cells performed in tamoxifen-inducible tissue-specific Cre-deleter strains (Figure III in the online-only Data Supplement) had no effect on regeneration (Figure 4B and 4C). To exclude a potential role of platelets in the process, mice were made thrombocytopenic by treatment with anti-CD42 antibody, and although this lowered the platelet count by >90%, it did not affect 1,25-VitD3–stimulated vascular regeneration (Figure IVA and IVB in the online-only Data Supplement). To further define the nature of the LysM-positive cells, individual cell populations were depleted. Depletion of polymorphonuclear neutrophils by anti-polymorphonuclear neutrophil antibody had no effect on 1,25-VitD3–induced reendothelialization, whereas clodronate, which predominantly depletes macrophages and related cells, prevented the 1,25-VitD3–induced reendothelialization (Figure IVC through IVF in the online-only Data Supplement). Because these data suggest myeloid-derived monocytic cells as the mediator of the effects of 1,25-VitD3 and because 1,25-VitD3 increased the number of AMCs, a possible involvement of AMCs in carotid artery repair was studied. Homing of AMCs depends on the CXC4 receptor, and indeed the mRNA of CXC4 was increased after 3 days but not after 1 day of vascular injury (Figure V in the online-only Data Supplement), suggesting a homing of CXC4-positive myeloid cells to the site of injury. In vivo blockade of CXC4 with AMD3100 drastically reduced the 1,25-VitD3–induced reendothelialization (Figure 4D), suggesting that AMCs are...
indeed essential for the beneficial effect of 1,25-VitD3 on vascular regeneration.

**Stromal Cell–Derived Factor 1 Mediates the Regenerative Effect of 1,25-VitD3**

CXCR4 is the receptor for stromal cell–derived factor 1 (SDF1), making this chemokine a potential mediator of the 1,25-VitD3 effects. Indeed, SDF1 mRNA expression was increased in the carotid artery of 1,25-VitD3–treated mice within 24 hours after injury, whereas injury alone or 1,25-VitD3 alone had no effect on SDF1 expression (Figure 4E). To address whether tissue-resident cells mediate this effect, experiments were repeated in isolated murine vascular segments. Similar to the in vivo setting, ex vivo stimulation with a fairly low concentration of 1,25-VitD3 (10 nmol/L) increased SDF1 mRNA only in vessels that were previously injured ex vivo by detergent application (Figure 4F). The increase in SDF1 mRNA also resulted in protein formation and secretion of the chemokine, as detected by a significant increase in SDF1 protein in the conditioned medium of the injured vessels (Figure 4G). To test whether SDF1 released from the 1,25-VitD3–treated injured vessel mediates the chemotatic stimulus on myeloid cells, Boyden chamber experiments were performed with conditioned medium prepared from vascular segments. Medium conditioned with injured 1,25-VitD3–treated vessels had the greatest chemotactic effect (Figure 4H), and, importantly, the chemotactic effect was blocked by an SDF1-neutralizing antibody (Figure 4I). To directly visualize this chemotactic effect in vivo, homing was studied. Bone marrow mononuclear cells were labeled ex vivo and injected into mice. Without injury, no positive cells were observed in the carotid artery, whereas injury induced homing, and this effect was stimulated by 1,25-VitD3 pretreatment (Figure 4J). Collectively, these data demonstrate that tissue-resident cells express and release SDF1 on the combined stimulation of injury and 1,25-VitD3, which then attracts AMCs to the vessel.

**Myeloid Cells Are the Source of Injury and 1,25-VitD3–Induced SDF1 Production**

To identify the cellular sources of SDF1, VDR-conditional knockout mice were studied. Interestingly, an increase in SDF1 plasma level in response to 1,25-VitD3 was not observed in VDR<sup>lox/lox</sup> mice crossed with the LysM-Cre deleter strain (Figure 5A), raising the possibility that myeloid
cells are the source of SDF1. To address this, human AMCs, which are LysM positive, were generated from human blood by density gradient centrifugation and subsequently cultured in endothelial growth medium supplemented with epidermal growth factor on fibronectin-coated dishes for 3 days. To mimic the situation of vascular injury, cultured AMCs, after pretreatment with 1,25-VitD3 (24 hours, 10 nmol/L), were stimulated with the combination of tumor necrosis factor-α and interleukin-1β, 2 cytokines produced at sites of vascular injury. Similar to the data obtained in the carotid artery, only the combination of cytokines and 1,25-VitD3 induced a marked upregulation of SDF1 mRNA (Figure 5B) that was sufficient to also increase the SDF1 protein concentration in the supernatant of the cells (Figure 5C). Similar data were obtained in the human monocytic cell line THP-1 (Figure VI in the online-only Data Supplement). Because these data suggest myeloid cells as the source of SDF1, the consequence of conditional VDR deletion on SDF1 mRNA expression was studied in the carotid artery. Genetic deletion of VDR in LysM-positive cells but not in endothelial or smooth muscle cells prevented the induction of SDF1 in response to 1,25-VitD3 and injury, which then, either by directly stimulating the endothelium or by attracting AMCs to the site of injury, promote vascular regeneration.

The Potentiating Effect of 1,25-VitD3 on SDF1 Is Mediated by HIF1α

The stimulating effect of 1,25-VitD3 on injury-induced SDF1 expression was present on the protein as well as on mRNA level, suggesting an induction or stabilization of SDF1 mRNA. The SDF1 promoter harbors binding sites for several transcription factors. In cytokine-primed human AMCs, 1,25-VitD3, however, downregulated JAK2 and heme oxygenase-1 expression (Figure VIIA and VIIB in the online-only Data Supplement), arguing against a role of the signal transducer and activator (STAT) and nuclear factor erythroid–derived 2 system, which leaves the transcription factors nuclear factor-κB (NF-κB) and HIF 1 or 2 as likely mediators. Pharmacological inhibition of NF-κB with 2 different inhibitors did not affect the 1,25-VitD3–stimulated SDF1 induction in cytokine-primed AMCs (Figure VIIC in the online-only Data Supplement), whereas the HIF1α inhibitor acriflavine completely blocked the effect of 1,25-VitD3 of SDF1 mRNA (Figure 6A). Importantly, also when administered to mice in vivo, acriflavine (given as reported previously)2 prevented the 1,25-VitD3–stimulated carotid artery repair (Figure 6B). To seek molecular confirmation

Figure 3. 1,25-Dihydroxy-vitamin D3 (1,25-VitD3) restores vascular repair in type 1 diabetic mice. Evans blue staining of the injured carotid artery (A) and spleen colony-forming unit assay (B) from healthy and diabetic mice 60 days after streptozotocin application with (100 ng/kg per day) and without (CTL) 5 days of pretreatment with 1,25-VitD3 3 days after carotid injury are shown (n=6; *P<0.05). C through F, Angiogenesis in the femoral artery ligation model of normal and diabetic mice with or without 1,25-VitD3 treatment (100 ng/kg per day, given as 5 daily injections before the procedure followed by injection every second day for the next 14 days). Exemplary laser Doppler images on day 14 (C) and statistical analysis (D) for hind limb perfusion on days 7 and 14 after operation are shown: *P<0.05, day 14 vs nontreated control group and vs 1,25-VitD3–treated diabetic group. #*P<0.05, day 7 to day 14 for identical treatment group (E and F). Exemplary histology (F) and statistical analysis (E) of capillary density as determined by immunohistochemistry and confocal microscopy 2 weeks after operation are shown. *P<0.05 vs healthy control group and vs 1,25-VitD3–treated diabetic group (n=7; *P<0.05). Green indicates CD31-positive endothelial cells; red, laminin-positive skeletal muscle cells. n=4; *P<0.05, CTL vs 1,25-VitD3 and healthy vs diabetic.
of these pharmacological data, conditional LysM-specific HIF knockout mice were studied. Combined deletion of HIF1α and HIF2α in LysM-positive cells prevented the 1,25-VitD3–induced vascular healing and SDF1 induction (Figure 6C and 6D). To differentiate between the involvement of HIF1α and HIF2α, ex vivo experiments were performed in tissue from LysM-specific HIF1α single knockout mice. 1,25-VitD3 failed to induce SDF1 induction in injured vascular preparations of the knockouts ex vivo as well as in cytokine-primed murine spleen-derived monocytic cells of these LysM-Cre+/–,HIF1αflox/flox mice (Figure 6E and 6F). Collectively, these data suggest that 1,25-VitD3 acts through HIF1α in LysM-positive cells to induce SDF1.

**1,25-VitD3 Induces HIF1α**

Cytokines and injury are known to increase HIF1α protein by attenuating HIF1α protein degradation.23 The fact that 1,25-VitD3 had an additive effect to cytokines/injury suggests that the hormone acts through a different mechanism.
Vitamin D deficiency is common, but its medical consequences, apart from bone disease, are insufficiently understood. The possible vascular effects of vitamin D supplementation have been discussed controversially. In the present study, we report that 1,25-VitD3 supplementation boosts the number of circulating AMCs both in healthy humans and in mice with carotid injury. We demonstrate that 1,25-VitD3 promotes vascular regeneration in healthy mice and restores normal vascular repair and AMC function in diabetic mice. By acting on myeloid cells, 1,25-VitD3 increases the level of SDF1 in the plasma and at sites of vascular injury through the induction of HIF1α. SDF1, by stimulating CXCR4-positive cells, subsequently mediates the regenerative effect of 1,25-VitD3.

In the present study, we found little evidence for a direct effect of 1,25-VitD3 on the vascular endothelium because genetic deletion of VDR in endothelial cells did not affect SDF1 plasma levels or vascular regeneration. These findings were unexpected given that several studies documented effects of 1,25-VitD3 on endothelial cells in culture such as an anti-inflammatory action in human coronary arterial endothelial cells and inhibition of tumor necrosis factor-α–induced adhesion molecule expression. It has also been suggested that vitamin D acutely induces endothelial NO formation by altering phosphorylation of endothelial NO
In our hands, genetic deletion of endothelial NO synthase, however, was without effect on 1,25-VitD3–induced vascular repair (M.S.K. Wong, PhD, unpublished data). Although endothelial cells have been reported to express the VDR, reverse transcription quantitative polymerase chain reaction revealed that the level of receptor mRNA in these cells is >1000 times lower than that in monocytes or AMCs (M.S.K. Wong, PhD, unpublished data, 2013). Obviously, cultured cells, being devoid of most physiological stimuli, respond more vigorously than cells in an...
organ context, and thus the direct endothelial effects of vitamin D might be restricted to the culture situation. Indeed, in a placebo-controlled, randomized, double-blind trial study of 90 patients with documented coronary artery disease and low vitamin D level, treatment with 50,000 IU weekly for 12 weeks had no impact on reactive hyperemia peripheral arterial tonometry and levels of inflammatory cytokines in the blood, and vitamin D also failed to improve flow-mediated dilatation in diabetic patients. Despite this notion, it should be emphasized that in spontaneously hypertensive rats, 1,25-VitD3 supplementation improved endothelial function and reduced endothelium-dependent contraction. However, it is unclear whether this effect is a consequence of a global change in stress level or local inflammation that affects the endothelium or a direct endothelial effect associated with prolonged treatment.

Several studies documented an anti-inflammatory effect of 1,25-VitD3 in a variety of cell types, including endothelial cells, dendritic cells, T cells, and macrophages, which was in part linked to an inhibition of NF-kB activation and signaling. It was therefore unexpected that 1,25-VitD3 increased the expression of the cytokine SDF1 in response to injury in vivo and in culture in response to the combination of tumor necrosis factor-α and interleukin-1β, 2 cytokines that are increased after injury and that were used to mimic the injury scenario. The SDF1 promoter does not contain putative binding sites for VDR but does contain them for inflammatory transcription factors and STAT transcription factors. Nevertheless, tumor necrosis factor-α/interleukin-1β or injury alone had little influence on SDF1 expression, and 1,25-VitD3 was also without effect. After the combined application of these stimuli, SDF1 expression was induced, but this response was much delayed compared with responses mediated directly by activator protein 1 or NF-kB, which suggests an indirect mechanism. Indeed, inhibition of NF-kB in the present study was without effect on 1,25-VitD3–mediated induction of SDF1, and elements of the STAT pathway were downregulated by 1,25-VitD3 rather than induced. As the mechanism driving SDF1 in the present study, we identified HIF1α and observed that 1,25-VitD3 directly increases HIF1α mRNA transcription and that VDR binds directly to its promoter. This also explains why, in acute experiments in the absence of cytokines, 1,25-VitD3 had little effect on SDF1: Without a stabilizing signal like inflammation or hypoxia, increases in HIF1α mRNA have basically no effect on HIF1α protein levels.

It is a potential weakness of our study that we did not identify in detail the nature of the LysM-positive, 1,25-VitD3– sensitive cell in the vessel wall. LysM is expressed in all myeloid cells including macrophages, monocytes, and granulocytes but also in AMCs, which possess similarities to the myeloid lineage. Tissue-resident myeloid cells as well as progenitor cells are present in healthy vessels and are in part LysM positive. The deletion experiments in the present study indicate that potentially tissue-resident, adventitial-localized, clodronate-sensitive macrophages are the source of SDF1 in response to injury and 1,25-VitD3. Although we could exclude granulocytes as mediators of healing, we did not further differentiate the subpopulations of clodronate-sensitive cells that may comprise dendritic cells, tissue-resident progenitors, or macrophages.

Our study contrasts early observations suggesting that vitamin D limits angiogenesis. The context of that study, however, was different because a tumor environment and high doses of 1,25-VitD3 were studied. Given the antitumor effects of vitamin D, it is conceivable that part of the antiangiogenic effect of the compound is indirect.

An important finding in the present study was that vitamin D supplementation in humans increased the number of circulating AMCs. The particular design of our study favors a positive outcome because healthy subjects in the winter months were studied, but the small sample size and the open and consecutive design lead us to interpret the data with caution. Nevertheless, the dramatic effects on 24-hydroxylase observed in the present study indicate the high efficiency of the treatment and the compliance of the study population. Thus, studies on the effect of vitamin D supplementation for vascular repair in humans, for example after balloon angioplasty, are warranted.

Collectively, the present work advocates vitamin D treatment as a novel and effective approach to promote vascular regeneration after injury in healthy and diabetic subjects.

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Disclosures
None.

References


CLINICAL PERSPECTIVE

Active vitamin D hormone 1,25-dihydroxycholecalciferol (1,25-VitD3) enhances intestinal calcium uptake and elicits complex effects on the immune system. 1,25-VitD3 promotes differentiation of myeloid cells and is thought to limit inflammation. Supraphysiological doses of vitamin D are used in tumor therapy and have antiangiogenic effects. Endogenous vitamin D formation requires oral intake of its precursor or sufficient sunlight exposure to the skin. This leads to 1,25-VitD3 deficiency in up to 70% in some population groups, which is thought to be associated with autoimmune disease and neoplasia. A low vitamin D status is also associated with hypertension in blacks, and an association of low vitamin D with heart failure has been discussed. On this basis, we hypothesized that vitamin D promotes vascular regeneration. In healthy volunteers, supplementation of vitamin D increased the number of circulating angiogenic myeloid cells. In mice, active 1,25-VitD3 had a similar effect and also promoted vascular repair after carotid artery injury and angiogenesis in diabetic mice. Mechanistically, 1,25-VitD3 increased the formation of the cytokine stromal cell–derived factor 1 in tissue-resident myeloid cells at sides of vascular injury through a pathway involving the induction of the transcription factor hypoxia-inducible factor-1α. Stromal cell–derived factor 1 subsequently recruited angiogenic myeloid cells to the site of injury, where they are thought to release growth factors that ultimately promote vascular regeneration. Given the high prevalence of vitamin D deficiency, these observations advocate clinical studies on the effect of vitamin D supplementation on vascular repair and angiogenesis in subjects with impaired vascular regeneration such as diabetics.
Vitamin D Promotes Vascular Regeneration

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Supplemental Material

Vitamin D promotes vascular regeneration

Michael Sze Ka Wong PhD, Matthias S. Leisegang PhD, Christoph Kruse MSc, Juri Vogel MSc, Christoph Schürmann PhD, Nathalie Dehne PhD, Andreas Weigert PhD, Eva Herrmann PhD, Bernhard Brüne PhD, Ajay M. Shah MD, PhD, Dieter Steinhalber, Stefan Offermanns PhD, Geert Carmeliet MD PhD, Klaus Badenhoop MD, Katrin Schröder PhD, Ralf P. Brandes MD

Supplemental Figures

Figure S1: Effect of different treatment protocols of 1,25-VitD3 on carotid artery regeneration as determined by Evans blue staining 3 days after injury. (A) High dose (1 µg/kg/d) for 5 days, (B) low dose (100 ng/kg/d) over 28 days every other day before carotid injury, (C) high dose (1 µg/kg/d) single dose at day of carotid artery injury. n>3, *p<0.05 CTL vs. 1,25-VitD3

Figure S2: 1,25-VitD3 plasma level as determined by ELISA in the serum of control and streptozotocin-diabetic mice with and without 1,25-VitD3 supplementation (100 ng/kg/d). n=5, *p<0.05 CTL vs. 1,25-VitD3

Figure S3: Efficiency of tamoxifen-stimulated Cre-mediated excision of the vitamin D receptor (VDR). (A) RT-qPCR of the VDR in endothelial cells cultured from the lung of VDR-Flox-Flox-Cdh5-Cre-ERT2/0 and VDR-Flox-Flox-Cdh5-Cre-ERT2Cre0/0 mice treated with tamoxifen 10 days prior to sacrifice. (B) RT-qPCR of the VDR in the carotid artery of VDRFlox-Flox-SMMHC-Cre-ERT2/0 and VDRFloxFloxSMMHC-Cre-ERT2Cre0/0 mice treated with tamoxifen 10 days prior to sacrifice. n=3, *p<0.05 Cre- vs. Cre+
Figure S4: Contribution of circulating cells to the effect of 1,25-VitD3 on vascular repair. (A) Whole blood platelet count of mice treated with (platelet depleted) or without (CTL) purified rat monoclonal antibodies directed against mouse GPIb (CD42b, 2 μg/g bodyweight, i.v.) and (B&D&D&F) Evans blue staining of carotid artery injury in mice with or without (A&B) platelet depletion, (C&D) macrophage depletion, (E&F) PMN depletion and with or without 5 days of 1,25-VitD3 pretreatment. n>3. C: Exemplary liver stain for the macrophage marker F4/80 in Kupffer cells (brown). E: Exemplary whole blood count for PMNs. *p<0.05 CTL vs. 1,25-VitD3

Figure S5: Carotid artery CXCR4 expression is not increased 1 day after injury. Relative mRNA expression of CXCR4 from the mouse carotid artery 1 day (left) and 3 days (right) after carotid artery injury in animals with or without a 5 days pretreatment of 1,25-VitD3. n=3, *p<0.05 CTL vs. 1,25-VitD3

Figure S6: Combined stimulation with 1,25-VitD3 and cytokines induces SDF1 secretion from THP1 cells. SDF1 ELISA from the conditioned medium of THP1 cells stimulated with or without 1,25-VitD3 (10 nM, 24 hours) prior to TNFα (10ng/ml) and interleukin 1β (10 ng/ml, 16 hours). n=4, *p<0.05.
Figure S7: Pathway analysis activated by the combined stimulation with 1,25-VitD3 and cytokines. Human blood-derived AMCs were stimulated with or without 1,25-VitD3 (10 nM, 24 hours) prior to TNFα (10 ng/mL) and interleukin 1β (10 ng/mL, 16 hours). n=4, *p<0.05.

(A&B) Effect on heme oxygenase 1 and JAK2 mRNA expression. (C&D) Effect of NFκB inhibitor or PDTC (Pyrrolidine dithiocarbamate; 10 μmol/L) on 1,25-VitD3-induced SDF1 (C) and HIF1α (D) mRNA expression in response to cytokines. *p<0.05 CTL vs. 1,25-VitD3, n>3.
Supplemental Material

Vitamin D promotes vascular regeneration

Michael Sze Ka Wong PhD, Matthias S. Leisegang PhD, Christoph Kruse MSc, Juri Vogel MSc, Christoph Schürmann PhD, Nathalie Dehne PhD, Andreas Weigert PhD, Eva Herrmann PhD, Bernhard Brüne PhD, Ajay M. Shah MD, PhD, Dieter Steinbüchel, Stefan Offermanns PhD, Geert Carmeliet MD PhD, Klaus Badenhoop MD, Katrin Schröder PhD, Ralf P. Brandes MD

Expanded Material & Methods Section

Experimental animals and animal procedures

All experimental procedures were approved by the local governmental authorities (approval numbers: F28/14, F28/30) and were performed in accordance with the animal protection guidelines exclusively in male mice. C57BL/6 mice were purchased from Charles Rivers (Deisenhofen, Germany). HIF1αfloxflox- LysM-Cre<sup>lox</sup>-TG and HIF1αfloxflox-IF1αfloxflox-HIF2αfloxflox-TG were provided by one of the co-authors (N.D.). HIF1αfloxflox<sup>1</sup> and HIF2αfloxflox<sup>2</sup> mice were initially kindly provided by R.S. Johnson (San Diego, USA). A previously LysM-Cre deleter line was used for conditional gene knockout in monocytic cells like macrophages and leukocytes<sup>3</sup>. The generation and general characterization of VDRe<sup>lox/flox</sup> and SMMHC-CreERT2 mice<sup>5</sup> has been described previously. Cdh5-CreERT2 mice<sup>6</sup> were kindly provided by Ralf Adams, MPI Münster. Activation of CreERT2 was achieved by tamoxifen (40 mg/kg dissolved in autoclaved sunflower oil injected intraperitoneally) on 3 consecutive days followed by a “wash-out” phase of 2 weeks. In all experiments cre positive (denoted as Cre+) as well as the cre negative (denoted as Cre-) control animals received tamoxifen to exclude direct effects of this anti-estrogen. Breeding of the Cre-lines was carried out by crossing Cre<sup>+/-</sup> and Cre<sup>0/0</sup> animals such that Cre<sup>+/-</sup> and Cre<sup>0/0</sup> littermates could always be compared side by side. Mice were housed in a specified pathogen-free facility with 12/12 hours day/night cycle and free access to chow (25 µg vitamin D per kg chow) and water.

In some animals, compounds were used to delete different cell populations. To delete polymorphonuclear leukocytes (PMNs), a rabbit polyclonal anti-mouse PMN antibody (MyBiosource, MB5535278) was administered i.p. 1:100 dilution in 100 µl at time of first 1,25-VitD3 application as single injection. Platelets were depleted by an anti-CD42 antibody (rat monoclonal anti-mouse GPIb - CD42b antibody given as 2 µg/g bodyweight, i.v., Abcam) given daily and macrophages were removed by clodronate liposomes (www.clodronateliposomes.com) injected i.p. (100 µl) at the days of 1,25-VitD3 injection.

Streptozotocin diabetes type I model

Diabetes was induced by four consecutive daily injections of streptozotocin (STZ; 70 mg/kg body weight; Sigma-Aldrich, Munich, Germany) dissolved in 100 mmol/L sodium citrate buffer (pH 4.5). Citrate buffer only was administered to control animals. STZ-treated mice also received a subcutaneous insulin slow extended release implant (Linplant; Linshin, Ontario, Canada) to prevent early ketoadisis. Plasma glucose levels were measured before induction of diabetes and monitored for at least 60 days (Haemo-Glukotest; Roche Diagnostics).

Carotid artery injury model

Electric-injury was performed under general anesthesia by ketamin/rompun as described<sup>9</sup>. Briefly, the carotid artery was exposed to electric injury using a haemostatic electric coagulation forceps (ERBOTOM ICC 50 HF, ERBE, Tübingen, Germany) twice juxtapose for 3 seconds to induce a 3 mm wide denudation. Wounds were closed by staples and animals were allowed to recover for 3 days followed by Evans blue staining for determination of the denuded area. Evans blue solution (2%, 200 µL) was injected via the tail vein and allowed to circulate for 10 minutes. Subsequently, animals were perfused transcardially with NaCl 0.9% to remove the excessive dye. Carotid arteries were isolated and imaged by an infrared-based laser fluorescence scanner (Odyssey, Licor, Bad Homburg, Germany) or photographed on a macro stage. In some experiments, mice were injected i.v. with 100 µL Alexa Fluor® 647-conjugated Griffonia simplicifolia isoelectin B4 (0.25 mg/mL, Life Technologies, Grand Island, NY) 30 minutes before sacrifice. Followed by fixation and DAPI staining, isoelectin en face staining in the carotid artery was performed. For this, 3D-tile scans were recorded with a x20 objective in a LSM510 Meta and subsequently reconstructed for surface presentation. The lectin negative area was measured and subjected to statistics.

Femoral artery ligation model

Neovascularization capacity was investigated in a murine model of severe hind limb ischemia in 6 to 8-week old mice. The deep femoral artery was ligated with an electric coagulator (ERBOTOM ICC50, ERBE, Tübingen, Germany) and subsequently the superficial femoral artery and vein as well as the epigastric arteries were completely excised. The overlying skin was closed with 3 surgical staples. Relative blood flow was determined by laser Doppler imaging (Laser Doppler Perfusion Imager System, Wilmingtom, Germany) at 7 and 14 days post-ligation. The perfusion of the ischemic and non-ischemic limb was calculated on the basis of colored histogram pixels. To minimize variables including ambient light...
and temperature and to maintain a constant body temperature, mice were exposed to infrared light for 10 minutes before laser Doppler scans. During the scan, mice were positioned supine on a heating pad with their legs stretched and fixed.

To determine capillary density, cross-sections of adductor and semimembranosus muscles embedded in Tissue Tek (Sakura, Heppenheim, Germany) were stained. After fixation in phosphate buffer (100 mmol/L, pH 7.3) containing 4% formalin the tissue was blocked with 3% bovine serum albumin and permeabilized with 0.5% Triton X-100 followed by incubation with anti-CD31 (BD Pharmingen, Heidelberg, Germany) and anti-laminin (Abcam, Cambridge, UK) and imaged by confocal microscopy.

**Active vitamin D supplementation**

1α,25-Dihydroxy-vitamin D3 (1,25-VitD3, 100 ng / kg / day) was dissolved in a solvent mixture of water, propylene glycol and ethanol in a 5:4:1 ratio. For the carotid artery injury model, 1,25-VitD3 or solvent were injected intraperitoneally in a series of 5 days followed by electric-injury. For the femoral artery ligation model, animals received additional injections every other day after the operation.

**FACS analyses of angiogenic myeloid cells (AMCs)**

Murine circulating AMCs were determined in the blood obtained by cardiac puncture in heparinized syringes. Human AMCs were isolated from whole blood of healthy donors. Plasma was obtained by centrifugation at 800 g for 10 minutes. The remaining blood fraction was diluted with 1 ml PBS containing 0.5% BSA and 2 mmol/L EDTA and was overlaid on top of 3 mL LSM 1077 lymphocyte separation medium (PAA Laboratories, Pasching, Austria) and subjected to density gradient centrifugation (800 g, 20 minutes). Murine bone marrow-derived mononuclear cells were obtained by flushing the femurs with 1 mL PBS containing 0.5% BSA and 2 mmol/L EDTA and subsequent density gradient centrifugation as mentioned above. Mononuclear cells were counted and stained for CD45, CD117, Sca-1 and Flk-1 surface expression using PE, APC, FITC or eFluor® 605NC-labeled antibodies in a dilution of 1:100 in PBS buffer after blocking non-specific Fc receptor binding. CD45, CD117 and Flk-1 antibodies were purchased from BD Pharmingen (Heidelberg, Germany), while the Sca-1 antibody was purchased from eBioscience, San Diego, CA. Gating strategies were designed using fluorescence minus one (FMO) controls and samples were acquired using a LSRIIFortessa flow cytometer and FACSDiva software 6.0 (BD Biosciences, Heidelberg, Germany).

**Colony forming units (CFU)**

Murine spleens were isolated, homogenized and filtered through 100 µm cell strainer (BD Falcon, Franklin Lakes, USA),followed by subsequent density gradient centrifugation as mentioned above. 1x10⁵ cells were plated per 3.5 cm dish in methylcellulose (Methocult GF H4535, Stem Cell Technologies). CFUs were counted after 14 days.

**Determination of vitamin D, its metabolites and cytokine plasma-level**

ELISA kits from IDS were used to determine the plasma concentrations of 1,25-VitD3 and 25-hydroxy-vitamin D3 according to the instructions contained in the kit. SDF-1 plasma level was determined by ELISA-kits purchased from R&D following the manufacturer’s instructions.

**Cell culture and transfection**

White blood cells were plated in a density of 4x10⁶ per cm² on fibronectin-coated plates in endothelial growth medium (EGM) containing 2% fetal calf serum (FCS). Medium was changed every other day until day 7.

For RNA induction or ELISA experiments, angiogenic myeloid cells (AMCs) or THP1 macrophages were treated with DMSO (Sigma-Aldrich, Munich, Germany) or 1,25-VitD3 (10 nM; Sigma-Aldrich, Munich, Germany) for 24 hours followed by the supplemental incubation with TNFα (10 ng/mL; Peprotech, Hamburg, Germany) and IFNγ (10 ng/mL; Peprotech, Hamburg, Germany) for 16 hours. For inhibition of hypoxia inducible factors, acriflavine (10 µmol/L) was used for 16 hours (Sigma-Aldrich, Munich, Germany).

AMCs were identified by their ability to stain positive for Ulex europaeus lectin and to take up 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (DiLAc-LDL). For this, cells were incubated with DiLAc-LDL (2.4 µg/mL) at 37°C for 1 hour and subsequently exposed to fluorescein-5-isothiocyanate (FITC)-labelled Ulex europaeus agglutinin I (lectin, 10 µg/mL; Sigma-Aldrich, Munich, Germany) for 1 hour. Double positive cells were judged as AMCs and 99% of all cells in the culture were positive for both dyes. Murine blood AMCs were isolated and cultured in a similar way.

**Homing experiments**

Bone marrow was obtained from the femurs of healthy, untreated donor mice. Monocytic cells were treated ex vivo with cell tracker orange (Invitrogen, Carlsbad, CA, USA) and cells (5x10⁶ cells) were resuspended in 100 µL of prewarmed PBS and injected into recipient mice (i.v. via tail vein) 3 hours after the carotid artery injury. The recipient mice were sacrificed 1 day after the injury and the carotid arteries were harvest, sectioned, counter stained with DAPI and imagined by laser microscopy. The number of positive cells in 10 consecutive sections was added per artery and used for statistics.

**Luciferase assay and site-directed mutagenesis**

AMCs were transiently transfected with the original or Vitamin D responsive element (VDRE) mutated pGL3-HIF1α promoter (821 nt) plasmid in pGL3 7
with Transpass D2 according to the manufacturer’s protocol (New England Biolabs, Frankfurt, Germany). 24 hours after transfection cells were stimulated with DMSO or 1,25-VitD3 (10 nmol/L) for 24 hours, followed by the stimulation with TNFα (10 ng/mL) and HIF1α (10 ng/mL) for 16 hours. Luciferase activity was determined by the assay kit from Promega (Mannheim, Germany) following the manufacturer’s instructions in a Berthold LB9505 luminometer (Bad Wildbad, Germany) and normalized to the empty pGL3 control vector (Promega). VDREs were predicted with the aid of the JASPAR database. The VDREs of the human HIF1α-promoter in the pGL3-HIF1α plasmid were mutated at sites 348 (tggatctcgag to tgtatAGGAag), 626 (agggcgaca tggct to agTcTaTITTc) and 648 (ccgggcac to ccttac) with the Quickchange II XL Site Directed Mutagenesis Kit from Agilent (Santa Clara, USA) following the manufacturer’s instructions (Mannheim, Germany) following the Quikchange II XL Site Directed Mutagenesis Kit from Agilent (Santa Clara, USA) according to the manufacturer’s protocol. For primer sequences, see supplementary table 1.

**Chromatin immuno-precipitation (ChIP)**
HEK293 cells were transiently transfected with pSG5 vectors expressing the retinoid x receptor (RXR) and the vitamin D receptor (VDR) with Fugene6 (Roche, Mannheim, Germany) according to the manufacturer’s protocol, and subsequently stimulated as indicated and subjected to ChIP as follows: Cell preparation, crosslinking and nuclei isolation was performed with the truCHIP™ Chromatin Shearing Kit (Covaris, Woburn, USA) according to the manufacturer’s protocol. Afterwards, the lysates were sonicated with the truCHIP™ Chromatin Shearing Kit (Covaris, Woburn, USA) according to the manufacturer’s protocol. Subsequently, the lysates were diluted 1:3 in dilution buffer (20 mmol/L Tris/HCl pH 7.4, 2 mmol/L EDTA, 0.5% Triton X-100 and protease inhibitors). After preclearing with 30 µL DiaMag protein A and protein G coated magnetic beads slurry (Diagenode, Seraing, Belgium) for 30 minutes at 4°C, samples were incubated over night at 4°C with 5 µL of anti-vitamin D receptor antibody (Sc-1008, Santa Cruz Biotechnology, Heidelberg, Germany), and 5% of the samples served as input. The VDR complexes were collected with 50 µL DiaMag protein A and protein G coated magnetic beads (Diagenode, Seraing, Belgium) for 3 hours at 4°C, subsequently washed twice for 5 minutes with each of the wash buffers 1-3 (Wash Buffer 1: 20 mmol/L Tris/HCl pH 7.4, 100 mmol/L NaCl, 2 mmol/L EDTA, 0.5% Triton X-100 and protease inhibitors). After preclearing with 30 µL DiaMag protein A and protein G coated magnetic beads slurry (Diagenode, Seraing, Belgium) for 30 minutes at 4°C, samples were incubated over night at 4°C with 5 µL of anti-vitamin D receptor antibody (Sc-1008, Santa Cruz Biotechnology, Heidelberg, Germany), and 5% of the samples served as input. The VDR complexes were collected with 50 µL DiaMag protein A and protein G coated magnetic beads (Diagenode, Seraing, Belgium) for 3 hours at 4°C, subsequently washed twice for 5 minutes with each of the wash buffers 1-3 (Wash Buffer 1: 20 mmol/L Tris/HCl pH 7.4, 100 mmol/L NaCl, 2 mmol/L EDTA, 0.5% Triton X-100; Wash Buffer 2: 20 mmol/L Tris/HCl pH 7.4, 500 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100; Wash Buffer 3: 10 mmol/L Tris/HCl pH 7.4, 250 mmol/L lithium chloride, 1% Nonidet, 1% sodium deoxycholate, 1 mmol/L EDTA) and finally washed with TE-buffer pH 8.0. Elution of the beads was done with elution buffer (0.1 M NaHCO3, 1% SDS containing 1proteinase K (Diagenode, Seraing, Belgium) and shaking at 600 rpm for 1 hour at 55°C, 1 hour at 62°C and 10 minutes at 95°C. After removal of the beads, the eluate was purified with the QiaQuick PCR purification kit (Qiagen, Hilden, Germany) and subjected to qPCR analysis. As negative control, primer targeting the β-actin promoter were used. Primers amplifying the protomor region of 25-hydroxy vitamin D3-24 hydroxylase (CYP24A1) served as positive control. For primer sequences, see supplementary table 1.

**Ex vivo vascular injury model**
Mice were killed by exsanguination in deep isoflurane anesthesia and the remaining blood was then removed by transcatheter perfusion with Hanks buffer. Subsequently, the aorta was isolated, cleaned of adhering tissue under sterile condition, cut into two equal-size pieces. One was injured by perfusion with CHAPS (1% in 5% glucose). Both vessel segments were extensively washed afterwards in sterile Hanks buffer and transferred into separate wells of a 24 well plate filled with medium EGM containing 2% FCS and antibiotic without 1,25-VitD3. After 24 hours, the conditioned medium was used for Boyden chamber experiments and ELISA assays. The vessels were subjected to qRT-PCR.

**Cell migration assay**
Murine blood AMCs were kept in culture for 3 days. After detaching the cells with EDTA/Versene, 2x10⁴ cells in endothelial growth medium (EGM) with 2% FCS were plated into the insert of a Boyden chamber (8 µm pore size). The lower part medium was supplemented with VEGF165 (50 ng/ml; R&D) or conditioned medium. EGM with 2% FCS without cytokine supplement served as negative control, reflecting basal migration. Cells were allowed to migrate for 24 hours and then migration was stopped by adding paraformaldehyde to the chamber. Cells at the upper side of the insert-membrane were removed and cells at the lower side were stained with DAPI. Positive cells in three different fields of view were counted and migration in response to the cytokines was calculated relative to basal migration. Conditioned medium was generated from mouse aortas dissected and cleaned of surrounding tissue. After preparation, the aortas were cut into 8 pieces of equal length and treated with or without CHAPS (2%) in order to remove the endothelium. Two segments were pooled and incubated in 400 µL EGM with 2% FCS with or without 1,25-VitD3 (10 nM) in a 48 well plate. Medium without aortic segments incubated on the same plate served as controls.

**qRT-PCR**
RNA was isolated from AMCs using the RNA Miniprep Kit (Bio&Sell, Feucht, Germany). cDNA synthesis was carried out with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primers; semiquantitative real-time PCR was performed with Fast Plus EvaGreen Master Mix for qPCR w/Low Rox (2x, 100 rxn) (Biotium, Hayward, CA, USA) in a Mx3005 cycler (Stratagene) with the indicated primers. Relative expressions of
target genes were normalized, analyzed by the delta-delta-CT method and given as ratio compared to control experiments. For primer sequences, see supplemental table 1.

**Vitamin D treatment in healthy volunteers**
A pilot study in 6 healthy volunteers (4 male, 2 female, age 32.5 ± 4.9 years, mean ± SD) who gave informed written consent, was performed. This was part of the ViDDA1 trial (registered under EUDRACT 2010-022677-34, “Influence of high dose vitamin D substitution on humoral immunity and lymphocyte function in patients with newly diagnosed and recent onset type 1 diabetes mellitus or Addison’s disease”), which was approved by the Ethical Committee of Frankfurt Medical School. Blood was taken weekly in the morning over a period of 12 weeks and vitamin D was supplemented from week 4 to 10 by a daily oral intake of 4,000 IU cholecalciferol (Vigantol Öl, Merck Serono).

**Statistical analysis**
Unless otherwise indicated, data are given as means ± standard error of mean (SEM). Calculations were performed with BiAS 10.12 with the exception of the linear mixed effect model. This was processed in R 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria) utilizing the extension package nlme 3.1-111 and multcomp 1.2. Processing and the specific model accounting for inner-individual dependencies, variance heterogeneity and suitable contrasts as well as check of normality were programmed by one of the authors (E.H.). In case of multiple testing, significance correction of the linear mixed model or Bonferroni correction was applied. A p-value of <0.05 was considered as significant.

Linear mixed effect model: Fig 1A, 1B, 3D
Paired T-test: Fig 1C, 1D
Unpaired T-test: Fig 2E, 2F, 6B, 6C, 6D, 6I, 6J, S1A, S1B, S1C, S3A, S3B, S4F, S7C, S7D
Mann-Whitney test: 2A, 2B, 2C, 2D, 2F, 4G, 6H, S4A, S4D
ANOVA followed by post hoc Bonferroni T-test for selected groups: Fig 3A, 3B, 4A, 4B, 4C, 4D, 4E, 4F, 4H, 4I, 5A, 5B, 5C, 5D, 6A, 6E, 6F, 6G, S2, S4B, S4, S5, S7, S7A, S7B

Reference List

### Primer for qRT-PCR

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### Primer for site-directed mutagenesis:

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