Original Paper

1 α ,25(OH)₂D₃ Induces Actin Depolymerization in Endometrial Carcinoma Cells by Targeting RAC1 and PAK1

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Key Words

Actin polymerization • RAC1 • PAK1 • Endometrium • Tumour cells

Abstract

Background: Cell proliferation and motility require actin reorganization, which is under control of various signalling pathways including ras-related C3 botulinum toxin substrate 1 (*RAC1*), p21 protein-activated kinase 1 (*PAK1*) and actin related protein 2 (*ARP2*). Tumour cell proliferation is modified by 1 α ,25-Dihydroxy-Vitamin D₃ (1 α ,25(OH)₂D₃), a steroid hormone predominantly known for its role in calcium and phosphorus metabolism. The present study explored whether 1 α ,25(OH)₂D₃ modifies actin cytoskeleton in Ishikawa cells, a well differentiated endometrial carcinoma cell line. **Methods:** To this end, actin cytoskeleton was visualized by confocal microscopy. Globular over filamentous actin ratio was determined utilizing Western blotting and flow cytometry, transcript levels by qRT-PCR and protein abundance by immunoblotting. **Results:** A 24 hour treatment with 1 α ,25(OH)₂D₃ (100 nM) significantly decreased *RAC1* and *PAK1* transcript levels and activity, decreased *ARP2* protein levels and depolymerized actin. The effect of 1 α ,25(OH)₂D₃ on actin polymerization was mimicked by pharmacological inhibition of *RAC1* and *PAK1*. **Conclusions:** 1 α ,25(OH)₂D₃ leads to disruption of *RAC1* and *PAK1* activity with subsequent actin depolymerization of endometrial carcinoma cells.

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Introduction

1 α ,25(OH)₂D₃, the biologically active metabolite of Vitamin D [1], is predominantly known for its role in mineral metabolism [2]. 1 α ,25(OH)₂D₃ is, in addition, a powerful inhibitor of malignancy [3], including endometrial cancer [4].

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Vitamin D is a fat soluble secosteroid which can be produced endogenously or obtained exogenously from dietary or supplementary sources [1]. The two major forms are vitamin D₂ (ergocalciferol; C₂₈H₄₄O) and D₃ (cholecalciferol; C₂₇H₄₄O) [1]. Vitamin D₃ is endogenously produced by UVB (ultraviolet B) radiation (wavelength 290-315 nm) converting 7-dehydrocholesterol into vitamin D₃ [2] which is transported to the liver by vitamin D-binding protein [1]. Exogenous dietary vitamin D exists as either vitamin D₂ or D₃ [5]. Both endogenous and exogenous vitamin D are converted into 25-hydroxy vitamin D (25(OH)D; C₂₇H₄₄O₂) in a reaction catalysed by 25-hydroxylase in the liver [6]. 25-hydroxy vitamin D is hydroxylated in the kidney to 1α,25-dihydroxy vitamin D₃ (1α,25(OH)₂D₃; C₂₇H₄₄O₃), the biologically active form, by the enzyme 1α-hydroxylase. The renal 1α-hydroxylase is under tight regulation by parathyroid hormone (PTH), calcium and phosphate [2]. The biological actions of 1α,25(OH)₂D₃ are achieved by binding to the vitamin D receptor (VDR) [7].

VDR expression has been identified in a variety of tissue types, including: adipose tissue, brain, breast cancer cells, colon, liver, lung, muscle, ovary, pancreas, prostate, skin, stomach, and thyroid [8]. Expression of PTH and calcium insensitive 1α-hydroxylase has been identified in cancer and non-cancer cells, such as prostate, colon, lung, endothelial, brain, pancreatic β-cells and monocytes [1]. Current research suggests that vitamin D and its metabolites may reduce progression of many types of cancers [1]. Despite the large amount of recent research that has investigated the associations between vitamin D and cancer [9, 10], the exact mechanism of the protective effect of vitamin D remained incompletely understood.

Effects of 1α,25(OH)₂D₃ include reorganization of actin cytoskeleton [11-14]. Apparently depending on the cell type, 1α,25(OH)₂D₃ may either increase [11, 12, 14] or decrease [13] actin polymerisation. Early and/or late actin restructuring, following modifications of actin polymerization dynamics equilibrium, is a cellular response initiated by various signals including growth factors and cytokines [15-17], hormones [18-21] and ions [22, 23]. Alterations of actin organization, in turn, govern crucial cellular functions and outcomes such as cell volume regulation [24], survival [25], migration and cell motility [26, 27], secretion [28], cell growth and proliferation [29] as well as apoptosis [30, 31].

Key regulators of the actin cell cytoskeleton include ras-related C3 botulinum toxin substrate 1 (RAC1) [32], which stimulates formation of lamellipodia [33], a prerequisite for cell motility [34]. The regulatory proteins of the actin cytoskeleton play a pivotal role for the motility of cancer cells and contribute to most steps during cancer progression [35, 36]. The ability of cancer cells to invade the surrounding tissue, crossing the endothelial barrier to metastasize at a secondary site requires a highly dynamic reorganization of the actin cytoskeleton [37]. RAC1 and other Rho GTPases were found to be overexpressed in many types of cancer [38, 39]. Down-regulation of RAC1 activity suppresses tumor growth and RAC1 was therefore identified as a potential therapeutic target for cancer cell treatment [40, 41].

Here we report that treatment of human endometrial carcinoma cells with 1α,25(OH)₂D₃ leads to a dramatic decrease in RAC1 activity and p21 protein-activated kinase 1 (PAK1) phosphorylation leading to actin reorganization.

Material and Methods

Cell culture

A well differentiated endometrial carcinoma cell line, Ishikawa cells (Sigma; routinely tested every 6 months for mycoplasma. The authenticity of the cells were confirmed by qPCR every 10th passage) were cultured in DMEM/F12 without phenol red media, containing 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic solution and 0.25% L-Glutamine (Invitrogen, Karlsruhe, Germany) in a humidified atmosphere at 37°C and 5% CO₂. Cells were treated as described with 1α,25(OH)₂D₃ (#D1530; 100 nM; Sigma Aldrich, München, Germany), RAC1 inhibitor NSC23766 trihydrochloride (#SML0952; 100nM; Sigma Aldrich,

München, Germany), or PAK1 inhibitor IPA 3 (#3622; 50 nM; Tocris bioscience, Germany) for 24 hours.

Quantitative Real-time PCR

Total RNA was extracted from Ishikawa cultures using Trizol (Invitrogen) based on a phenol-chloroform extraction protocol. Equal amounts of total RNA (1 µg) were reverse transcribed with the Superscript III First-Strand synthesis system for RT-PCR (Invitrogen) primed using random hexamers, and the resulting cDNA used as template in qRT-PCR analysis. The gene-specific primer pairs were designed using the Primerblast (NCBI) software. Detection of gene expression was performed with KapaFast-SYBR Green (Peqlab, Erlangen, Germany) and quantitative RT-PCR was performed on a BioRad iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, München, Germany). The expression levels of the samples were calculated by the $2^{-\Delta\Delta Ct}$ method using *L19* to normalize for variances in input cDNA and expressed as arbitrary units. All measurements were performed in duplicate. Melting curve analysis and agarose gel electrophoresis confirmed amplification specificity.

Measurement of the G/F actin ratio by Triton X-100 fractionation

To quantify actin polymerization in Ishikawa cells, cells were incubated in 100 µl of Triton X-extraction buffer containing 0.3% Triton X-100, 5 mM Tris [pH 7.4], 2 mM EGTA, 300 mM sucrose, 2 µM phalloidin, 1 mM PMSF, 10 µg/ml leupeptin, 20 µg/ml aprotinin, 1 mM sodium orthovanadate, and 50 mM NaF for 5 min on ice. The supernatant containing the soluble proteins was removed by aspiration. The Triton X-insoluble pellet was scraped from the plate directly into 100 µl of RIPA buffer. Any remaining insoluble material was removed by centrifugation. Equal volumes of each fraction were boiled in Roti-Load1 Buffer (Carl Roth, Karlsruhe, Germany) at 100°C for 10 min. Proteins were separated on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (Amersham Biosciences, UK). Non-specific binding sites were blocked by 1 hour at room temperature incubation with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween. The membranes were incubated 2 hours at room temperature with monoclonal rabbit anti-Pan-Actin (D18C11)-HRP conjugated antibody (#12748, 1:1000, Cell Signalling, Frankfurt, Germany). Antibody binding was detected with the Novex ECL Chemiluminescent Substrate Reagen Kit (Invitrogen) and densitometry was performed on scanned images using ImageJ software.

Western Blotting

Whole cell protein extracts were obtained by direct lysis in Lammelli's Buffer and boiled at 100°C for 10 min. Proteins were separated on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (Amersham Biosciences, UK). Non-specific binding sites were blocked for 1 hour at room temperature with 5% non-fat dry milk in TBS-T. Membranes were incubated overnight at 4°C with anti-Rac1/Cdc42 (#4651), anti-Phospho-Rac1/Cdc42 (Ser71) (#2461), anti-ARP2 (#3128), anti-PAK1/2/3 (#2604), anti-Phospho-PAK1 (Thr423)/PAK2 (Thr402) (#2601), and anti-GAPDH (#2118) (Cell Signalling, Leiden, The Netherlands). All primary antibodies were used at 1:1000, washed 3 times with TBS-T, followed by incubation with HRP-conjugated anti-rabbit secondary antibodies (#7074, 1:1000, Cell Signalling, Leiden, The Netherlands). Protein complexes were visualized with a chemiluminescent detection kit (Novex ECL) and bands were quantified using ImageJ software.

Flow Cytometry of G- and F-actin

Ishikawa cells ($\approx 1.0 \times 10^5$ cells) were first fixed with 4% paraformaldehyde (PFA) for 15 min, then permeabilized with 1× Permeabilization buffer (eBioscience, Frankfurt, Germany) and stained with 1.0 µl of fluorescent DNaseI-Alexafluor-488 (50mg/ml) for G-actin detection and fluorescent Phalloidin-eFluor 660 (1000×) (eBioscience, Frankfurt, Germany) for F-actin detection for 15 min. The abundance of the respective labels was measured using green (FL-1) and red channel (FL-4) on FACSCalibur (BD Biosciences, Heidelberg, Germany) and analyzed using Flowjo software (Flowjo LLC, Oregon, USA). G- and F-actin geometric mean values were determined from the respective fluorescence and the ratio of G/F calculated from the geometric mean values.

Table 1.

Gene	Sequence
<i>L19</i>	Forward (5'-3'):GCAGCCGGCGCAAA Reverse (5'-3'):GCGGAAGGGTACAGCCAAT
<i>RAC1</i>	Forward (5'-3'):TGCAGACACTTGCTCTCTATGTAG Reverse (5'-3'):GAGTTCAATGGCAACGCTTCA
<i>PAK1</i>	Forward (5'-3'):GGGAGTTTACGGGAATGCCA Reverse (5'-3'):CCTGCGGGTTTTCTTCTGC

Confocal Microscopy

Cells were treated and grown in sterile 4-well tissue culture chambers (Sarstedt, Nümbrecht, Germany). They were washed with PBS once before fixed with 4% PFA for 15 min at room temperature, then washed with PBS for 3 times and permeabilized in 0.1% Triton X-100/PBS for 15 min. The slides were blocked with 5% goat serum in 0.1% Triton X-100/PBS for 1 hour at room temperature. Actin was stained with eflour660-phalloidin (1:200, Invitrogen) for 1 hour and with SYTOXGreen dye (1:5000, Invitrogen) for nuclei staining for 30 min in the dark. The slides were mounted with ProLong Gold antifade reagent (Invitrogen). Confocal microscopy was performed with a confocal laser-scanning microscope (LSM 5 Exciter, Carl Zeiss, Oberkochen, Germany) with a C-Apochromat 63/1.3 NA DIC water immersion objective. The mean fluorescence from six related cells of each picture was quantified by ZEN software (Carl Zeiss).

Statistics

Data are provided as means $\pm$ SEM, *n* represents the number of independent experiments. All data were tested for significance using Student's two-tailed t-test or two-way ANOVA followed by Sidak's multiple comparisons test as appropriate. Only results with *P*<0.05 were considered statistically significant.

Results

Effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on actin polymerization

Our study addressed the role of the organization of the actin cytoskeleton in Ishikawa cells in response to 24 hours treatment with 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$. First, we determined the changes in the actin polymerization dynamics by Western blotting. Fig. 1A illustrates that treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ resulted in a dramatic depolymerization of the actin cytoskeleton in Ishikawa cells as apparent from the substantial decrease of F-actin content and increase of the G to F actin ratio. This effect was further confirmed by FACS analysis (Fig. 1B) indicating a persistent effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on actin reorganization. Confocal laser

Fig. 1. Dramatic effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on actin polymerization in Ishikawa cells after 24h treatment with or without $1\alpha,25(\text{OH})_2\text{D}_3$ (100 nM). (A) Representative original Western Blot of G- over F-actin in Ishikawa cells with or without a 24h treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ (100 nM) (left). Arithmetic means $\pm$ SEM (*n*=8) of G- and F- actin ratio (right). (B) Representative original FACS histograms of G- and F- actin in Ishikawa cells with (red, vitamin D) or without (blue, Control) a 24h treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ (100nM) (left) and graph showing arithmetic means $\pm$ SEM (*n*=12) of G- and F- actin ratio (right). (C) Representative original confocal images (left) of eflour660-phalloidin binding to F-actin (red) and SYTOXGreen for nuclei (blue) in Ishikawa cells that remained untreated (upper panel) or with a 24h treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ (lower panel) and a bar graph showing the arithmetic means $\pm$ SEM (*n*=4) of actin fluorescence (F-actin) in Ishikawa cells. **P*<0.05, ***P*<0.01, *****P*<0.0001 indicate statistically significant difference from control cells using Student's t-test.

Fig. 2. $1,25(\text{OH})_2\text{D}_3$ reduces *RAC1* transcript levels and total RAC1, Phospho RAC1 and ARP2 protein levels in Ishikawa cells after 24h treatment with $1,25(\text{OH})_2\text{D}_3$ (100 nM). (A) Arithmetic means $\pm$ SEM (n=4) of *RAC1* normalized to *L19* transcript levels in Ishikawa cells. Data are depicted as fold induction relative to transcript levels of untreated samples. (B) Representative original Western Blot of phospho-RAC1, RAC1, ARP2 and GAPDH in Ishikawa cells that remained untreated or with a 24h treatment with $1,25(\text{OH})_2\text{D}_3$. (C) Arithmetic means $\pm$ SEM (n=4) of RAC1, phospho-RAC1, and ARP2 ratio normalized to GAPDH in Ishikawa cells. *P<0.05; **P<0.01; ***P<0.001 indicate statistically significant difference from control cells using Student's t-test.

scanning microscopy fully supported these findings, demonstrating a pronounced actin cytoskeleton restructuring with significant reduction of stress fibres (Fig. 1C).

Effect of $1,25(\text{OH})_2\text{D}_3$ on RAC1 and ARP2

Small G protein or GTPase RAC1 can regulate actin organization and polymerization [32]. In search for a cellular mechanism accounting for the reorganization of the actin filaments, the effect of $1,25(\text{OH})_2\text{D}_3$ on RAC1 expression and activity was tested. As shown in Fig. 2A, $1,25(\text{OH})_2\text{D}_3$ treatment (100 nM for 24 h) was followed by a significant decline of *RAC1* transcript levels normalized to *L19* transcript levels (P = 0.025). Moreover, $1,25(\text{OH})_2\text{D}_3$ treatment was followed by a significant decline of total-RAC1, phospho-RAC1 and ARP2 protein levels (Fig. 2B, C).

Effect of $1,25(\text{OH})_2\text{D}_3$ on actin polymerization in absence or presence of RAC1 inhibitor

A further series of experiments tested whether the effect of $1,25(\text{OH})_2\text{D}_3$ on RAC1 indeed contributed to the depolymerization following treatment with $1,25(\text{OH})_2\text{D}_3$ (100 nM) for 24 h. As illustrated in Fig. 3, Western blot (Fig. 3A) and FACS analysis (Fig. 3B) revealed that application of the RAC1 inhibitor NSC23766 trihydrochloride (NSC; 100 nM) for 24 hours was followed by a significant increase of soluble G-actin over F-actin in human endometrial cancer Ishikawa cells (Fig. 3A, P = 0.0015; Fig. 3B, P = 0.0291), thus mimicking the effect of $1,25(\text{OH})_2\text{D}_3$ treatment (Fig. 3A, P < 0.0001; Fig. 3B, P = 0.0297). In the presence of the RAC1 inhibitor the additional administration of $1,25(\text{OH})_2\text{D}_3$ (100 nM) led to a significant further increase of the soluble G-actin over F-actin ratio in Ishikawa cells (Fig. 3A, P = 0.0003; Fig. 3B, P = 0.0003).

Effect of $1,25(\text{OH})_2\text{D}_3$ on actin polymerization in absence or presence of PAK1 inhibitor

RAC1 is known to trigger phosphorylation of PAK1, which in turn regulates the actin network [31]. As shown in Fig. 4, application of $1,25(\text{OH})_2\text{D}_3$ for 24 hours decreased PAK1

Fig. 3. $1,25(\text{OH})_2\text{D}_3$ reduces actin polymerization in Ishikawa cells after 24h treatment with or without $1,25(\text{OH})_2\text{D}_3$ (100 nM) in absence or presence of RAC1 inhibitor NSC23766 trihydrochloride (100 nM). (A) Ishikawa cells were treated with $1,25(\text{OH})_2\text{D}_3$ either in the absence or presence of RAC1 inhibitor NSC23766 trihydrochloride (NSC,100 nM) or remained untreated. Cells were harvested for G- and F- actin. Representative Western Blot of G- and F-actin in Ishikawa cells (left). Arithmetic means $\pm$ SEM (n=4) of G- and F-actin ratio (right). (B) In parallel cultures, cells were harvested and stained for G- (DNase1) and F- actin (phalloidin) and subjected to FACS. Representative FACS plot of G- and F- actin in Ishikawa cells, upper and lower panels respectively. Arithmetic means $\pm$ SEM (n=12) of G- and F- actin ratio (lower). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 indicate statistically significant difference from control cells first by Student's t-test.

transcript (Fig. 4A, $P=0.032$) and protein (Fig. 4B, $P=0.0001$) levels, pointing to a decrease of PAK1 activity in Ishikawa cells. Pharmacological inhibition of PAK1 with IPA3 (50 nM) again mimicked the destabilizing effect of inhibited RAC1 activity on the actin network (Fig. 5). Similar to inhibition of RAC1, inhibition of PAK1 was followed by a significant increase of soluble G-actin over F-actin in human endometrial cancer Ishikawa cells (Fig. 5A, $P=0.006$; Fig. 5B, $P=0.011$), an effect thus mimicking the effect of $1,25(\text{OH})_2\text{D}_3$ treatment (Fig. 5A, $P=0.0009$; Fig. 5B, $P=0.045$). In the presence of the PAK1 inhibitor the additional administration of $1,25(\text{OH})_2\text{D}_3$ also led to a significant further increase of the soluble G-actin over F-actin ratio in Ishikawa cells (Fig. 5B, $P=0.0003$).

Discussion

The present study describes a completely novel function of $1,25(\text{OH})_2\text{D}_3$ in downregulating the expression and activity of the small G-protein RAC1 and of the kinase PAK1 with subsequent actin depolymerisation.

RAC1 acting downstream of FAK/PI3K signaling [18, 42] is a key regulator of actin cytoskeleton organization [32], and down-regulation of RAC1 expression and activity following $1,25(\text{OH})_2\text{D}_3$ treatment contributes to or even accounts for the observed actin

Fig. 4. *PAK1* transcript levels and protein levels are significantly reduced after a 24h treatment with $1\alpha,25(\text{OH})_2\text{D}_3$. (A) Ishikawa cells were treated with $1\alpha,25(\text{OH})_2\text{D}_3$ for 24h. Cells were harvested for mRNA and subjected to qRT-PCR. Arithmetic means $\pm$ SEM (n=4) of *PAK1* normalized to *L19* transcript levels in Ishikawa cells. Data are depicted as fold induction relative to transcript levels of untreated samples. (B) Ishikawa cells were treated with $1\alpha,25(\text{OH})_2\text{D}_3$ for 24h. Cells were harvested for protein. Representative original Western Blot of PAK1, phospho-PAK1 and GAPDH in Ishikawa cells. (C) Arithmetic means $\pm$ SEM (n=4) of PAK1 and phospho-PAK1 ratio normalized to GAPDH in Ishikawa cells. *P<0.05, ***P<0.001 indicate statistically significant difference from control cells using Student's t-test.

Fig. 5. Effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on actin polymerization in Ishikawa cells in absence or presence of PAK1 inhibitor. (A) Ishikawa cells remained untreated or were treated with $1\alpha,25(\text{OH})_2\text{D}_3$ either in the presence or absence of PAK1 (IPA3) inhibitor. Representative Western Blot of G- and F-actin in Ishikawa cells (left). Arithmetic means $\pm$ SEM (n=4) of G- and F-actin ratio normalized to control G- and F- ratio (right). (B) In parallel cultures, cells were harvested and stained for G- (DNase1) and F- actin (phalloidin) and subjected to FACS. Representative FACS plot of G- and F- actin in Ishikawa cells, upper and lower panels respectively. A bar graph shows the arithmetic means $\pm$ SEM (n=12) of G- and F- actin ratio. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 indicate statistically significant difference from control cells first by Student's t-test.

reorganization. RAC1 activation is known to regulate actin polymerization interacting with the ARP2/3-mediated actin nucleation pathway rather than with the formin-mediated F-actin polymerization [43]. On the other hand, it has been reported that the long formin-

mediated F-actin accounts for the cortical elasticity of cells rather than the short ARP2/3-mediated F-actin. In our study however, we observed a 1 α ,25(OH)₂D₃ induced decrease of RAC1 phosphorylation and ARP2, indicating deactivation of these signaling effectors. On the other hand PAK1 has also been identified as a downstream target of the small GTPases CDC42 and RAC1 [44, 45]. Upon binding to these GTPases, PAK1 is activated thus increasing its catalytic activity [44], resulting in peripheral actin cytoskeleton reorganization that may include cortical actin polymerization as well as formation of filopodia and membrane ruffles [46, 47]. In line with this, we observed reorganization of the actin filaments network towards substantial depolymerization of F-actin filaments. The present results are in line with previous observations in endothelial cells, whereby PAK1 phosphorylates actin, an effect resulting in actin polymerization [48]. In our results we show that treatment with 1 α ,25(OH)₂D₃ reduces both RAC1 and PAK1 activity. Inhibition of these two key molecules is further expected to decrease of lamellipodia formation, which could decrease the cell area and prevent migration of cancerous cells.

In conclusion, the present study demonstrates that 1 α ,25(OH)₂D₃ down-regulates expression and function of the small G-protein RAC1, ARP2 and of PAK1 with subsequent depolymerisation of the actin filaments. The reorganization of the actin filaments could decrease cancer cell survival resulting in increased cell death, as this has been reported in various tumor cells [29, 49, 50]. Moreover, given that the actin cytoskeleton significantly changes upon cellular transformation and the correlative changes in actin filament architecture in drug resistant cells, drugs that target tumor-specific actin filaments could be utilized in combination with routine therapies to enhance their effectiveness in patients. Thus, this new role of 1 α ,25(OH)₂D₃ may provide new avenues to pursue the development of novel cancer therapeutics.

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Disclosure Statement

All authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

References

- 1 Feldman D, Krishnan AV, Swami S, Giovannucci E, Feldman BJ: The role of vitamin D in reducing cancer risk and progression. *Nat Rev Cancer* 2014;14:342-357.
- 2 Holick MF: Vitamin D deficiency. *N Engl J Med* 2007;357:266-281.
- 3 Thorne J, Campbell MJ: The vitamin D receptor in cancer. *Proc Nutr Soc* 2008;67:115-127.
- 4 Salazar-Martinez E, Lazcano-Ponce E, Sanchez-Zamorano LM, Gonzalez-Lira G, Escudero-DE Los Rios P, Hernandez-Avila M: Dietary factors and endometrial cancer risk. Results of a case-control study in Mexico. *Int J Gynecol Cancer* 2005;15:938-945.
- 5 Stokes CS, Lammert F: Vitamin D supplementation: less controversy, more guidance needed. *F1000Res* 2016;5:

- 6 Zhu J, DeLuca HF: Vitamin D 25-hydroxylase - Four decades of searching, are we there yet? Arch Biochem Biophys 2012;523:30-36.
- 7 Haussler MR, Norman AW: Chromosomal receptor for a vitamin D metabolite. Proc Natl Acad Sci U S A 1969;62:155-162.
- 8 Wang Y, Zhu J, DeLuca HF: Where is the vitamin D receptor? Arch Biochem Biophys 2012;523:123-133.
- 9 Cheung FS, Lovicu FJ, Reichardt JK: Current progress in using vitamin D and its analogs for cancer prevention and treatment. Expert Rev Anticancer Ther 2012;12:811-837.
- 10 Krishnan AV, Trump DL, Johnson CS, Feldman D: The role of vitamin D in cancer prevention and treatment. Endocrinol Metab Clin North Am 2010;39:401-418.
- 11 Fajol A, Honisch S, Zhang B, Schmidt S, Alkahtani S, Alarifi S, Lang F, Stournaras C, Foller M: Fibroblast growth factor (Fgf) 23 gene transcription depends on actin cytoskeleton reorganization. FEBS Lett 2016;590:705-715.
- 12 Young MR, Lozano Y: Inhibition of tumor invasiveness by 1alpha,25-dihydroxyvitamin D3 coupled to a decline in protein kinase A activity and an increase in cytoskeletal organization. Clin Exp Metastasis 1997;15:102-110.
- 13 Ramirez AM, Wongtrakool C, Welch T, Steinmeyer A, Zugel U, Roman J: Vitamin D inhibition of pro-fibrotic effects of transforming growth factor beta1 in lung fibroblasts and epithelial cells. J Steroid Biochem Mol Biol 2010;118:142-150.
- 14 Brackman D, Trydal T, Lillehaug JR, Aarskog D: Reorganization of the cytoskeleton and morphological changes induced by 1,25-dihydroxyvitamin D3 in C3H/10T1/2 mouse embryo fibroblasts: relation to inhibition of proliferation. Exp Cell Res 1992;201:485-493.
- 15 Papakonstanti EA, Stournaras C: Tumor necrosis factor-alpha promotes survival of opossum kidney cells via Cdc42-induced phospholipase C-gamma1 activation and actin filament redistribution. Mol Biol Cell 2004;15:1273-1286.
- 16 Moustakas A, Heldin CH: Dynamic control of TGF-beta signaling and its links to the cytoskeleton. FEBS Lett 2008;582:2051-2065.
- 17 Konstantinidis G, Moustakas A, Stournaras C: Regulation of myosin light chain function by BMP signaling controls actin cytoskeleton remodeling. Cell Physiol Biochem 2011;28:1031-1044.
- 18 Papakonstanti EA, Kampa M, Castanas E, Stournaras C: A rapid, nongenomic, signaling pathway regulates the actin reorganization induced by activation of membrane testosterone receptors. Mol Endocrinol 2003;17:870-881.
- 19 Castoria G, Lombardi M, Barone MV, Bilancio A, Di Domenico M, De Falco A, Varricchio L, Bottero D, Nanayakkara M, Migliaccio A, Auricchio F: Rapid signalling pathway activation by androgens in epithelial and stromal cells. Steroids 2004;69:517-522.
- 20 Kirsch T, Beese M, Wyss K, Klinge U, Haller H, Haubitz M, Fiebeler A: Aldosterone modulates endothelial permeability and endothelial nitric oxide synthase activity by rearrangement of the actin cytoskeleton. Hypertension 2013;61:501-508.
- 21 Schmidt S, Schneider S, Yang W, Liu G, Schmidt EM, Schmid E, Mia S, Brucker S, Stournaras C, Wallwiener D, Brosens JJ, Lang F: TGFbeta1 and SGK1-sensitive store-operated Ca2+ entry and Orai1 expression in endometrial Ishikawa cells. Mol Hum Reprod 2014;20:139-147.
- 22 Papakonstanti EA, Emmanouel DS, Gravanis A, Stournaras C: Na+/Pi co-transport alters rapidly cytoskeletal protein polymerization dynamics in opossum kidney cells. Biochem J 1996;315:241-247.
- 23 Theodoropoulos PA, Stournaras C, Stoll B, Markogiannakis E, Lang F, Gravanis A, Haussinger D: Hepatocyte swelling leads to rapid decrease of the G-/total actin ratio and increases actin mRNA levels. FEBS Lett 1992;311:241-245.
- 24 Papakonstanti EA, Vardaki EA, Stournaras C: Actin cytoskeleton: a signaling sensor in cell volume regulation. Cell Physiol Biochem 2000;10:257-264.
- 25 Stournaras C, Gravanis A, Margioris AN, Lang F: The actin cytoskeleton in rapid steroid hormone actions. Cytoskeleton (Hoboken) 2014;71:285-293.
- 26 Pollard TD, Cooper JA: Actin, a central player in cell shape and movement. Science 2009;326:1208-1212.
- 27 Gu S, Papadopoulou N, Nasir O, Foller M, Alevizopoulos K, Lang F, Stournaras C: Activation of membrane androgen receptors in colon cancer inhibits the prosurvival signals Akt/bad in vitro and in vivo and blocks migration via vinculin/actin signaling. Mol Med 2011;17:48-58.

- 28 Schmidt EM, Schmid E, Munzer P, Hermann A, Eylich AK, Russo A, Walker B, Gu S, vom Hagen JM, Faggio C, Schaller M, Foller M, Schols L, Gawaz M, Borst O, Storch A, Stournaras C, Lang F: Chorein sensitivity of cytoskeletal organization and degranulation of platelets. *FASEB J* 2013;27:2799-2806.
- 29 Hatzoglou A, Kampa M, Kogia C, Charalampopoulos I, Theodoropoulos PA, Anezinis P, Dambaki C, Papakonstanti EA, Stathopoulos EN, Stournaras C, Gravanis A, Castanas E: Membrane androgen receptor activation induces apoptotic regression of human prostate cancer cells in vitro and in vivo. *J Clin Endocrinol Metab* 2005;90:893-903.
- 30 Leadsham JE, Kotiadis VN, Tarrant DJ, Gourlay CW: Apoptosis and the yeast actin cytoskeleton. *Cell Death Differ* 2010;17:754-762.
- 31 Foller M, Hermann A, Gu S, Alesutan I, Qadri SM, Borst O, Schmidt EM, Schiele F, vom Hagen JM, Saft C, Schols L, Lerche H, Stournaras C, Storch A, Lang F: Chorein-sensitive polymerization of cortical actin and suicidal cell death in chorea-acanthocytosis. *FASEB J* 2012;26:1526-1534.
- 32 Bosco EE, Mulloy JC, Zheng Y: Rac1 GTPase: a "Rac" of all trades. *Cell Mol Life Sci* 2009;66:370-374.
- 33 Nobes CD, Hall A: Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 1995;81:53-62.
- 34 Small JV, Stradal T, Vignal E, Rottner K: The lamellipodium: where motility begins. *Trends Cell Biol* 2002;12:112-120.
- 35 Parri M, Chiarugi P: Rac and Rho GTPases in cancer cell motility control. *Cell Commun Signal* 2010;8:23.
- 36 Vega FM, Ridley AJ: Rho GTPases in cancer cell biology. *FEBS Lett* 2008;582:2093-2101.
- 37 Yamaguchi H, Condeelis J: Regulation of the actin cytoskeleton in cancer cell migration and invasion. *Biochim Biophys Acta* 2007;1773:642-652.
- 38 Kamai T, Yamanishi T, Shirataki H, Takagi K, Asami H, Ito Y, Yoshida K: Overexpression of RhoA, Rac1, and Cdc42 GTPases is associated with progression in testicular cancer. *Clin Cancer Res* 2004;10:4799-4805.
- 39 Schnelzer A, Prechtel D, Knaus U, Dehne K, Gerhard M, Graeff H, Harbeck N, Schmitt M, Lengyel E: Rac1 in human breast cancer: overexpression, mutation analysis, and characterization of a new isoform, Rac1b. *Oncogene* 2000;19:3013-3020.
- 40 Yoshida T, Zhang Y, Rivera Rosado LA, Chen J, Khan T, Moon SY, Zhang B: Blockade of Rac1 activity induces G1 cell cycle arrest or apoptosis in breast cancer cells through downregulation of cyclin D1, survivin, and X-linked inhibitor of apoptosis protein. *Mol Cancer Ther* 2010;9:1657-1668.
- 41 Bid HK, Roberts RD, Manchanda PK, Houghton PJ: RAC1: an emerging therapeutic option for targeting cancer angiogenesis and metastasis. *Mol Cancer Ther* 2013;12:1925-1934.
- 42 Kallergi G, Agelaki S, Markomanolaki H, Georgoulas V, Stournaras C: Activation of FAK/PI3K/Rac1 signaling controls actin reorganization and inhibits cell motility in human cancer cells. *Cell Physiol Biochem* 2007;20:977-986.
- 43 Goley ED, Welch MD: The ARP2/3 complex: an actin nucleator comes of age. *Nat Rev Mol Cell Biol* 2006;7:713-726.
- 44 Manser E, Leung T, Salihuddin H, Zhao ZS, Lim L: A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* 1994;367:40-46.
- 45 Bagrodia S, Taylor SJ, Creasy CL, Chernoff J, Cerione RA: Identification of a mouse p21Cdc42/Rac activated kinase. *J Biol Chem* 1995;270:22731-22737.
- 46 Dharmawardhane S, Bokoch GM: Rho GTPases and leukocyte cytoskeletal regulation. *Curr Opin Hematol* 1997;4:12-18.
- 47 Daniels RH, Bokoch GM: p21-activated protein kinase: a crucial component of morphological signaling? *Trends Biochem Sci* 1999;24:350-355.
- 48 Papakonstanti EA, Stournaras C: Association of PI-3 kinase with PAK1 leads to actin phosphorylation and cytoskeletal reorganization. *Mol Biol Cell* 2002;13:2946-2962.
- 49 Gu S, Papadopoulou N, Gehring EM, Nasir O, Dimas K, Bhavsar SK, Foller M, Alevizopoulos K, Lang F, Stournaras C: Functional membrane androgen receptors in colon tumors trigger pro-apoptotic responses in vitro and reduce drastically tumor incidence in vivo. *Mol Cancer* 2009;8:114.
- 50 Papadopoulou N, Charalampopoulos I, Anagnostopoulou V, Konstantinidis G, Foller M, Gravanis A, Alevizopoulos K, Lang F, Stournaras C: Membrane androgen receptor activation triggers down-regulation of PI-3K/Akt/NF-kappaB activity and induces apoptotic responses via Bad, FasL and caspase-3 in DU145 prostate cancer cells. *Mol Cancer* 2008;7:88.