



Sphingosine kinase-1 mediates androgen-induced osteoblast cell growth

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ABSTRACT

Herein we report that the lipid kinase sphingosine kinase-1 (SphK1) is instrumental in mediating androgen-induced cell proliferation in osteoblasts. Dihydrotestosterone (DHT) triggered cell growth in steroid-deprived MC3T3 cells, which was associated with a rapid stimulation of SphK1 and activation of both Akt and ERK signaling pathways. This mechanism relied on functional androgen receptor/PI3K/Akt nongenotropic signaling as pharmacological antagonists could block SphK1 stimulation by DHT and its consequences. Finally, SphK1 inhibition not only abrogated DHT-induced ERK activation but also blocked cell proliferation, while ERK inhibition had no impact, suggesting that SphK1 was critical for DHT signaling yet independently of the ERK.

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Introduction

Androgens are essential for skeletal development and maintenance of bone metabolism throughout adult life. The central role of estrogen and androgen deficiency on bone loss provided a major stimulus to investigate the mechanism of the sex hormone actions on the skeleton and significant improvements in the clinical management of patients with osteoporosis over the last two decades. Androgens increase bone mass in specific skeletal compartments enhancing osteoblast activity but inhibiting that of osteoclasts [1].

Sphingosine 1-phosphate (S1P) has emerged as a critical lipid mediator playing a major role in cell proliferation, differentiation, apoptosis or angiogenesis [2]. The balance between S1P and its metabolic precursors ceramide and sphingosine is regarded as a switch that could determine whether a cell proliferates or undergoes apoptosis or growth arrest [3]. The master regulator of this balance is sphingosine kinase-1 (SphK1), the enzyme converting sphingosine into S1P, which has been established to be responsible for S1P effects on cell proliferation and survival [4]. S1P has been reported to trigger cell proliferation in various osteoblastic cell models [5–10]. Regarding the relationship between sex steroids and SphK1, previous reports have indicated that SphK1 mediates

estrogen signaling in breast cancer cells [11], while we demonstrate a crucial role for SphK1 in mediating the effects of androgens in prostate cancer [12] supporting the idea that SphK1 could be a crucial mediator of sex steroids biological effects in target tissues, such as the bone.

The important role of androgens on bone homeostasis and the emerging role of SphK1/S1P in regulating the effects of sex steroids spurred us to determine whether SphK1 activity could play a part in the crucial control of bone physiology exerted by androgens.

Materials and methods

Cell line and culture conditions. MC3T3-E1 mouse calvaria osteoblasts obtained from DSMZ (Germany) were cultured in α -MEM (Lonza) supplemented with 5% fetal bovine serum (FBS). For experiments, cells were plated at 10,000 cells/cm² and cultured for 48 h, then medium was replaced by serum-free medium overnight to starve the cells before the indicated treatments.

Reagents. FBS and charcoal-stripped serum (CSS) were from Hyclone (Perbio). 5 α -Dihydrotestosterone (DHT), hydroxyflutamide and bicalutamide were from Sigma. SKI-2 and LY294002 were from Calbiochem. [γ -³²P]-ATP was purchased from Perkin-Elmer and silica gel 60 high-performance TLC plates were from VWR.

Cell counting. Cells were harvested by trypsinization and resuspended in culture medium. Aliquots were diluted in Isoton II (Coulter Corp.), and 200 μ l duplicates were counted in a Coulter particle counter and averaged.

MTT assay. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye

Abbreviations: AR, androgen receptor; CSS, charcoal-stripped serum; DHT, dihydrotestosterone; PI3K, phosphoinositide-3 kinase; SFM, serum-free conditions; SphK1, sphingosine kinase-1.

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reduction assay [13]. Cells were incubated with MTT reagent (5 mg/ml) for 3 h before solubilization (20% SDS in 50% *N,N*-dimethylformamide), and formazan was quantified using a microplate reader (Bio-Rad).

SphK1 assay. SphK1 activity was quantified as described previously [14] in presence of 50 μ M sphingosine, 0.25% Triton X-100 and [γ - 32 P] ATP containing 10 mM MgCl₂. The labeled S1P was separated by thin layer chromatography on silica gel 60 with 1-butanol/ethanol/acetic acid/water (80:20:10:10, v/v) and visualized by autoradiography. Activity was expressed as picomoles of S1P formed/min/mg of protein.

Immunoblotting. Western blotting was carried out as previously described [15]. Rabbit anti-SphK1 (gift from Dr. Pitson, Adelaide, Australia), rabbit anti-Akt/phospho Ser473 (CST # 9271), rabbit anti-MAPKinase p44/42 (CST # 9106) were used as primary antibodies. Protein were detected by ECL detection system (Perbio) using a HRP-conjugated anti-rabbit or anti-mouse secondary antibody. Equal loading was confirmed by the mouse anti-tubulin antibody (Sigma).

RNA interference. Transient interference was achieved by double-stranded human SphK1-specific siRNA [16] or aleatory sequence siScr (Eurogentec). Transfection was performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Statistical analysis. The statistical significance of differences between the means was evaluated using the one-way analysis of variance (ANOVA) test. All statistical tests were two-sided and the

level of significance was set at $P < 0.05$. Calculations were performed using Instat (Graphpad Software). Densitometry quantitation was determined using the ImageJ software.

Results

DHT stimulates proliferation of MC3T3-E1

The murine calvarial MC3T3-E1 osteoblasts express high levels of AR [17,18] and are responsive to androgens [17,19,20]. As compared to CSS medium, which is deprived of all steroids inducing osteoblastic growth, the addition of DHT increased cell proliferation by approximately 90% at 96 h, which represented about 60–70% of the growth rate observed in FBS (Fig. 1A). The proliferative and prosurvival ERK1/2 and Akt pathways have been previously associated with serum and DHT-induced osteoblastic growth or survival [20–22]. Reflecting its effect on cell proliferation (Fig. 1A), DHT treatment triggered both ERK1/2 and Akt phosphorylation in steroid-deprived conditions (Fig. 1B). This effect was rapid in accordance with a nongenotropic reported mechanism [20,21].

DHT stimulates SphK1 through AR and the PI3 kinase/Akt pathway

We next investigated whether DHT could impact SphK1. Under CSS conditions, the addition of DHT was associated with an

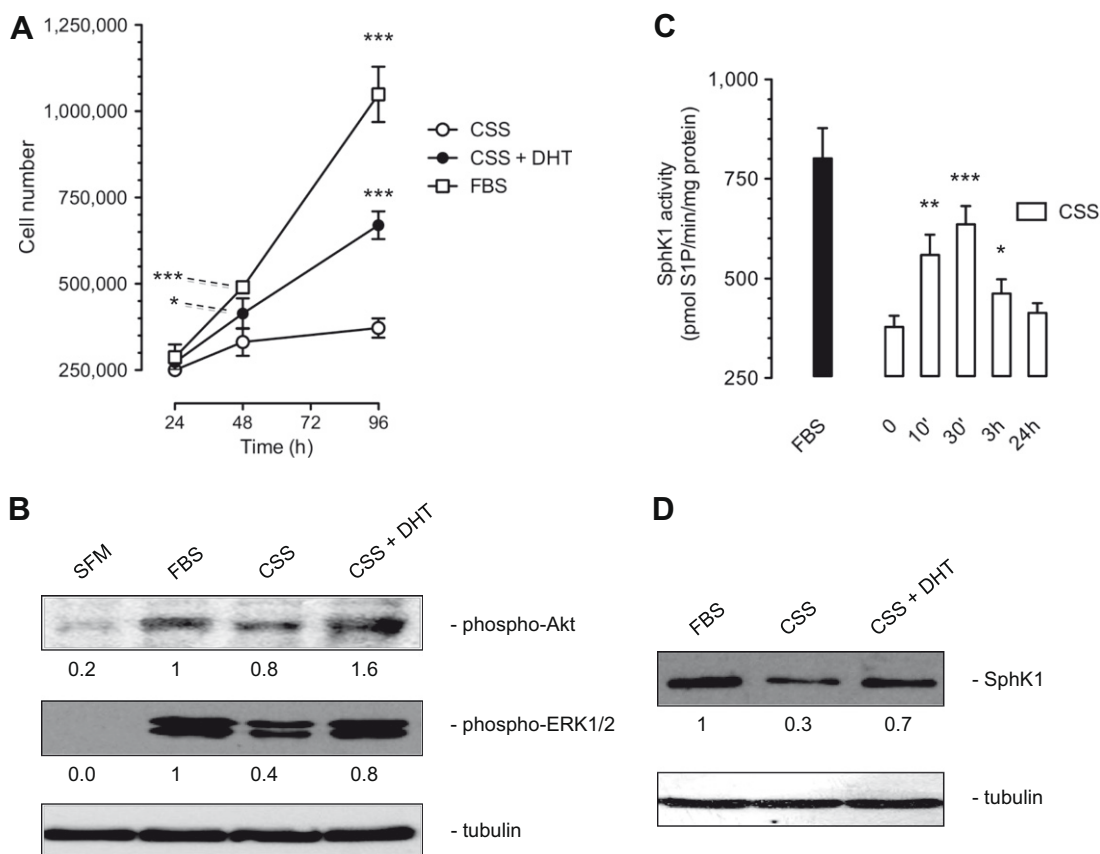


Fig. 1. DHT stimulates growth signaling pathways and SphK1 activity in MC3T3-E1 osteoblasts. MC3T3-E1 cells were incubated in presence of 5% FBS, 5% CSS, or 5% CSS in presence of 20 nM DHT. (A) Cell counting measurements were performed at the indicated times. Columns, mean of at least five independent experiments; bars, SD. The P values between the means are as follows: *** $P < 0.001$; * $P < 0.05$ (compared to CSS conditions). (B) Osteoblasts were incubated for 30 min with the indicated conditions and cell lysates were assayed for phospho-ERK and phospho-Akt expression by Western blot analysis. Similar results were obtained in three independent experiments. (C) SphK1 activity was quantified from MC3T3-E1 cells incubated under 5% CSS conditions and treated with 20 nM DHT for the indicated times. Columns, mean of at least six independent experiments; bars, SD. The P values between the means are as follows: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ (compared to T0). (D) Cell lysates from cells incubated for 30 min with or without 20 nM DHT were assayed for SphK1 expression by Western blot analysis. Similar results were obtained in three independent experiments.

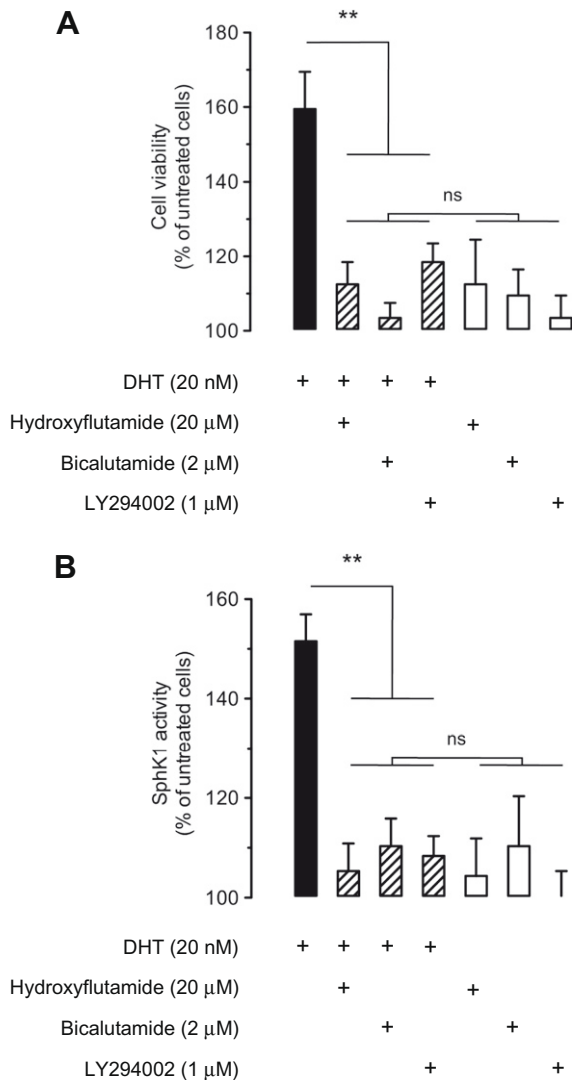


Fig. 2. Androgen receptor and PI3K inhibition blocks cell growth and SphK1 stimulation induced by DHT. MC3T3-E1 cells were incubated in presence of 5% CSS with or without hydroxyflutamide, bicalutamide or LY294002 in presence or not of 20 nM DHT for 72 h to assess cell proliferation by MTT test (A) or for 30 min to quantify SphK1 activity (B). Columns, mean of three to five independent experiments; bars, SD. The *P* values between the means are as follows: ***P* < 0.01; ns, non significant.

early but pronounced activation of SphK1 peaking at 30 min (>1.6-fold increase) after which SphK1 activity returned to basal levels (Fig. 1C). Mirroring its activity, SphK1 expression was strongly down-regulated in CSS-treated cells compared to FBS conditions whereas DHT treated cells exhibited a significantly higher expression yet not to the same extent as FBS treated cells (Fig. 1D).

Nongenotropic effects produced by androgens have been shown to involve the PI3K/Akt cascade through a direct interaction between membrane associated AR and PI3K in epithelial prostate cells [23,24] and MC3T3-E1 osteoblasts [20]. As anticipated, MC3T3-E1 cell proliferation response at 20 nM DHT was totally blunted by AR antagonists hydroxyflutamide and bicalutamide (Fig. 2A). Noteworthy, the DHT-triggered stimulation of SphK1 was also completely abolished in presence of the AR antagonists (Fig. 2B). Similarly, the inhibition of PI3K activity by LY294002 suppressed DHT-mediated cell proliferation (Fig. 2A) and SphK1 activation (Fig. 2B).

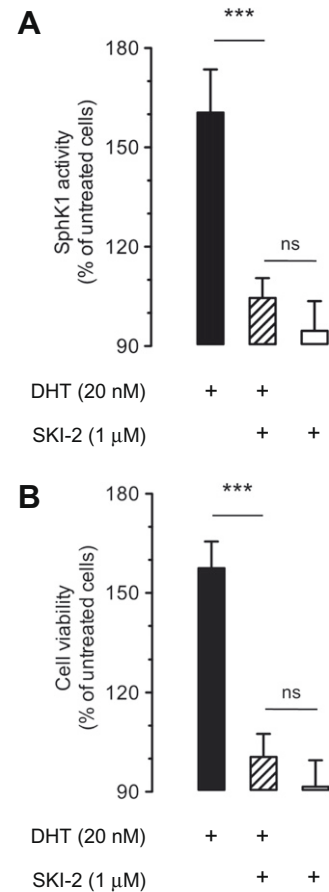


Fig. 3. SphK1 inhibition prevents cell growth induced by DHT. MC3T3-E1 cells were incubated in presence of 5% CSS with or without 1 μ M SKI-2 in presence or not of 20 nM DHT for 30 min to quantify SphK1 activity (A) or for 72 h to assess cell proliferation by MTT test (B). Columns, mean of three to five independent experiments; bars, SD. The *P* values between the means are as follows: ****P* < 0.001; ns, non significant.

SphK1 is instrumental for the proliferative effect of DHT independently of the ERK signaling

MC3T3-E1 osteoblasts were treated with SKI-2, a specific pharmacological inhibitor of SphK1 [25], to determine whether SphK1 stimulation was instrumental for the action of androgens. As previously established in other cell systems [16,26], SKI-2 displayed a dose-dependent inhibitory effect on SphK1 activity (data not shown). A low micromolar concentration of SKI-2 abrogated the stimulation of SphK1 by DHT (Fig. 3A). Cell proliferation stimulated by DHT was also completely reversed when cells were co-treated with 1 μ M SKI-2 (Fig. 3B) thus suggesting that SphK1 activity was indeed critical to mediate the effect of androgens. A similar result was obtained by SphK1 knock-down with siRNA targeted against SphK1 (data not shown).

We next studied the relationship between the SphK1 and the ERK1/2 pathways both stimulated upon androgen addition to MC3T3-E1 osteoblasts. Interestingly, SphK1 inhibition with SKI-2 (Fig. 4A) or knock-down by siRNA (data not shown) could markedly suppress ERK1/2 activation by DHT. On the contrary, the interruption of the ERK1/2 signaling pathway by the MEK inhibitor UO126 did not have any impact on SphK1-induced stimulation by DHT (Fig. 4B) indicating that MEK/ERK1/2 cascade was downstream of SphK1. More importantly and in contrast to SphK1, the MEK/ERK1/2 pathway was not essential to the growth stimulating effects ascribed to DHT since the MEK inhibitor UO126 did not

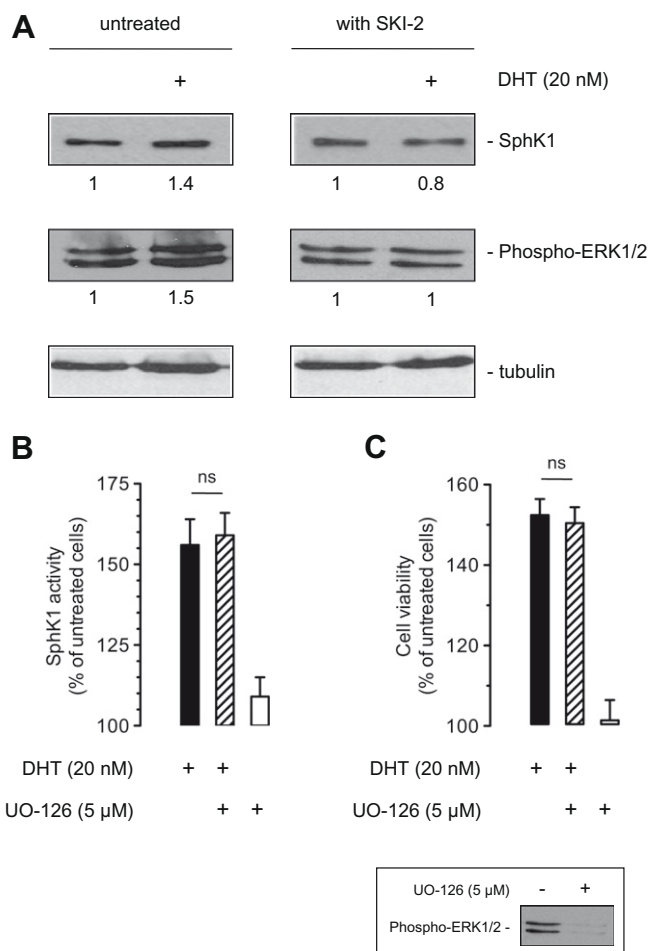


Fig. 4. The MEK/ERK cascade does not transmit DHT-induced osteoblast growth. (A) MC3T3-E1 cells were incubated in presence of 5% CSS with or without 1 μM SKI-2 in presence or not of 20 nM DHT for 30 min. Cell extracts were assayed for phospho-ERK and SphK1 expression by Western blot analysis. Similar results were obtained in three independent experiments. MC3T3-E1 cells were incubated in presence of 5% CSS with or without 5 μM UO126 in presence or not of 20 nM DHT for 30 min to quantify SphK1 activity (B) or for 72 h to assess cell proliferation by MTT test (C). *Inset*, phospho-ERK1/2 expression in MC3T3-E1 cells incubated with 5 μM UO126 for 30 min under CSS conditions. *Columns*, mean of three to five independent experiments; *bars*, SD. The *P* values between the means are as follows: ns, non significant.

block DHT-induced cell proliferation in MC3T3-E1 cells (Fig. 4C) despite the fact that ERK1/2 phosphorylation was strongly inhibited by UO126 (Fig. 4C, inset).

Discussion

Compelling evidence has implicated the SphK1/S1P signaling in mediating cell growth in response to a wide array of stimuli including growth factors, cytokines, phorbol esters [27] but also sex steroid such as estradiol [11]. Sex steroids are major mediators in maintaining bone mass and preventing osteoporosis, the most prevalent degenerative disease in developed countries [28]. Androgens have a dual function on bone metabolism since they can be converted by aromatase to estrogens to inhibit bone resorption [29] or bind to AR to elicit bone formation through osteoblast proliferation [17,19,20].

The present study is the first demonstration that SphK1 is a mediator of the proliferative effect of androgens in a noncancerous cell line through a nongenotropic AR-dependent pathway. The action of DHT not only relied on functional AR signaling and stimula-

tion of the PI3K/Akt cascade as recently reported in prostate cancer cells [23,24] and MC3T3-E1 osteoblasts [20], but also on the downstream activation of SphK1. The blockade of SphK1 activity by AR antagonists, PI3K and SphK1 inhibitors could potentially inhibit both SphK1 stimulation and proliferation induced by DHT hence supporting the instrumental role of SphK1. Similar observations have been made in the LNCaP prostate cancer cell model [12], suggesting a canonical SphK1-dependent pathway in androgen-mediated effects.

The mechanism by which SphK1 transmits cell proliferation in response to DHT remains undefined but does not appear to involve the MEK/ERK signaling pathway. Although SphK1 blockade prevented ERK1/2 activation upon DHT treatment, the inhibition of the MEK/ERK signaling did not impact cell growth thus suggesting a ERK-independent signaling, in line with observations made in AR transfected MC3T3-E1 model [30]. S1P may exert its effects either intracellularly or extracellularly in a paracrine fashion through S1P receptors present on the surface of the cell [2] as previously shown with exogenous S1P in various osteoblastic models [5–10].

We therefore conclude that through a nongenotropic pathway, SphK1 is a crucial mediator of DHT-proliferative effect on the osteoblast and a key target in bone degenerative diseases and bone metastasis.

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References

- [1] K.M. Wires, Androgens and bone growth: it's location, location, location, *Curr. Opin. Pharmacol.* 5 (2005) 626–632.
- [2] S. Spiegel, S. Milstien, Sphingosine-1-phosphate: an enigmatic signalling lipid, *Nat. Rev. Mol. Cell Biol.* 4 (2003) 397–407.
- [3] O. Cuvillier, G. Pirianov, B. Kleuser, P.G. Vanek, O.A. Coso, S. Gutkind, S. Spiegel, Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate, *Nature* 381 (1996) 800–803.
- [4] A. Olivera, T. Kohama, L. Edsall, V. Nava, O. Cuvillier, S. Poulton, S. Spiegel, Sphingosine kinase expression increases intracellular sphingosine-1-phosphate and promotes cell growth and survival, *J. Cell Biol.* 147 (1999) 545–558.
- [5] R. Dziak, B.M. Yang, B.W. Leung, S. Li, N. Marzec, J. Margarone, L. Bobek, Effects of sphingosine-1-phosphate and lysophosphatidic acid on human osteoblastic cells, *Prostaglandins Leukot. Essent. Fatty Acids* 68 (2003) 239–249.
- [6] A. Grey, X. Xu, B. Hill, M. Watson, K. Callon, I.R. Reid, J. Cornish, Osteoblastic cells express sphingolipid receptors and phosphatases and proliferate in response to sphingosine-1-phosphate, *Calcif. Tissue Int.* 74 (2004) 542–550.
- [7] R. Liu, M.C. Farach-Carson, N.J. Karin, Effects of sphingosine derivatives on MC3T3-E1 pre-osteoblasts: psychosine elicits release of calcium from intracellular stores, *Biochem. Biophys. Res. Commun.* 214 (1995) 676–684.
- [8] L.C. Carpio, E. Stephan, A. Kamer, R. Dziak, Sphingolipids stimulate cell growth via MAP kinase activation in osteoblastic cells, *Prostaglandins Leukot. Essent. Fatty Acids* 61 (1999) 267–273.
- [9] O. Kozawa, M. Niwa, H. Matsuno, H. Tokuda, M. Miwa, H. Ito, K. Kato, T. Uematsu, Sphingosine 1-phosphate induces heat shock protein 27 via p38 mitogen-activated protein kinase activation in osteoblasts, *J. Bone Miner. Res.* 14 (1999) 1761–1767.
- [10] J.M. Lyons, N.J. Karin, A role for G protein-coupled lysophospholipid receptors in sphingolipid-induced Ca^{2+} signaling in MC3T3-E1 osteoblastic cells, *J. Bone Miner. Res.* 16 (2001) 2035–2042.
- [11] O.A. Sukocheva, L. Wang, N. Albanese, S.M. Pitson, M.A. Vadas, P. Xia, Sphingosine kinase transmits estrogen signaling in human breast cancer cells, *Mol. Endocrinol.* 17 (2003) 2002–2012.
- [12] A. Dayon, L. Brizuela, C. Mazerolles, N. Pirot, N. Doumerc, L. Nogueira, M. Golzio, J. Teissié, G. Serre, P. Rischmann, B. Malavaud, O. Cuvillier, Sphingosine kinase-1 is central to androgen-regulated prostate cancer growth and survival, *PLoS One* 4 (2009) e8048.
- [13] O. Cuvillier, T. Levade, Sphingosine 1-phosphate antagonizes apoptosis of human leukemia cells by inhibiting release of cytochrome c and Smac/DIABLO from mitochondria, *Blood* 98 (2001) 2828–2836.
- [14] E. Bonhoure, D. Pchejetski, N. Aouali, H. Morjani, T. Levade, T. Kohama, O. Cuvillier, Overcoming MDR-associated chemoresistance in HL-60 acute

- myeloid leukemia cells by targeting sphingosine kinase-1, *Leukemia* 20 (2006) 95–102.
- [15] O. Cuvillier, D.S. Rosenthal, M.E. Smulson, S. Spiegel, Sphingosine 1-phosphate inhibits activation of caspases that cleave poly(ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes, *J. Biol. Chem.* 273 (1998) 2910–2916.
- [16] I. Ader, L. Brizuela, P. Bouquerel, B. Malavaud, O. Cuvillier, Sphingosine kinase 1: a new modulator of hypoxia inducible factor 1 α during hypoxia in human cancer cells, *Cancer Res.* 68 (2008) 8635–8642.
- [17] Y. Nakano, I. Morimoto, O. Ishida, T. Fujihira, A. Mizokami, A. Tanimoto, N. Yanagihara, F. Izumi, S. Eto, The receptor, metabolism and effects of androgen in osteoblastic MC3T3-E1 cells, *Bone Miner.* 26 (1994) 245–259.
- [18] R. Gruber, K. Czerwenka, F. Wolf, G.M. Ho, M. Willheim, M. Peterlik, Expression of the vitamin D receptor of estrogen and thyroid hormone receptor α - and β -isoforms, and of the androgen receptor in cultures of native mouse bone marrow and of stromal/osteoblastic cells, *Bone* 24 (1999) 465–473.
- [19] A. Masuyama, Y. Ouchi, F. Sato, T. Hosoi, T. Nakamura, H. Orimo, Characteristics of steroid hormone receptors in cultured MC3T3-E1 osteoblastic cells and effect of steroid hormones on cell proliferation, *Calcif. Tissue Int.* 51 (1992) 376–381.
- [20] H.Y. Kang, C.L. Cho, K.L. Huang, J.C. Wang, Y.C. Hu, H.K. Lin, C. Chang, K.E. Huang, Nongenomic androgen activation of phosphatidylinositol 3-kinase/Akt signaling pathway in MC3T3-E1 osteoblasts, *J. Bone Miner. Res.* 19 (2004) 1181–1190.
- [21] S. Kousteni, T. Bellido, L.I. Plotkin, C.A. O'Brien, D.L. Bodenner, L. Han, K. Han, G.B. DiGregorio, J.A. Katzenellenbogen, B.S. Katzenellenbogen, P.K. Roberson, R.S. Weinstein, R.L. Jilka, S.C. Manolagas, Nongenotropic sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity, *Cell* 104 (2001) 719–730.
- [22] Y. Engelbrecht, H. Wet, K. Horsch, C.R. Langeveldt, F.S. Hough, P.A. Hulley, Glucocorticoids induce rapid up-regulation of mitogen-activated protein kinase phosphatase-1 and dephosphorylation of extracellular signal-regulated kinase and impair proliferation in human and mouse osteoblast cell lines, *Endocrinology* 144 (2003) 412–422.
- [23] M. Sun, L. Yang, R.I. Feldman, X.M. Sun, K.N. Bhalla, R. Jove, S.V. Nicosia, J.Q. Cheng, Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85 α , androgen receptor, and Src, *J. Biol. Chem.* 278 (2003) 42992–43000.
- [24] S. Baron, M. Manin, C. Beaudoin, L. Leotoing, Y. Communal, G. Veyssiere, L. Morel, Androgen receptor mediates non-genomic activation of phosphatidylinositol 3-OH kinase in androgen-sensitive epithelial cells, *J. Biol. Chem.* 279 (2004) 14579–14586.
- [25] K.J. French, R.S. Schrecengost, B.D. Lee, Y. Zhuang, S.N. Smith, J.L. Eberly, J.K. Yun, C.D. Smith, Discovery and evaluation of inhibitors of human sphingosine kinase, *Cancer Res.* 63 (2003) 5962–5969.
- [26] D. Pchejetski, M. Golzio, E. Bonhoure, C. Calvet, N. Doumerc, V. Garcia, C. Mazerolles, P. Rischmann, J. Teissie, B. Malavaud, O. Cuvillier, Sphingosine kinase-1 as a chemotherapy sensor in prostate adenocarcinoma cell and mouse models, *Cancer Res.* 65 (2005) 11667–11675.
- [27] S. Spiegel, O. Cuvillier, L. Edsall, T. Kohama, R. Menzeleev, A. Olivera, D. Thomas, Z. Tu, J. Van Brocklyn, F. Wang, Roles of sphingosine-1-phosphate in cell growth, differentiation, and death, *Biochemistry (Mosc)* 63 (1998) 69–73.
- [28] P. Sambrook, C. Cooper, Osteoporosis, *Lancet* 367 (2006) 2010–2018.
- [29] S. Harada, G.A. Rodan, Control of osteoblast function and regulation of bone mass, *Nature* 423 (2003) 349–355.
- [30] K.M. Wiren, A.R. Toombs, X.W. Zhang, Androgen inhibition of MAP kinase pathway and Elk-1 activation in proliferating osteoblasts, *J. Mol. Endocrinol.* 32 (2004) 209–226.