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Vitamin K2 binds 17β-hydroxysteroid dehydrogenase 4 and modulates estrogen metabolism

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Abstract

Vitamin K is a cofactor for γ-glutamyl carboxylase, an enzyme that is important for blood coagulation. Recent studies have shown that vitamin K has other roles, in addition to post-transcriptional modification, such as bone metabolism and antitumoral actions; these findings have indicated that there might be unknown intracellular binding proteins that are specific for vitamin K. In this study, vitamin K-binding proteins were characterized by pull-down experiment using a chemically synthesized biotynylated vitamin K followed by mass spectrometric identification of the pull-downed components. The results indicated that 17β hydroxy steroid dehydrogenase 4, apolipoportein E, and 40S ribosomal proteins S7 and S13 might be the candidates of the vitamin K-binding proteins. Subsequent experiments showed that vitamin K2 binds 17β hydroxysteroid dehydrogenase 4 and decreases the intracellular estradiol:estrone ratio, which resulted in the inhibition of the

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amount of estrogen receptor α-binding to its target DNA. These results suggest a possible novel role for vitamin K in modulating estrogen function.

Keywords: Vitamin K; 17β-HSD4; Estrogen; Mass spectrometry

Introduction

Vitamin K is crucial for blood coagulation. The role of vitamin K as a cofactor in normal blood coagulation involves the post-translational modification of several plasma proteins including prothrombin, coagulation factors, and proteins C, S, and Z. The enzymatic carboxylation of these proteins by γ-glutamyl carboxylase depends on the vitamin K-dependent γ carboxylase, which requires the reduced hydroquinone form of vitamin K as a cofactor (Dahlback, 2000).

In addition to the aforementioned functions of vitamin K, recent studies by us and others have revealed that vitamin K plays a role in several functions that do not involve post-transcriptional modification, such as acting as a cofactor in bone metabolism and having antitumor actions in various cancer cells (Habu et al., 2004; Otsuka et al., 2004; Lamson and Plaza, 2003; Zittermann, 2001). Although the precise mechanisms that underlie these functions of vitamin K are largely unknown, these functions suggest that there might be specific intracellular binding proteins for vitamin K (Carlberg, 1999). The purpose of this study was to elucidate the intracellular binding proteins of vitamin K2.

Materials and methods

Chemicals

Vitamin K2 (menatetrenone), spacer-conjugated vitamin K2, and biotinylated vitamin K2 were synthesized chemically by a commercial vendor (Eisai, Tsukuba, Japan).

Cell lines

The human hepatoblastoma cell line HepG2 and the human embryonic kidney cell line 293T were obtained from the Riken cell bank (Tsukuba Science City, Japan) and were maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum.

Effect of vitamin K2 on des-γ-carboxy prothrombin production

To compare the effects of biotinylated vitamin K2 with those of natural (non-biotinylated) vitamin K2, des-γ-carboxy prothrombin (DCP) production by HepG2 cells was measured after the addition of the aforementioned vitamins. On the first day, 5×10^4 cells/well were seeded onto 6-well plates. The

culture medium was changed the next day for medium that contained 0, 10, or 50 μM vitamin K2. Two days later, the concentration of DCP in the medium was measured using the commercial services of SRL laboratories (Tokyo, Japan).

Precipitation and peptide sequencing

To examine the binding proteins of vitamin K2, 4×10^5 HepG2 cells were incubated for 6 h with or without 50 μM biotinylated vitamin K2. Cell extracts were prepared in 200 μl of precipitation buffer that contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin. Proteins were precipitated by the addition of 20 μl NeutrAvidin (Pierce, Rockford, IL), which is an avidin derivative with exceptionally low nonspecific binding properties. After 4 washes with precipitation buffer, the precipitated proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and were visualized with silver staining, as described previously (Ichimura et al., 1995). The protein bands that appeared only in the vitamin K2-treated sample lane were excised from the gel, and in-gel digested as described previously (Ichimura et al., 1995; Taoka et al., 2003). The peptide mixture was applied to a direct nano-flow liquid chromatography - high-resolution quadrupole time-of -flight hybrid mass spectrometer (Q-TOF; Micromass) system, and assigned to proteins by database search as described (Natsume et al., 2002).

Construction of mammalian expression plasmids for 17β-hydroxysteroid dehydrogenase 4

The mammalian expression plasmid pcDNA3-h17β-HSD4 that was used for recombinant expression of human full-length 17β-hydroxysteroid dehydrogenase 4 (17β-HSD4) was provided by Prof. Adamski (GSF-National Research Center, Neuherberg, Germany) (Adamski and Jakob, 2001). A 17β-HSD4 protein expression plasmid (pTri-HA-17β-HSD4) tagged at the N-terminal with hemagglutinin (HA) (YPYDVPDYA) was made by subcloning a polymerase chain reaction (PCR)-amplified 17β-HSD4 cDNA fragment obtained by digestion with *Sma*I and *Not*I into the pTriEx-1.1 vector (Novagen, San Diego, CA). pcDNA3-h17β-HSD4 was used as the template. The reconstructed plasmids were sequenced using an autosequencer (Applied Biosystems, Foster City, CA) to confirm appropriate gene integration. Expression of the cloned gene was confirmed by immunoblotting extracts of the transfected 293T cells with an anti-HA antibody (Roche, Mannheim, Germany).

Transfection, precipitation, and Western blot

Precipitation and Western blot analysis was performed to confirm the results of the peptide sequencing. HepG2 cells were seeded onto 6-well plates (5×10^4 cells/well). One day later, the cells were transfected with 1 μg of the pTri-HA-17β-HSD4 or pTri vector using the FuGENE 6 Transfection Reagent (Roche), according to the manufacturer's instructions. After 36 h, cells were incubated for 6 h with or without 50 μM biotinylated vitamin K2 before being harvested. Cell extracts were prepared in 200 μl of precipitation buffer, and were precipitated by the addition of 20 μl NeutrAvidin. After washing with precipitation buffer, the precipitated proteins were probed by

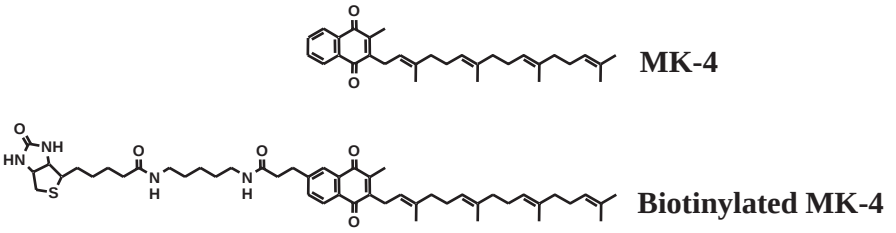

Fig. 1. Structures of chemically synthesized biotinylated vitamin K2. Biotinylated vitamin K2 was synthesized based on the structure of natural vitamin K2.

immunoblotting with an anti-HA antibody (Roche). For reference, a fraction (1/20; 10 μl) of the lysate was used as the 'input'.

Effect of vitamin K2 on intracellular concentrations of estrone and estradiol

Because 17β-HSD4 inactivates estradiol (E2) by converting it to estrone (E1) (Mindnich et al., 2004), we were able to examine the effect of vitamin K2 on 17β-HSD4 function by quantifying the concentrations of E2 and E1. HepG2 cells (5×10^4 cells/well) were seeded onto 6-well plates. One day later, the cells were incubated for 6 h with 50 μM vitamin K2. Cell extracts were then prepared in 200 μl of precipitation buffer and the concentrations of E1 and E2 were determined using the commercial services of SRL laboratories.

Estrogen receptor-mediated transcriptional activity

To examine the modulation of estrogen receptor (ER)-mediated transcriptional activity by vitamin K2, DNA binding by ER was determined using an enzyme-linked immunosorbent assay for transcriptional activity (BD TransFactor chemiluminescent kit; BD Clontech, Palo Alto, CA). Briefly, 5×10^4 HepG2 cells were treated with vitamin K2 for 6 h, and nuclear extracts were prepared using a mini-nuclear extraction method that has been described previously (Schreiber et al., 1989). The nuclear extracts were incubated in wells coated with oligonucleotides that contained the consensus binding sequences for the ER, after which the bound transcriptional factors were detected with an anti-ERα antibody. A horseradish peroxidase-conjugated secondary antibody was then used to detect the bound primary antibody, and the absorbance was measured with a spectrophotometer.

Table 1
The effect of natural and biotinylated vitamin K2 on cellular production of alpha-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP)

		MK-4 (μM)		Biotinylated MK-4 (μM)	
	0	10	50	10	50
AFP (ng/ml)	7390	8830	6040	8520	7070
PIVKAII (maU/ml)	17200	1930	1460	9140	2830

Results

Biotinylated vitamin K2 maintained γ-carboxylase cofactor activity

To examine whether the synthetic biotinylated vitamin K2 maintained γ-carboxylase cofactor activity (a well-known function of natural vitamin K2), the conversion of DCP to prothrombin was measured by incubating DCP-producing HepG2 cells with natural or biotinylated vitamin K2 (Fig. 1). As shown in Table 1, biotinylated vitamin K2 decreased the production of DCP but did not affect alpha-

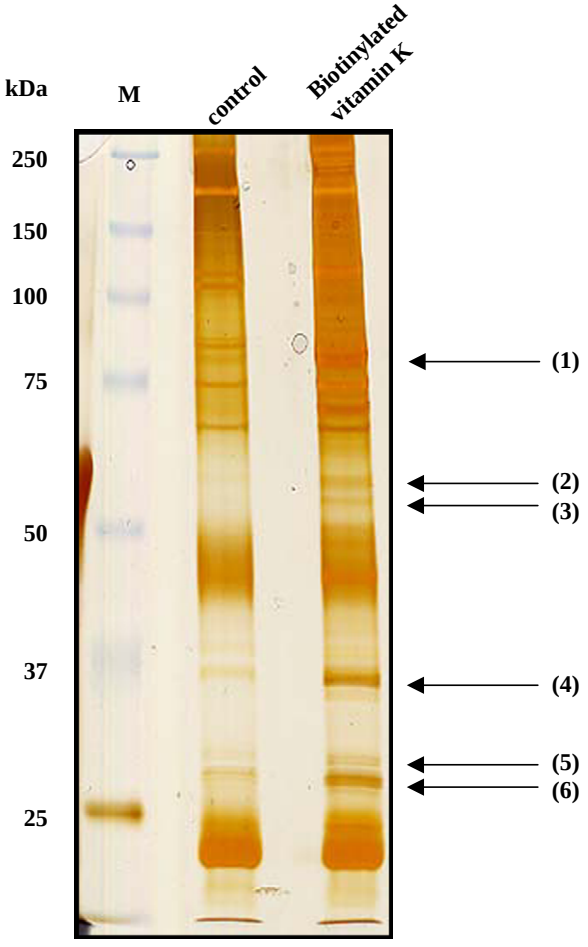

Fig. 2. The bands for mass spectrometric analysis. HepG2 cells were incubated for 6 h with or without 50 μM biotinylated vitamin K2. Cell lysates were precipitated, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized with silver staining. Six protein bands that appeared only in the samples from vitamin K2-treated cells were excised and analyzed by the LC-MS system. Arrows indicate the bands that were sequenced. The bands corresponded to the following proteins: (1) 17β-hydroxysteroid dehydrogenase 4 (17β-HSD4); (2) apolipoprotein E; (3) 17β-HSD4; (4) 17β-HSD4; (5) 40S ribosomal protein S7; and (6) 40S ribosomal protein S1.

fetoprotein (AFP) production; both of these effects resembled the effects of natural vitamin K2. Therefore, we concluded that biotinylated vitamin K2 mimicked the effects of natural vitamin K2.

Identification of vitamin K2-binding proteins

Precipitation and mass spectrometric peptide analysis were carried out to identify vitamin K2-binding proteins. We noted six protein bands that appeared only in the protein extracts from cells treated with biotinylated vitamin K2 (Fig. 2). Sequencing of each of these bands and comparison of the sequences with a protein sequence database revealed that the bands corresponded to 17β-HSD4, apolipoprotein E, 40S ribosomal protein S7 and S13. Among these proteins, 17β-HSD4 was examined further.

17β-HSD4 was identified from the bands (1), (3) and (4) (Fig. 2). The data base analysis of the MS/MS spectrum of the peptide obtained from band (1) showed that it corresponded to the sequence LLGTIYTAAEEIEAVGGK, ATSTATSGFAGAIGQK, and KVNAVFEWHITK of 17β-HSD4 at residues 50–67, 316–331, and 645–656, respectively. They covered 6.3% of the full-length 17β-HSD4 amino acids. From band (3) and (4), the peptides corresponding to the sequence

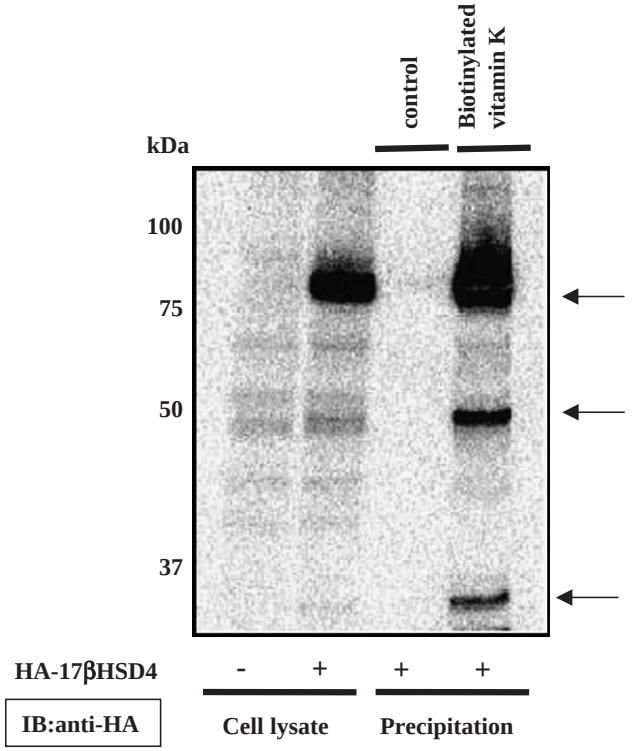

Fig. 3. Confirmation of binding of vitamin K2 and 17β-HSD4 using heterologous expression. HepG2 cells were transfected with pTri-HA-17β-HSD4 (see Methods) and were incubated with or without 50 μM biotinylated vitamin K2 for 6 h. Cell lysates were prepared and precipitated proteins were resolved by immunoblotting with an anti-HA antibody. For reference, a fraction (1/20) of the lysate from cells that did and cells that did not express 17β-HSD4 were used as a reference. Arrows indicate HA-17β-HSD4.

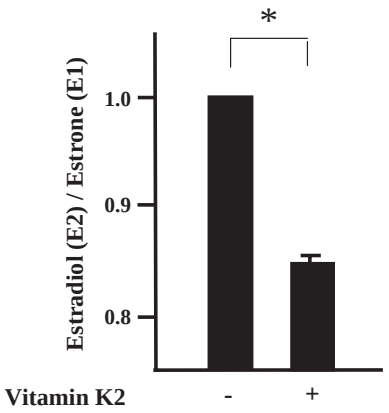

Fig. 4. Intracellular concentrations of estrone and estradiol after treatment with vitamin K2. HepG2 cells were exposed to vitamin K2 for 6 h before cell extracts were prepared to measure the concentrations of estrone (E1) and estradiol (E2). Each experiment was carried out in duplicate. *, $p < 0.05$.

GALVVVNDLGGDFKGVGK, IIMTASASGIYGNFGQANYSAAK, and LGLLGLANSLAIEGR of 17β-HSD4 at residues 33–50, 146–168, and 169–183, respectively, were obtained. They covered 5.6% and 2.0% of the full length 17β-HSD4 amino acids, respectively.

To confirm the MS/MS identification, an HA-tagged 17β-HSD4 expression plasmid was transfected into HepG2 cells, which were incubated in the presence of biotinylated vitamin K2. After precipitation, proteins were probed with an anti-HA antibody. As shown in Fig. 3, 17β-HSD4 was confirmed to bind vitamin K2. Consistent with a previous report (Carstensen et al., 1996), 17β-HSD4 was present in three bands: one band was at 80 kDa, while the other two bands were at ~46 and ~32 kDa. Each of the three forms of 17β-HSD4 bound vitamin K2.

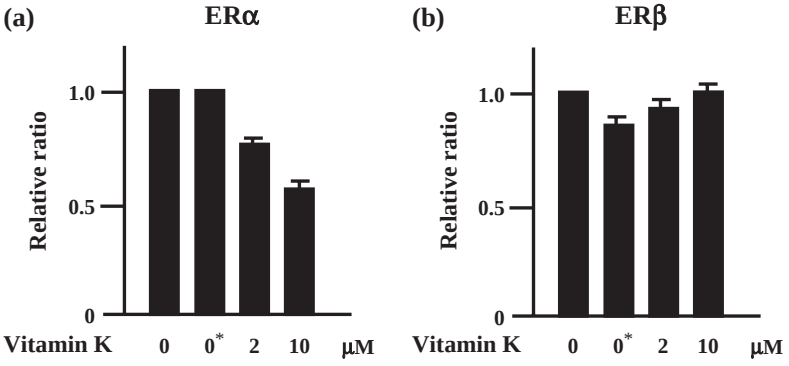

Fig. 5. Vitamin K2 decreased the amount of estrogen receptor α-DNA binding. HepG2 cells were treated with vitamin K2 for 6 h and nuclear extracts were incubated with oligonucleotides containing consensus binding sequences of the estrogen receptor. The bound transcriptional factors were incubated with an anti-ERα antibody and binding was quantified using chemiluminescence. The absorbance of the control sample was defined as 1.0. Each experiment was carried out in duplicate. *, control (vehicle; ethanol). **, $p < 0.05$.

Vitamin K2 decreased intracellular estrogen concentrations

Because 17β-HSD4 converts E2 to E1, we quantified intracellular estrogen concentrations in HepG2 cells following treatment with vitamin K2 by measuring the E2:E1 ratio. As shown in Fig. 4, 10 μM vitamin K2 decreased the E2:E1 ratio in cells by ~25%.

Vitamin K2 decreased the amount of ERα-DNA binding

Because E2 binds the ER, and the activated ER binds to the E2 response element (ERE) in the promoter regions of target genes (Buller and O'Malley, 1976), we examined whether vitamin K2 modulated the amount of ER-DNA binding. As shown in Fig. 5, vitamin K2 inhibited ERα-binding to its target DNA in a dose-dependent manner.

Discussion

In the present study, we used chemically synthesized vitamin K2 and peptide sequencing to identify novel vitamin K2-binding proteins. We demonstrated that vitamin K2 bound 17β-HSD4 and decreased the E2:E1 ratio in vitro, which resulted in an inhibition of ERα-DNA binding ability.

Vitamin K is an essential nutrient associated mainly with the clotting cascade, in which it acts as a cofactor for γ-glutamyl carboxylase. In addition, recent studies have revealed that vitamin K is also involved in bone metabolism and has an inhibitory effect on the growth of cancerous cells. Because these functions do not require that vitamin K act as a cofactor for γ-glutamyl carboxylase, it was suggested that there might be unknown proteins that bind vitamin K. Indeed, we identified several novel intracellular vitamin K2-binding proteins.

Our strategy to identify the vitamin K2-interacting molecules was based on the tandem affinity purification of the newly synthesized biotinylated vitamin K2 complex with a followed by protein identification by mass spectrometry. The method enabled us to isolate a sufficient amount of the vitamin K2 complex for characterization by nanospray tandem mass spectrometry. This affinity-tag technique coupled with mass spectrometry proved to be a useful method to detect novel protein interactions.

Among the several proteins identified as vitamin K2 binding proteins, we examined 17β-HSD4 further in this study. From the results of peptide sequences and immunoblots after biotinylated vitamin K2 precipitation, vitamin K seemed to bind each of the known three forms of 17β-HSD4. However, there remains a possibility that the smaller proteins were produced during the precipitation procedures.

17β-HSD4 is a key enzyme that regulates important redox reactions at the C17 position of steroid hormones, converting E2 to E1 (Adamski and Jakob, 2001). ERα is an E2-activated transcriptional factor that binds to the ERE in the promoter regions of target genes. We found in the present study that vitamin K2 decreased E2:E1 ratio and inhibited the amount of ERα-DNA binding in a dose-dependent manner, although the precise relationship between the binding and functional modulation of vitamin K2 by 17β-HSD4 remains to be elucidated. These results may provide new knowledge of the linkage between vitamin K and estrogen function.

Recently, vitamin K was reported to have a beneficial effect on the outcome of hepatocellular carcinoma (Habu et al., 2004; Otsuka et al., 2004). Recent studies have shown that locally elevated estrogen formation in malignant human liver tissues and cells may have a role in the development and/or

maintenance of human HCC (Castagnetta et al., 2003; Tanaka et al., 2000). Our finding using HepG2 cells, a hepatocellular carcinoma cell line, may provide one of the clues to the mechanisms of beneficial vitamin K-effect on the outcome of hepatocellular carcinoma.

On the other hand, vitamin K is also reported to be useful for osteoporosis (Zittermann, 2001). This phenomenon seems to be inconsistent to the results that vitamin K decreased E2:E1 ratio in this study. However, as recently reported, estrogen related biological functions are highly divergent in different tissues (Hurst et al., 2004; Matthews and Gustafsson, 2003; Kian Tee et al., 2004). Therefore, based on our finding that vitamin K might modulate the metabolism of estrogen, clarifying the effect of vitamin K on estrogen related function in various cells and tissues might provide some opportunities for the use of vitamin K in the treatment of estrogen-mediatgenic states.

Conclusion

We newly identified 17β-HSD4 as an intracellular vitamin K binding protein using a chemically synthesized biotynylated vitamin K followed by mass spectrometric analysis. These results may provide new knowledge of the linkage between vitamin K and estrogen function.

References

Adamski, J., Jakob, F.J., 2001. A guide to 17beta-hydroxysteroid dehydrogenases. Mol. Cell Endocrinol. 171 (1–2), 1–4.

Buller, R.E., O'Malley, B.W., 1976. The biology and mechanism of steroid hormone receptor interaction with the eukaryotic nucleus. Biochem. Pharmacol. 25 (1), 1–12.

Carlberg, C., 1999. Lipid soluble vitamins in gene regulation. Biofactors 10 (2–3), 91–97.

Carstensen, J.F., Tesdorpf, J.G., Kaufmann, M., Markus, M.M., Husen, B., Leenders, F., Jakob, F., de Launoit, Y., Adamski, J., 1996. Characterization of 17 beta-hydroxysteroid dehydrogenase IV. J. Endocrinol. 150, S3–S12.

Castagnetta, L.A., Agostara, B., Montalto, G., Polito, L., Campisi, I., Saetta, A., Itoh, T., Yu, B., Chen, S., Carruba, G., 2003. Local estrogen formation by nontumoral, cirrhotic, and malignant human liver tissues and cells. Cancer Res. 63 (16), 5041–5045.

Dahlback, B., 2000. Blood coagulation. Lancet 355 (9215), 1627–1632.

Habu, D., Shiomi, S., Tamori, A., Takeda, T., Tanaka, T., Kubo, S., Nishiguchi, S., 2004. Role of vitamin K2 in the development of hepatocellular carcinoma in women with viral cirrhosis of the liver. JAMA 292 (3), 358–361.

Hurst, A.G., Goad, D.W., Mohan, M., Malayer, J.R., 2004. Independent downstream gene expression profiles in the presence of estrogen receptor {alpha} or {beta}. Biol. Reprod. 71 (4), 1252–1261.

Ichimura, T., Uchiyama, J., Kunihiro, O., Ito, M., Horigome, T., Omata, S., Shinkai, F., Kaji, H., Isobe, T., 1995. Identification of the site of interaction of the 14-3-3 protein with phosphorylated tryptophan hydroxylase. J. Biol. Chem. 270 (48), 28515–28518.

Kian Tee, M., Rogatsky, I., Tzagarakis-Foster, C., Cvoro, A., An, J., Christy, R.J., Yamamoto, K.R., Leitman, D.C., 2004. Estradiol and selective estrogen receptor modulators differentially regulate target genes with estrogen receptors alpha and beta. Mol. Biol. Cell 15 (3), 1262–1272.

Lamson, D.W., Plaza, S.M., 2003. The anticancer effects of vitamin K. Altern. Med. Rev. 8 (3), 303–318.

Matthews, J., Gustafsson, J.A., 2003. Gustafsson, estrogen signaling: a subtle balance between ER alpha and ER beta. Mol. Interv. 3 (5), 281–292.

Mindnich, R., Moller, G., Adamski, J., 2004. The role of 17 beta-hydroxysteroid dehydrogenases. Mol. Cell Endocrinol. 218 (1–2), 7–20.

Natsume, T., Yamauchi, Y., Nakayama, H., Shinkawa, T., Yanagida, M., Takahashi, N., Isobe, T., 2002. A direct nanoflow liquid chromatography-tandem mass spectrometry system for interaction proteomics. Anal. Chem. 74 (18), 4725–4733.

Otsuka, M., Kato, N., Shao, R.X., Hoshida, Y., Ijichi, H., Koike, Y., Taniguchi, H., Moriyama, M., Shiratori, Y., Kawabe, T., Omata, M., 2004. Vitamin K2 inhibits the growth and invasiveness of hepatocellular carcinoma cells via protein kinase A activation. Hepatology 40 (1), 243–251.

Schreiber, E., Matthias, P., Muller, M.M., Schaffner, W., 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res. 17 (15), 6419.

Tanaka, K., Sakai, H., Hashizume, M., Hirohata, T., 2000. Serum testosterone: estradiol ratio and the development of hepatocellular carcinoma among male cirrhotic patients. Cancer Res. 60 (18), 5106–5110.

Taoka, M., Ichimura, T., Wakamiya-Tsuruta, A., Kubota, Y., Araki, T., Obinata, T., Isobe, T., 2003. V-1, a protein expressed transiently during murine cerebellar development, regulates actin polymerization via interaction with capping protein. J. Biol. Chem. 278 (8), 5864–5870.

Zittermann, A., 2001. Effects of vitamin K on calcium and bone metabolism. Curr. Opin. Clin. Nutr. Metab. Care 4 (6), 483–487.