Identification of simvastatin-regulated targets associated with JNK activation in DU145 human prostate cancer cell death signaling

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The results of this study show that c-Jun N-terminal kinase (JNK) activation was associated with the enhancement of docetaxel-induced cytotoxicity by simvastatin in DU145 human prostate cancer cells. To better understand the basic molecular mechanisms, we investigated simvastatin-regulated targets during simvastatin-induced cell death in DU145 cells using two-dimensional (2D) proteomic analysis. Thus, vimentin, Ras-related protein Rab-1B (RAB1B), cytoplasmic hydroxymethylglutaryl-CoA synthase (cHMGCS), thioredoxin domain-containing protein 5 (TXNDC5), heterogeneous nuclear ribonucleoprotein K (hnRNP K), N-myc downstream-regulated gene 1 (NDRG1), and isopentenyl-diphosphate Delta-isomerase 1 (IDI1) protein spots were identified as simvastatin-regulated targets involved in DU145 cell death signaling pathways. Moreover, the JNK inhibitor SP600125 significantly inhibited the upregulation of NDRG1 and IDI protein levels by combination treatment of docetaxel and simvastatin. These results suggest that NDRG1 and IDI could at least play an important role in DU145 cell death signaling as simvastatin-regulated targets associated with JNK activation. [BMB Reports 2017; 50(9): 466-471]

INTRODUCTION

Prostate cancer has become a major health issue in men due to relapse after androgen deprivation therapy, leading to incurable and highly aggressive castration-resistant prostate cancer (CRPC) (1). Epidemiological studies have shown that high blood-cholesterol levels are associated with increased risk of prostate cancer, and can be reduced by cholesterol-lowering drugs (statins) (2). In addition to cholesterol reduction, statins, such as simvastatin, lovastatin, fluvastatin, and atorvastatin, play an important role in the regulation of cell proliferation, apoptosis, autophagy, cell motility, invasion, and adhesion in various cancer cells (3, 4). Notably, it is known that castration-resistant progression in LNCap prostate cancer xenografts-bearing mice is delayed by oral administration of simvastatin (5), and the clinical use of statins after diagnosis decreases the risk of mortality in prostate cancer patients (6).

The c-Jun N-terminal kinase (JNK) belongs to the serine/threonine protein kinase family, and is activated through phosphorylation(s) by many stress stimuli, resulting in the phosphorylation of c-Jun transcription factor; this JNK activation signaling is markedly inhibited by the ATP-competitive inhibitor SP600125 (7). Previous studies have shown that JNK activation is involved in cell death signaling by simvastatin in rat C6 glioma cells (8), human leukemia cells (9), and breast cancer cells (10, 11). In addition, JNK activation is known to be associated with enhanced cytotoxicity by the combination of fluvastatin and sorafenib in melanoma cells (12), and the combined effect of statin and docetaxel has been studied in PC3 human CRPC cells (13, 14). However, the relevance of JNK activation signaling to statin-enhanced docetaxel-induced cell death has not yet been investigated. Since JNK activation signaling is related to statin-induced cell death in some cancer cell types (8-12), studies on a new JNK activation signaling network may contribute greatly to improving the chemotherapeutic effect of statins on various cancers, and remain to be investigated.

Simvastatin-regulated targets in the mitochondria fraction of rat adult brain tissue (15), acute phase of stroke in a rat embolic model (16), detergent-resistant membrane domains of rat embryonic neuronal cells (17), and mouse calvarial cells (18) and rat primary hepatocytes (19) using two-dimensional (2D) proteomic analysis after simvastatin treatment have already been known. However, simvastatin-regulated targets associated with JNK activation in DU145 human prostate cancer cell death signaling have not been reported. In this study, we investigated whether JNK activation is involved in

the enhancement of docetaxel-induced cytotoxicity caused by simvastatin in DU145 cells, identified novel targets associated with simvastatin-induced morphological changes and cell death using 2D proteomic analysis, and examined whether the simvastatin-regulated targets are implicated in the JNK activation signaling during cytotoxicity by combination of docetaxel and simvastatin, through the effect of SP600125.

RESULTS

JNK activation signaling is associated with the enhancement of docetaxel-induced cytotoxicity caused by simvastatin in DU145 cells

In this study, we used cell viability analysis to show that docetaxel-induced cytotoxicity was enhanced by simvastatin in a dose- (1, 5, and 10 μM) and time-dependent (24, 48, and 72 h) manner in DU145 human prostate cancer cells (Fig. 1A). To investigate whether JNK-dependent signaling is activated during cytotoxicity by combination treatment of docetaxel and simvastain, we performed Western blot analysis 48 h after drug treatment in DU145 cells. Fig. 1B shows that JNK phosphorylation was more upregulated by co-treatment of docetaxel and simvastatin compared to single treatment,

leading to activation of the JNK downstream target c-Jun, whereas phosphorylation of Akt and ERK was downregulated. In addition, protein levels of protein kinase Cζ (PKCζ) and IκB kinase α (IKKα), which play an important role in nuclear factor kappa B (NF-κB) activation signaling pathways (20, 21), were more downregulated by the co-treatment of docetaxel and simvastatin, compared to single treatment (Fig. 1B). These results indicate that, as well as Akt, ERK, PKCζ, and IKKα inactivation signaling pathways, JNK activation signaling is significantly associated with the enhancement of docetaxel-induced cytotoxicity caused by simvastatin.

Proteomic analysis of novel targets associated with simvastatin-induced morphological changes and cell death in DU145 cells

DU145 cell morphology was significantly altered by treatment with 10 μM simvastatin for 72 h: the attached cells had a modified cell body, and the floating cells were changed to a smaller round apoptotic phenotype, and were determined to be dead cells as a result of trypan blue staining (Fig. 2A and 2B). To identify novel targets associated with simvastatin-induced morphological changes and cell death, we performed 2D electrophoresis, CBB staining, and PDQuest image analysis
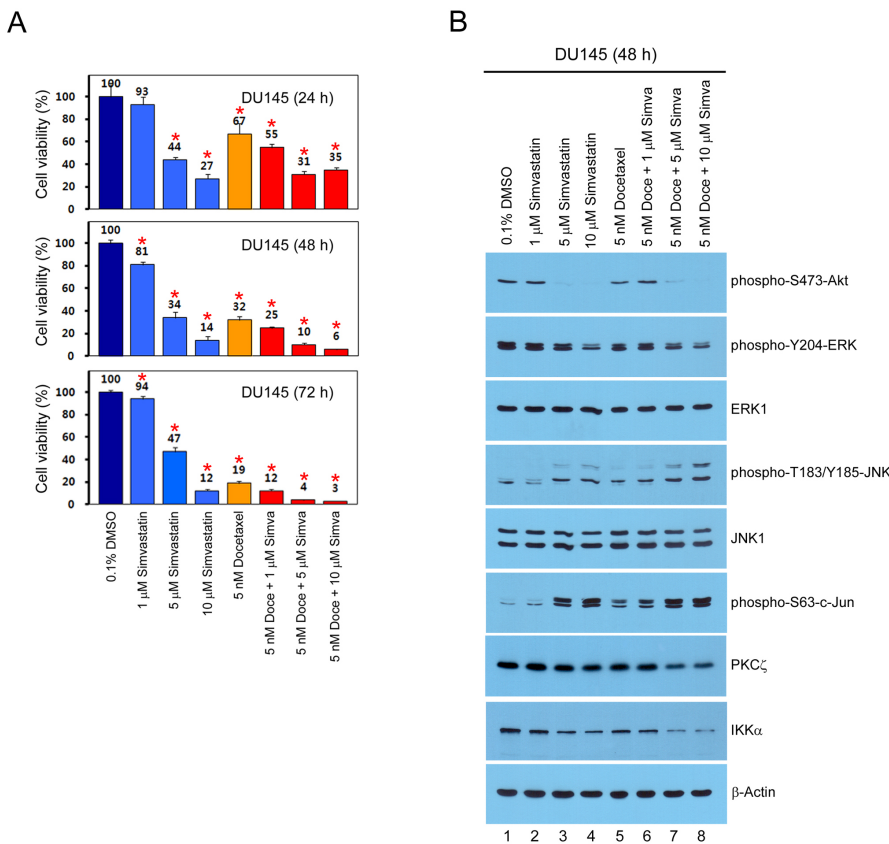

Fig. 1. JNK activation signaling is associated with the enhancement of docetaxel-induced cytotoxicity caused by simvastatin in DU145 cells. (A) DU145 cells were split in a 24-well dish, and treated with 0.1% DMSO, 1-10 μM simvastatin (Simva), and 5 nM docetaxel (Doce) for 24-72 h. The cell viability was determined by CCK-8 assay. Each bar represents the mean ± s.d. of three experiments, and the data significance was evaluated with Student's t-test, *P < 0.05. (B) Total proteins (20-50 μg each) were analyzed by Western blot using the indicated antibodies.
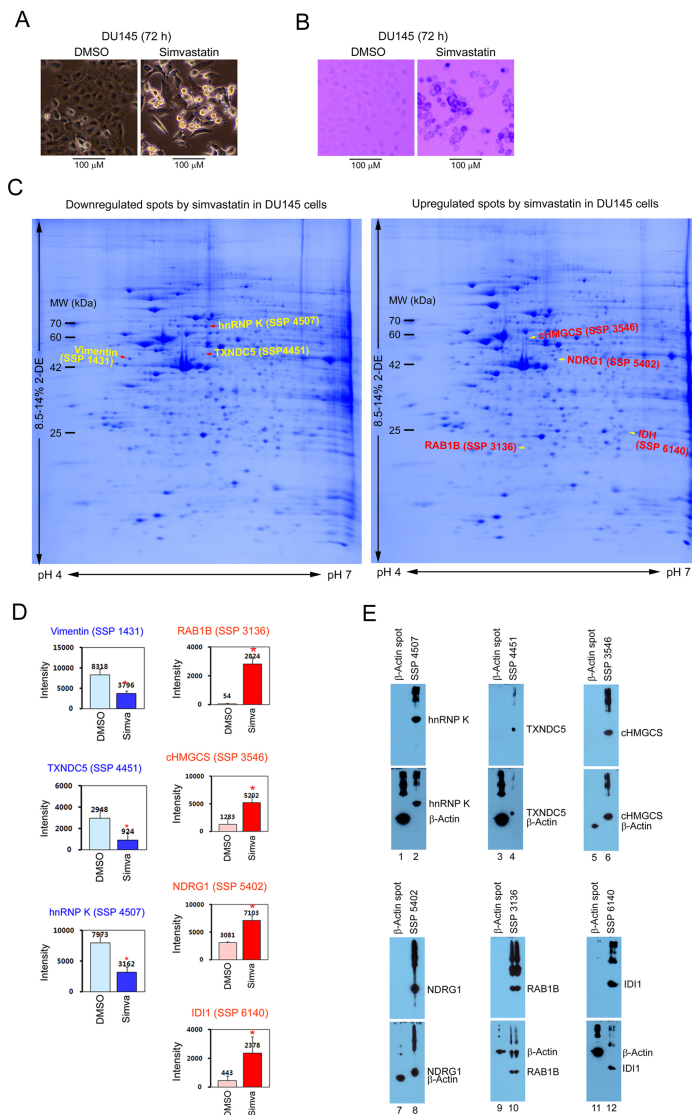
Fig. 2. Proteomic analysis of novel targets associated with simvastatin-induced morphological changes and cell death in DU145 cells. (A) The cell morphology was analyzed in a 6-well dish by phase-contrast light microscopy. (B) After trypan blue staining, dead cells were detected by bright-field phase-contrast light microscopy. (C) The 2D electrophoresis profile of simvastatin-regulated protein spots. DU145 cells were cultured on a 15 cm dish with 0.1% DMSO (control) and 10 μM simvastatin for 72 h, and were analyzed by 2D electrophoresis and CBB staining. The 3 downregulated and 4 upregulated protein spots by simvastatin were identified by MALDI-TOF/TOF mass spectrometric analysis. Each protein spot is indicated by an arrow and SSP number on a representative 2D image. (D) Quantitative analysis of simvastatin- regulated protein spots. The relative intensities of the protein spots were determined using PDQuest software. Each bar represents the mean ± s.d. of four independent experiments, and the data significance was evaluated by Student's *t*-test, *P < 0.05. (E) Verification of protein spots identified by MALDI-TOF/TOF mass spectrometric analysis. Each protein spot and a piece of β-actin spot were excised from CBB-stained 2D gels, and performed by 10% or 13.5% SDS-PAGE. The proteins were analyzed by Western blot using the indicated antibodies (upper panels). Without stripping, the membranes were reprobed by β-actin (lower panels).

72 h after 10 μM simvastatin treatment in DU145 cells. Protein spots that were more than two-fold upregulated or downregulated by simvastatin were identified using matrix-associated laser desorption/ionization (MALDI) time of flight/time of flight (TOF/TOF) mass spectrometry, and Table 1 and Fig. 2C show the statistically significant results (P < 0.05). Fig. 2D shows the quantification results for 3 simvastatin-downregulated and 4 simvastatin-upregulated spots analyzed by PDQuest software with statistical significance (*P < 0.05). To confirm the identified results, each protein spot and a piece of β-actin spot as a negative control were picked from CBB-stained 2D gels, and were analyzed by Western blot. As expected, SSP 4507, 4451, 3546, 5402, 3136, and 6140 protein spots were confirmed as heterogeneous nuclear ribonucleoprotein K

(hnRNP K), thioredoxin domain-containing protein 5 (TXNDC5), cytoplasmic hydroxymethylglutaryl-CoA synthase (cHMGCS), *N*-myc downstream-regulated gene 1 (NDRG1), Ras-related protein Rab-1B (RAB1B), and isopentenyl-diphosphate Delta-isomerase 1 (IDI1), respectively (Fig. 2E). Since vimentin (18) and NDRG1 (17) proteins are already known to be regulated by simvastatin, in this study we present five novel simvastatin-regulated targets, such as hnRNP K, TXNDC5, cHMGCS, RAB1B, and IDI1, suggesting a new role of the targets in the regulation of morphological changes and cell death caused by simvastatin.

Table 1. Simvastatin-regulated protein spots in DU145 prostate cancer cells

| Spot number	Protein identification by MALDI-TOF/TOF MS/MS	Tryptic fragment coverage/Matches	MASCOT probability score/Expect (*p*)	UniProtKB entry/Database	Theoretical Mr (Da)/p*I*
1431	Vimentin	19%/10 fragment	119/2.0e-007	B0YJC4/NCBInr	49680/5.19
3136	Ras-related protein Rab-1B (RAB1B)	17%/7 fragment	99/2.1e-005	Q9H0U4/NCBInr	22328/5.55
3546	Hydroxymethylglutaryl-CoA synthase, cytoplasmic (cHMGCS)	23%/11 fragment	93/8.7e-005	Q01581/NCBInr	57828/5.22
4451	Thioredoxin domain-containing protein 5 (TXNDC5)	13%/11 fragment	85/0.00055	Q8NBS9/NCBInr	48283/5.63
4507	Heterogeneous nuclear ribonucleoprotein K (hnRNP K)	15%/10 fragment	103/8.2e-006	P61978/NCBInr	51230/5.39
5402	Protein NDRG1 (NDRG1 *N*-myc downstream-regulated gene 1)	24%/13 fragment	82/0.00098	Q92597/NCBInr	43264/5.49
6140	Isopentenyl-diphosphate Delta-isomerase 1 (IDI1)	34%/10 fragment	128/2.6e-008	Q13907/NCBInr	26645/5.93
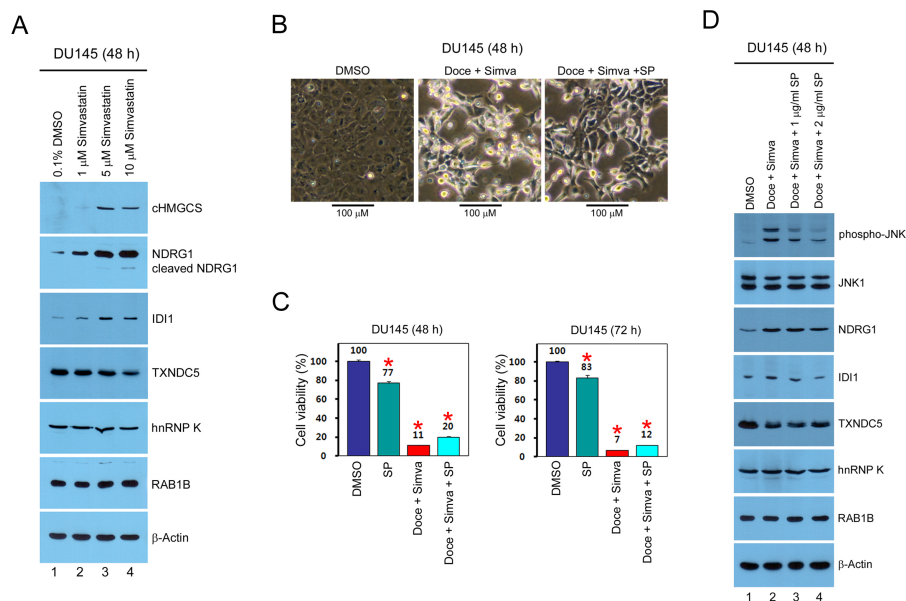
Fig. 3. Effect of JNK inhibitor SP600125 on the simvastatin- regulated targets during cytotoxicity by combination of docetaxel and simvastatin. (A) Total proteins (30-50 μg each) were analyzed by Western blot using the indicated antibodies. (B) DU145 cells were split in a 10 cm dish, and treated with 0.3% DMSO, 5 nM docetaxel, 10 μM simvastatin, and 2 μg/ml SP600125 (SP) for 48 h. The cell morphology was analyzed by phase-contrast light microscopy. (C) DU145 cells were split in a 24-well dish, and treated with 0.2% DMSO, 2 μg/ml SP600125, 5 nM docetaxel, and 5 μM simvastatin for 48 and 72 h. The cell viability was determined by CCK-8 assay. Each bar represents the mean ± s.d. of three experiments, and the data significance was evaluated by Student's t-test, *P < 0.05. (D) DU145 cells were split in a 10 cm dish, and treated with 0.3% DMSO, 5 nM docetaxel, 10 μM simvastatin, and 1-2 μg/ml SP600125 for 48 h. Total proteins (25 μg each) were analyzed by Western blot using the indicated antibodies.

Effect of JNK inhibitor SP600125 on the simvastatin-regulated targets during cytotoxicity by combination of docetaxel and simvastatin

To prove the results of the 2D proteomic analysis, we used Western blot analysis to investigate how the identified targets are regulated by simvastatin in whole cell extract of DU145 cells. The results showed that cHMGCS, NDRG1, and IDI1 protein levels were dose-dependently increased by simvastatin treatment for 48 h, whereas TXNDC5 was downregulated by simvastatin, although the molecular weight of TXNDC5 (~70 kDa) was higher than that in Fig. 2C (~48 kDa), suggesting a massive posttranslational modification of TXNDC5 in normal

proliferating DU145 cells (Fig. 3A). However for currently unknown reasons, no significant effect of simvastatin on the hnRNP K and RAB1B proteins was detected, suggesting an intracellular localization change of hnRNP K and RAB1B by simvastatin, followed by an alteration of solubility during 2D sample preparation (Fig. 3A).

Next, we used phase-contrast light microscopy and cell viability analysis to investigate how morphological changes and cytotoxicity by combination of docetaxel and simvastatin are regulated by JNK inhibitor SP600125 in DU145 cells. As a result, morphological changes and cytotoxicity due to combination of docetaxel and simvastatin were somewhat significantly, but not largely, inhibited by JNK inhibitor SP600125, indicating that both JNK-dependent and -independent signaling pathways are implicated in cell death mechanisms by docetaxel and simvastatin (Fig. 3B and 3C). Finally, we performed Western blot analysis in whole cell extract of DU145 cells to determine whether the identified targets are associated with JNK activation signaling by combination of docetaxel and simvastatin. Notably, upregulation of NDRG1 and IDI protein levels by co-treatment of docetaxel and simvastatin for 48 h was dose-dependently inhibited by SP600125, whereas downregulation of TXNDC5 had no significant effect on SP600125 (Fig. 3D). The hnRNP K and RAB1B protein levels were not altered by combination of docetaxel and simvastatin, and had no significant effect by SP600125 (Fig. 3D). These results suggest that the identified simvastatin-regulated targets may play an important role in the regulation of morphological changes and cell death by combination of docetaxel and simvastatin via JNK-dependent and -independent signal transduction pathways in various cancer cells.

DISCUSSION

Statins inhibit the mevalonate pathway, which is essential for the synthesis of various compounds, including cholesterol, through the suppression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR) (3). Simvastatin is one of the most commonly used statins, and is known to induce apoptosis in glioma (8), leukemia (9), and breast cancer cells (10, 11) through JNK activation signaling. Docetaxel is the most commonly prescribed anti-cancer agent in the standard first-line chemotherapy for metastatic CRPC (22). In this study, we demonstrated that JNK activation signaling was associated with the enhancement of docetaxel-induced cytotoxicity by simvastatin in DU145 human prostate cancer cells (Fig. 1).

It is known that downregulation of vimentin is associated with the inhibition of TGF-β1-induced epithelial-mesenchymal transition by simvastatin in DU145 prostate cancer cells (23); downregulation of RAB1B promotes the proliferation and migration of triple-negative breast cancer cells by activating TGF-β1/SMAD signaling (24); TXNDC5 overexpression is associated with non-small cell lung carcinoma (25) and CRPC tumor (26); NDRG1 mediates tumor-suppressive effects of the

iron chelator Dp44mT in prostate cancer cells through the PI3K/Akt, TGF-β and ERK pathways (27); and the intracellular localization and phosphorylation status of hnRNP K are implicated in the mechanisms that regulate androgen receptor expression and activity (28, 29). In addition, it is known that high mRNA levels of HMGCR and HMGCS1 are correlated with poor prognosis of primary breast cancer (30); IDI1 functions in the terpenomic diversity by catalyzing the conversion of isopentenyl diphosphate (IPP) into dimethylallyl diphosphate (DMAPP) in the mevalonate-isoprenoid biosynthesis (MIB) pathway (31); and downregulation of the MIB pathway is associated with the attenuation of colorectal cancer stem cell growth through the inhibition of insulin-like growth factor receptor/Akt/mammalian target of rapamycin signaling (32).

In this study, we used 2D proteomic analysis to identify five novel simvastatin-regulated targets (hnRNP K, TXNDC5, cHMGCS, RAB1B, and IDI1), and two previously known simvastatin-regulated targets (vimentin and NDRG1) (Table 1 and Fig. 2). Moreover, our results have shown that NDRG1 and IDI1 proteins are at least involved in JNK activation signaling during cytotoxicity by the combination of docetaxel and simvastatin (Fig. 3D). Taken together, we propose seven simvastatin-regulated targets that could play a crucial role in the regulation of cell morphology, death, and lipid metabolism through JNK-dependent and -independent signal transduction pathways. Future molecular and cellular studies of these simvastatin-regulated targets are expected to provide a new chemotherapeutic mechanism in various cancers.

MATERIALS AND METHODS

See supplementary information.

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CONFLICTS OF INTEREST

The authors have no conflicting financial interests.

REFERENCES

1. Katsogiannou M, Ziouziou H, Karaki S, Andrieu C, Henry de Villeneuve M and Rocchi P (2015) The hallmarks of castration-resistant prostate cancers. Cancer Treat Rev 41, 588-597
2. Krycer JR and Brown AJ (2013) Cholesterol accumulation in prostate cancer: A classic observation from a modern perspective. Biochim Biophys Acta 1835, 219-229
3. Osmak M (2012) Statins and cancer: Current and future

prospects. Cancer Lett 324, 1-12

4. Altwairgi AK (2015) Statins are potential anticancerous agents (Review). Oncol Rep 33, 1019-1039

5. Gordon JA, Midha A, Szeitz A et al (2016) Oral simvastatin administration delays castration-resistant progression and reduces intratumoral steroidogenesis of LNCaP prostate cancer xenografts. Prostate Cancer Prostatic Dis 19, 21-27

6. Yu O, Eberg M, Benayoun S et al (2014) Use of statins and the risk of death in patients with prostate cancer. J Clin Oncol 32, 5-11

7. Bogoyevitch MA and Arthur PG (2008) Inhibitors of c-Jun N-terminal kinases: JuNK no more? Biochim Biophys Acta 1784, 76-93

8. Koyuturk M, Ersoz M and Altiok N (2004) Simvastatin induces proliferation inhibition and apoptosis in C6 glioma cells via c-jun N-terminal kinase. Neurosci Lett 370, 212-217

9. Chen YJ and Chang LS (2014) Simvastatin induces NFκB/p65 down-regulation and JNK1/c-Jun/ATF-2 activation, leading to matrix metalloproteinase-9 (MMP-9) but not MMP-2 down-regulation in human leukemia cells. Biochem Pharmacol 92, 530-543

10. Koyuturk M, Ersoz M and Altiok N (2007) Simvastatin induces apoptosis in human breast cancer cells: p53 and estrogen receptor independent pathway requiring signalling through JNK. Cancer Lett 250, 220-228

11. Gopalan A, Yu W, Sanders BG and Kline K (2013) Simvastatin inhibition of mevalonate pathway induces apoptosis in human breast cancer cells via activation of JNK/CHOP/DR5 signaling pathway. Cancer Lett 329, 9-16

12. Zhang S, Doudican NA, Quay E and Orlow SJ (2011) Fluvastatin enhances sorafenib cytotoxicity in melanoma cells via modulation of AKT and JNK signaling pathways. Anticancer Res 31, 3259-3265

13. Goc A, Kochuparambil ST, Al-Husein B, Al-Azayzih A, Mohammad S and Somanath PR (2012) Simultaneous modulation of the intrinsic and extrinsic pathways by simvastatin in mediating prostate cancer cell apoptosis. BMC Cancer 12, e409

14. Chen X, Liu Y, Wu J et al (2016) Mechanistic study of inhibitory effects of atorvastatin and docetaxel in combination on prostate cancer. Cancer Genomics Proteomics 13, 151-160

15. Pienaar IS, Schallert T, Hattingh S and Daniels WM (2009) Behavioral and quantitative mitochondrial proteome analyses of the effects of simvastatin: implications for models of neural degeneration. J Neural Transm 116, 791-806

16. Campos-Martorell M, Salvador N, Monge M et al (2014) Brain proteomics identifies potential simvastatin targets in acute phase of stroke in a rat embolic model. J Neurochem 130, 301-312

17. Ponce J, Brea D, Carrascal M et al (2010) The effect of simvastatin on the proteome of detergent-resistant membrane domains: Decreases of specific proteins previously

18. related to cytoskeleton regulation, calcium homeostasis and cell fate. Proteomics 10, 1954-1965

18. Hwang R, Lee EJ, Kim MH et al (2004) Calcyclin, a Ca^{2+} ion-binding protein, contributes to the anabolic effects of simvastatin on bone. J Biol Chem 279, 21239-21247

19. Cho YE, Moon PG, Lee JE et al (2013) Integrative analysis of proteomic and transcriptomic data for identification of pathways related to simvastatin-induced hepatotoxicity. Proteomics 13, 1257-1275

20. Hirai T and Chida K (2003) Protein kinase Cζ (PKCζ): activation mechanisms and cellular functions. J Biochem 133, 1-7

21. Huang WC and Hung MC (2013) Beyond NF-κB activation: nuclear functions of IκB kinase α. J Biomed Sci 20, e3

22. Liu JJ and Zhang J (2013) Sequencing systemic therapies in metastatic castration-resistant prostate cancer. Cancer Control 20, 181-187

23. Xie F, Liu J, Li C and Zhao Y (2016) Simvastatin blocks TGF-β1-induced epithelial-mesenchymal transition in human prostate cancer cells. Oncol Lett 11, 3377-3383

24. Jiang HL, Sun HF, Gao SP et al (2015) Loss of RAB1B promotes triple-negative breast cancer metastasis by activating TGF-β/SMAD signaling. Oncotarget 6, 16352-16365

25. Vincent EE, Elder DJ, Phillips L et al (2011) Over-expression of the TXNDC5 protein in non-small cell lung carcinoma. Anticancer Res 31, 1577-1582

26. Wang L, Song G, Chang X et al (2015) The role of TXNDC5 in castration-resistant prostate cancer-involvement of androgen receptor signaling pathway. Oncogene 34, 4735-4745

27. Dixon KM, Lui GY, Kovacevic Z et al (2013) Dp44mT targets the AKT, TGF-β and ERK pathways via the metastasis suppressor NDRG1 in normal prostate epithelial cells and prostate cancer cells. Br J Cancer 108, 409-419

28. Barboro P, Salvi S, Rubagotti A et al (2014) Prostate cancer: Prognostic significance of the association of heterogeneous nuclear ribonucleoprotein K and androgen receptor expression. Int J Oncol 44, 1589-1598

29. Barboro P, Borzì L, Repaci E, Ferrari N and Balbi C (2013) Androgen receptor activity is affected by both nuclear matrix localization and the phosphorylation status of the heterogeneous nuclear ribonucleoprotein K in anti-androgen-treated LNCaP cells. PLoS One 8, e79212

30. Clendening JW, Pandyra A, Boutros PC et al (2010) Dysregulation of the mevalonate pathway promotes transformation. Proc Natl Acad Sci U S A 107, 15051-15056

31. Berthelot K, Estevez Y, Deffieux A and Peruch F (2012) Isopentenyl diphosphate isomerase: A checkpoint to isoprenoid biosynthesis. Biochimie 94, 1621-1634

32. Sharon C, Baranwal S, Patel NJ et al (2015) Inhibition of insulin-like growth factor receptor/AKT/mammalian target of rapamycin axis targets colorectal cancer stem cells by attenuating mevalonate-isoprenoid pathway in vitro and in vivo. Oncotarget 6, 15332-15347