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Knockdown of Akt isoforms by RNA silencing suppresses the growth of human prostate cancer cells *in vitro* and *in vivo*

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ABSTRACT

The serine/threonine kinase Akt has three highly homologous isoforms in mammals: Akt1, Akt2, and Akt3. Recent studies indicate that Akt is often constitutively active in many types of human malignancy. Here we investigated the expression and function of Akt isoforms in human prostatic carcinoma cells. Initially, we used Western blotting to examine Akt expression in four human prostate cancer cell lines. Next, small-interfering RNAs (siRNAs) specific for Akt isoforms were used to elucidate their role on the *in vitro* and *in vivo* growth of prostate cancer cells. Expression of Akt1 and Akt2 was detected in all cells tested, but Akt3 was expressed only in cancer cells that did not express androgen receptors. All synthetic siRNAs against Akt isoforms suppressed their expression and inhibited the growth of cancer cells *in vitro*. Furthermore, atelocollagen-mediated systemic administration of siRNAs significantly reduced the growth of tumors that had been subcutaneously xenografted. These results suggest that targeting Akt isoforms could be an effective treatment for prostate cancers.

1. Introduction

Despite advances in the detection and treatment of prostate cancer, the mortality rate remains high because current therapeutic strategies are limited in patients with advanced or recurrent disease. Hormone therapy in the form of medical or surgical castration remains the mainstay of systemic prostate cancer treatment; however, untreatable hormone-refractory prostate cancer can develop even after initial favorable responses to hormone therapy.

In androgen-independent cancer cells, the androgen receptor (AR) and its signaling remain intact, as demonstrated by the expression of prostate-specific antigen. Alterations in these cells include AR amplification, AR point mutations, and changes in the expression of AR co-regulatory proteins [1]. In addition, the AR can be activated in a ligand-independent fashion by compounds such as growth factors and cytokines [2]. The phosphatidylinositol 3-kinase (PI3K) pathway has also been implicated in prostate carcinogenesis and hormone resistance. Understanding the mechanism of androgen-independent prostate cancer development is therefore essential not only for the diagnosis but also for the provision of more effective therapy.

The PI3K pathway includes enzymes that are primarily involved in the phosphorylation of membrane inositol lipids, which mediate cellular signal transduction [3]. Akt is a well-characterized serine/threonine kinase that lies downstream of PI3K. Activated Akt promotes cell proliferation and survival by phosphorylating and modulating the activity of various transcription factors in the nucleus. Genetic and biochemical evidence suggests that aberrant activation of the PI3K/Akt pathway contributes to tumorigenesis, which is associated with a worse outcome of prostate cancer [4], with up-regulation of PI3K/Akt cascades being associated with advanced prostate cancer [5].

Three isoforms of Akt are expressed in mammals: Akt1, Akt2, and Akt3 [6]. These are encoded by three separate genes with more than 85% sequence identity. All isoforms are associated with tumorigenesis [7]. Akt1 is expressed at high levels in most tissues with the exception of kidney, liver, and spleen. Akt2 expression is tissue-dependent, being especially abundant in brown fat and, to a lesser extent, skeletal muscle and liver [8]. Akt3 is most strongly expressed in brain and testis, with lower expression levels in

Abbreviations: AR, androgen receptor; BLAST, Basic Local Alignment Search Tool; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog deleted from chromosome 10; RNAi, RNA interference; SD, standard deviation; SDS, sodium dodecyl sulfate; siGFP, siRNA specific for green fluorescent protein; siRNA, small-interfering RNA.

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intestinal organs and muscle tissues [9]. Increased expression and activation levels have been reported for Akt1 in prostate, breast, and ovarian cancers [10], for Akt2 in breast and ovarian cancers [11], and for Akt3 in prostate and breast cancers [12]. In the current study, we explore the participation of Akt in androgen-independent prostate cancer *in vitro* and *in vivo* using an isoform-specific small-interfering RNA (siRNA).

2. Materials and methods

2.1. Cells and cell culture

Four human prostate cancer cell lines were used in this study: two AR-positive cells, LNCaP and 22Rv1, and two AR-negative cells, PC-3 and DU145. The cells were maintained in RPMI1640 medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Biosource International, Camarillo, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), referred to here as complete medium, and were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.2. Western blot analysis

Cells were grown in monolayers, harvested at subconfluence, and lysed with CelLytic M cell lysis reagent (Sigma–Aldrich). Samples were centrifuged at 12,000×*g* for 15 min at 4 °C, and supernatants were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (BioRad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat dried milk (Wako, Osaka, Japan) and 1× T-TBS (25 mM Tris–HCl, 125 mM NaCl, and 0.1% Tween 20; Sigma–Aldrich) overnight at 4 °C. They were then probed with primary antibodies overnight at 4 °C, followed by horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immune complexes were visualized with the Enhanced Chemiluminescence Plus detection system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol. As an internal control, monoclonal mouse anti-β-tubulin antibody (BD Biosciences, San Jose, CA) or polyclonal goat anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used. Primary antibodies were as follows: polyclonal rabbit anti-AR antibody (N-20; Santa Cruz Biotechnology); monoclonal mouse anti-Akt1 antibody and polyclonal rabbit anti-Akt2 antibody (Cell Signaling, Beverly, MA); and polyclonal rabbit anti-Akt3 antibody (Millipore).

2.3. Design and transfection of synthetic siRNAs specific for Akt isoforms

We designed and synthesized five specific synthetic siRNAs for each Akt isoform (designated as siAkt1, siAkt2, and siAkt3, respectively). The target sequences were optimized for maximum target-gene silencing and to minimize sequence-specific cross reactivity (off-target effects) using B-Algo and siPRECISE (B-Bridge, Mountain View, CA) [13]. B-Algo is an adaptive learning algorithm that formulates rules based on complex empirical experiments, and siPRECISE is a high-performance Basic Local Alignment Search Tool (BLAST)-like search that can find sporadic mismatches in a target sequence. An siRNA specific for green fluorescent protein (siGFP) was used as a negative control. Cells were seeded at a density of 5×10^5 in a 60-mm culture dish in complete medium, and were transfected 24 h later with 1 nM siRNA using Lipofectamine 2000 Reagent (Invitrogen).

2.4. Cell-proliferation analysis

Cells (5×10^4) were seeded into 60-mm dishes in complete medium with synthetic siRNA (1 nM) and Lipofectamine 2000 Reagent and incubated for 6 days. They were recovered by treatment with 0.05% trypsin–0.53 mM ethylenediaminetetraacetic acid (EDTA; Wako) and counted with a Z1 Coulter® particle counter (Beckman Coulter, Fullerton, CA).

2.5. Xenograft model and tumor therapy

PC-3 cells (1×10^6) in 0.1 ml culture medium were injected subcutaneously at two sites in the flanks of male athymic nude mice (CLEA Japan, Tokyo, Japan). Two weeks later, tumor cell-bearing nude mice were randomly divided into eight treatment groups as follows: a no-treatment group; a group treated with siGFP with

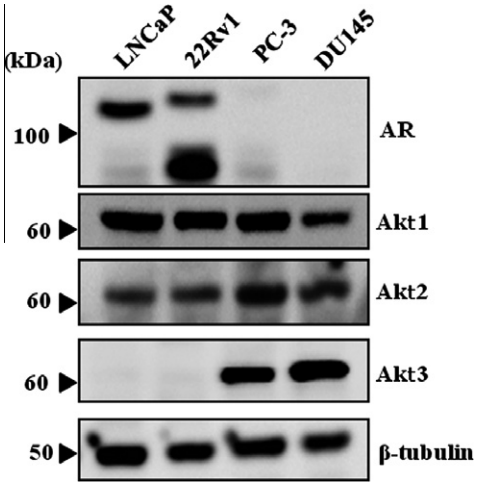

Fig. 1. Expression of Akt isoforms in human prostate cancer cells. Protein extracts from human prostatic carcinoma cells were analyzed for the expression of AR and Akt isoforms by Western blotting.

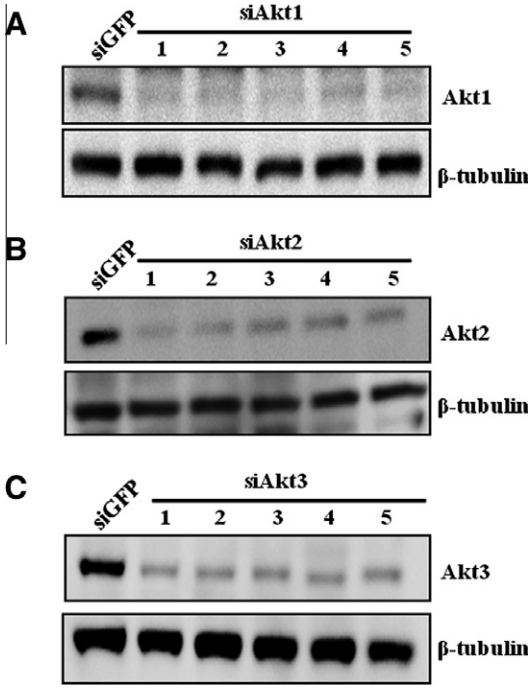

Fig. 2. RNAi effect of siAkt1 (A), siAkt2 (B), and siAkt3 (C) in human androgen-independent prostate cancer cells. PC-3 cells were transfected with 1 nM siRNAs in Lipofectamine 2000. The RNAi effect on Akt isoform protein expression was evaluated by Western blotting.

atelocollagen (AteloGene® Systemic Use; Koken, Tokyo, Japan); and six groups treated with either of two siRNAs specific for each Akt isoform complexed with atelocollagen. Each group consisted of three mice. The final concentration of siRNA was 40 μM in atelocollagen. These complexes were injected into the tail vein every 3 days. Tumor diameters were measured at regular intervals with digital calipers, and tumor volumes (mm^3) were calculated using the following formula: length × width × height × 0.523. After 15 days from the first administration of siRNA/atelocollagen complexes, PC-3 xenografts were dissected and examined for Akt isoform expression levels by Western blotting. Animal studies have been approved by the Ehime University Review Board.

2.6. Statistical analysis

The student's *t*-test was used to determine the significance of differences between the groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of Akt isoforms in human prostate cancer cells

We examined the expression of Akt isoforms in four human prostate cancer cell lines using Western blotting. Expression of

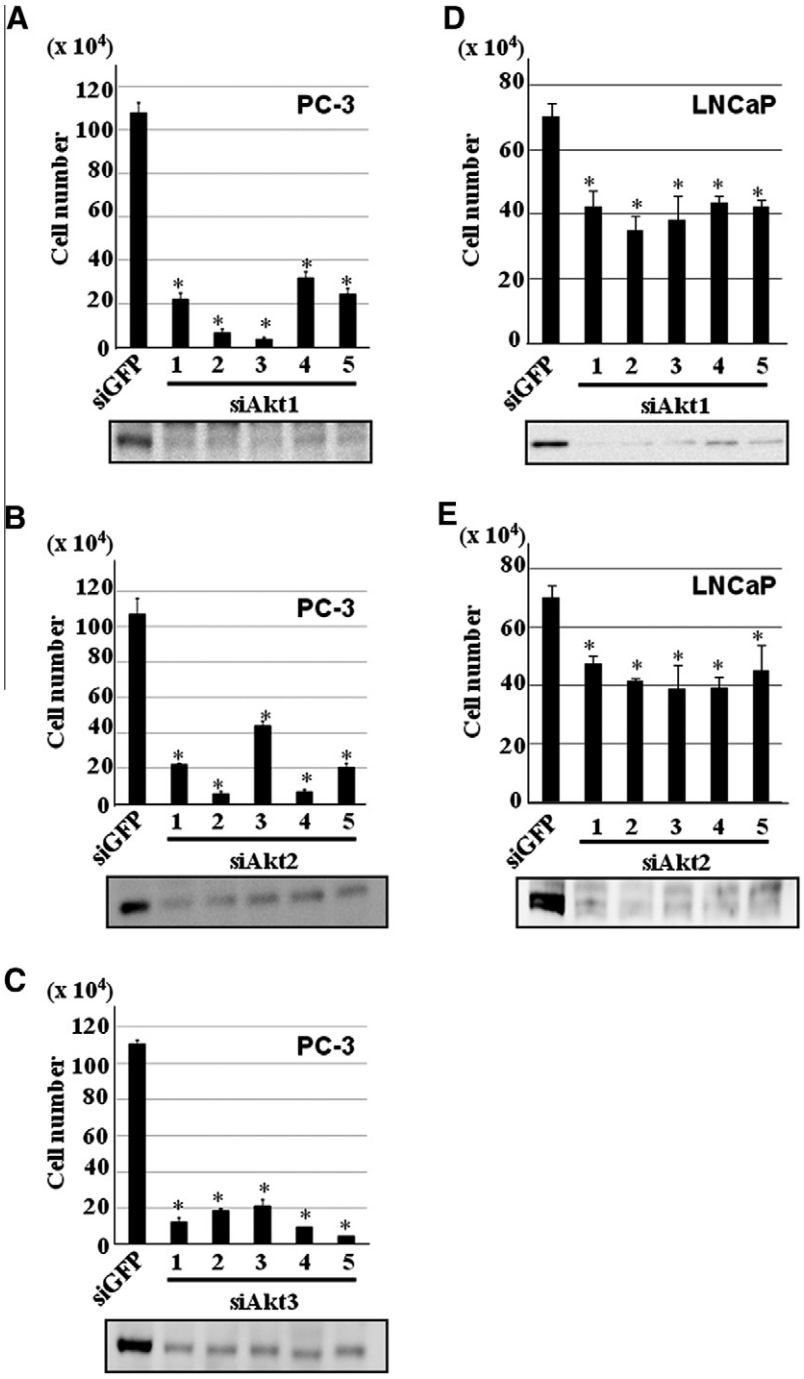

Fig. 3. Growth-inhibitory effect of siAkt1 (A and D), siAkt2 (B and E), and siAkt3 (C) on human prostate cancer cells *in vitro*. PC-3 (A–C) and LNCaP (D and E) cells were seeded in complete medium with synthetic siRNAs at the concentration of 1 nM. After 6 days, cells were recovered by treatment with 0.05% trypsin–0.53 mM EDTA and counted with a Z1 Coulter® particle counter. Bars denote standard deviation (SD) of samples performed in triplicate. * $P < 0.05$ compared with control culture.

Akt1 and Akt2 proteins was detected in all cells tested, whereas Akt3 protein was expressed only in the AR-negative cancer cell lines PC-3 and DU145 (Fig. 1).

3.2. RNA interference (RNAi) and growth-inhibitory effects of siAkt in prostate cancer cells

We used RNAi to clarify the function of Akt isoforms in the proliferation of human prostate cancer cells. Synthetic siAkt1, siAkt2, and siAkt3 were transfected into PC-3 cells expressing all Akt isoforms at a concentration of 1 nM to minimize off-target effects and interferon responses, and their effects were examined by Western blot analysis. Each siRNA reduced the expression of the corresponding Akt isoform by 36–63% (Fig. 2).

Subsequently, we tested the effects of siAkt1, siAkt2, and siAkt3 on the growth of PC-3 cells *in vitro*. The knockdown of Akt1 expression reduced the growth rate by 70–96% in PC-3 cells (Fig. 3A). siAkt2 and siAkt3 also suppressed the growth of these cells by 59–94% and 81–95%, respectively (Fig. 3B and C). Furthermore, siAkt1 and siAkt2 inhibited the androgen-dependent growth of LNCaP cells by 38–50% and 33–45%, respectively (Fig. 3D and E).

3.3. Effect of siAkt on the in vivo growth of androgen-independent prostate cancer cells

We assessed siAkt-mediated growth inhibition *in vivo* using a mouse model. We administered siAkt/atelocollagen complexes into mouse tail veins every 3 days for a total of five injections.

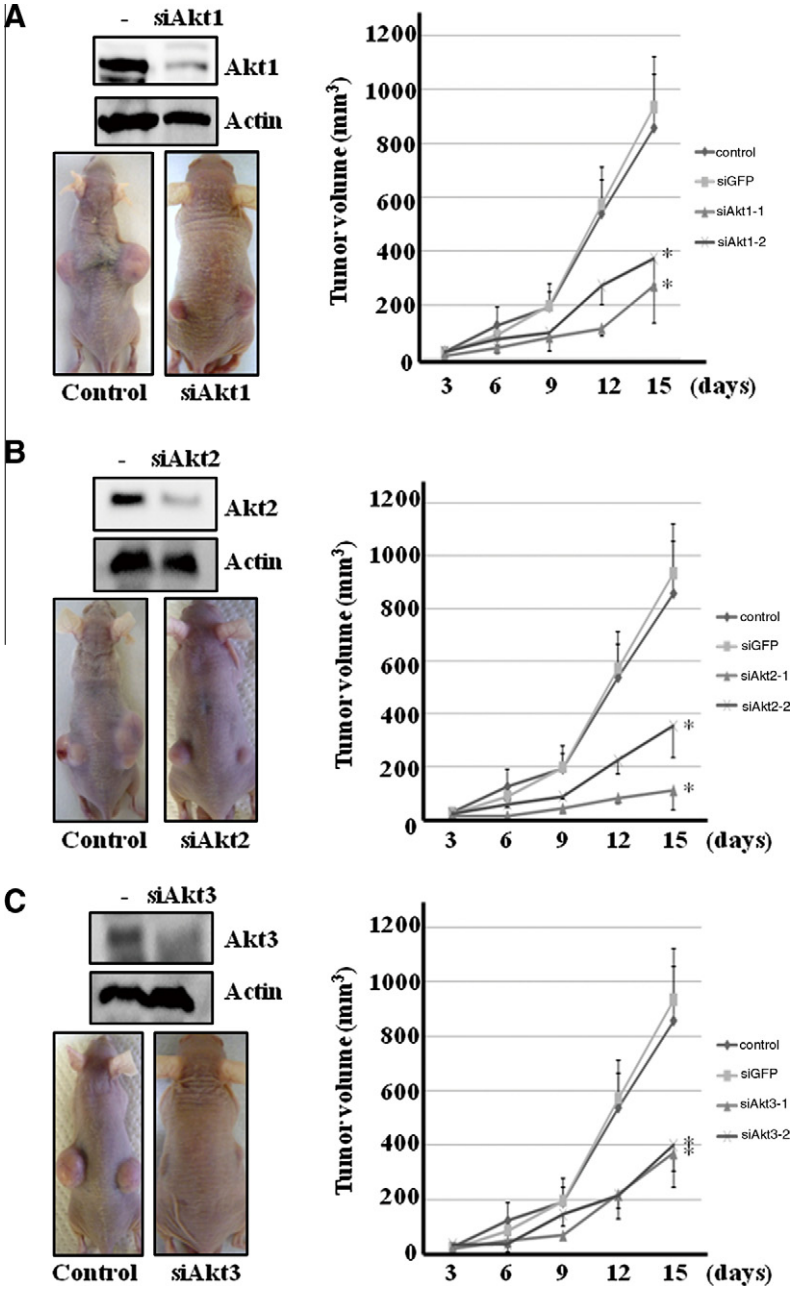

Fig. 4. Effect of siAkt1 (A), siAkt2 (B), and siAkt3 (C) with atelocollagen on the *in vivo* growth of human androgen-independent prostate cancer cells. PC-3 cells (1×10^6) were injected subcutaneously into the flank of nude mice. Synthetic siRNA (8 nmol)/atelocollagen complexes were intravenously administered into the tail vein every 3 days and tumor volume was measured at the same time. After 15 days, PC-3 xenografts were dissected and examined for expression levels of each Akt isoform by Western blotting.
* $P < 0.05$ compared with control.

We found that siAkt1, siAkt2, and siAkt3 significantly reduced the size of subcutaneously xenografted PC-3 tumors, compared with control groups. Furthermore, the expression of the corresponding Akt isoform in excised tumor tissues was notably suppressed in the groups administered siAkt/atelocollagen complex compared with the control groups (Fig. 4).

4. Discussion

The current study revealed that androgen-independent human prostate cancer cells did not express AR, but did express all Akt isoforms at high levels. To examine the oncogenic function of each Akt isoform in these cells, we designed and synthesized siAkt1, siAkt2, and siAkt3, which had RNAi effects at 1-nM concentrations, with little off-target effects. The targeting of any of the three Akt isoforms largely suppressed the growth of androgen-independent human prostate cancer cells both *in vitro* and *in vivo*. Therefore, all of the Akt isoforms appeared to be required for the androgen/AR-independent growth of human prostate cancer cells.

Akt lies downstream of PI3K, and is expressed and activated in many human malignancies. PI3K participates in various signal-transduction pathways that are implicated in many cellular responses [14]. Previous reports suggest that PI3K signaling might play a critical role, allowing prostatic cancer systems to maintain continued proliferation in low-androgen environments [15]. Knock-out mice carrying targeted deletions of PTEN which negatively regulates PI3K/Akt, demonstrate sequential neoplastic changes, namely, development of prostatic intraepithelial neoplasia, followed by invasive and metastatic adenocarcinoma. The tumor do respond to hormonal therapy but eventually progress to hormone-refractory cancer despite castration [16]. These results suggest that blockade of the PI3K/Akt pathway could be an effective approach for the treatment of androgen-independent prostate cancer.

The present study also demonstrated successful transfection of siRNA complexed with atelocollagen into xenografted tumor cells. Atelocollagen-mediated siRNA delivery has been reported to be effective in gene silencing following either local injection directly into tumors or intravenous systemic injection. This is because atelocollagen complexed with siRNA is resistant to nuclease and can be efficiently transduced into cells [17]. Previous reports showed that an siRNA can efficiently reach the target site *in vivo*, without being degraded by nucleases, if combined with an appropriate concentration of atelocollagen [18,19]. Furthermore, our recent study indicated that atelocollagen-mediated systemic administration of siRNAs specific for AR resulted in efficient inhibition of androgen-sensitive human prostate cancer cell growth without severe side effects such as lung, liver, or renal damage in nude mice [20].

In conclusion, any of the three mammalian Akt isoforms could be an appropriate target for the treatment of hormone-refractory prostate cancer. A promising future option for treatment might therefore be the combination of hormone therapy with inhibition of Akt signals.

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References

[1] M.E. Taplin, S.P. Balk, Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence, J. Cell Biochem. 91 (2004) 483–490.
[2] M.L. Zhu, N. Kyprianou, Androgen receptor and growth factor signaling cross-talk in prostate cancer cells, Endocr. Relat. Cancer 15 (2008) 841–849.
[3] I. Vivanco, C.L. Sawyers, The phosphatidylinositol 3-kinase AKT pathway in human cancer, Nat. Rev. Cancer 2 (2002) 489–501.
[4] E. Tokunaga, E. Oki, Y. Kimura, et al., Coexistence of the loss of heterozygosity at the PTEN locus and HER2 overexpression enhances the Akt activity thus leading to a negative progesterone receptor expression in breast carcinoma, Breast Cancer Res. Treat. 101 (2007) 249–257.
[5] J.I. Kreisberg, A.N. Malik, T.J. Prihoda, et al., Phosphorylation of Akt (Ser^{473}) is an excellent predictor of poor clinical outcome in prostate cancer, Cancer Res. 64 (2004) 5232–5236.
[6] E. Gonzalez, T.E. McGraw, The Akt kinases: isoform specificity in metabolism and cancer, Cell Cycle 8 (2009) 2502–2508.
[7] D.A. Altomare, J.R. Testa, Perturbations of the AKT signaling pathway in human cancer, Oncogene 24 (2005) 7455–7464.
[8] D.A. Altomare, G.E. Lyons, Y. Mitsuuchi, et al., Akt2 mRNA is highly expressed in embryonic brown fat and the AKT2 kinase is activated by insulin, Oncogene 16 (1998) 2407–2411.
[9] K. Nakatani, T. Sakaue, D.A. Thompson, et al., Identification of a human Akt3 (protein kinase B gamma) which contains the regulatory serine phosphorylation site, Biochem. Biophys. Res. Commun. 257 (1999) 906–910.
[10] M. Sun, G. Wang, J.E. Paciga, et al., AKT1/PKBα kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells, Am. J. Pathol. 159 (2001) 431–437.
[11] A. Bellacosa, D. de Feo, A.K. Godwin, et al., Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas, Int. J. Cancer 64 (1995) 280–285.
[12] K. Nakatani, D.A. Thompson, A. Barthel, et al., Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines, J. Biol. Chem. 274 (1999) 21528–21532.
[13] S. Hara, K. Nakashiro, H. Goda, et al., Role of Akt isoforms in HGF-induced invasive growth of human salivary gland cancer cells, Biochem. Biophys. Res. Commun. 370 (2008) 123–128.
[14] D.A. Fruman, S.B. Snapper, C.M. Yballe, et al., Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85α, Science 283 (1999) 393–397.
[15] D.J. Mulholland, S. Dedhar, H. Wu, et al., PTEN and GSK3β: key regulators of progression to androgen-independent prostate cancer, Oncogene 25 (2006) 329–337.
[16] S. Wang, J. Gao, Q. Lei, et al., Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer, Cancer Cell 4 (2003) 209–221.
[17] P. Mu, S. Nagahara, N. Makita, et al., Systemic delivery of siRNA specific to tumor mediated by atelocollagen: combined therapy using siRNA targeting Bcl^xL and cisplatin against prostate cancer, Int. J. Cancer 125 (2009) 2978–2990.
[18] T. Ochiya, Y. Takahama, S. Nagahara, et al., New delivery system for plasmid DNA *in vivo* using atelocollagen as a carrier material: the Minipellet, Nat. Med. 5 (1999) 707–710.
[19] Y. Minakuchi, F. Takeshita, N. Kosaka, et al., Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing *in vitro* and *in vivo*, Nucleic Acids Res. 32 (2004) e109.
[20] K. Azuma, K. Nakashiro, T. Sasaki, et al., Anti-tumor effect of small interfering RNA targeting the androgen receptor in human androgen-independent prostate cancer cells, Biochem. Biophys. Res. Commun. 391 (2010) 1075–1079.