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Membrane Fluidity, Invasiveness and Dynamic Phenotype of Metastatic Prostate Cancer Cells after Treatment with Soy Isoflavones

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Abstract Soy isoflavones represent hopeful unconventional remedies in the therapy of prostate cancer. The aim of our study was to determine the effects of genistein and daidzein on the parameters that reflect metastatic potential, membrane fluidity, invasiveness and dynamic phenotype in Matrigel of LNCaP and PC-3 prostate cancer cells. Cell viability tests, using a wide range of concentrations of soy isoflavones (6–75 μg/ml for 72 h), were conducted to determine their IC_{50} concentrations. Electron paramagnetic resonance investigations of prostate cancer cell membrane fluidity were performed at IC_{50} concentrations of genistein and daidzein (12.5 and 25 μg/ml, respectively, for 10 min). Genistein provoked significant increases in the membrane order parameter (which is reciprocally proportional to membrane fluidity) of 0.722 ± 0.006 (LNCaP), 0.753 ± 0.010 (LNCaP + genistein), 0.723 ± 0.007 (PC-3) and 0.741 ± 0.004 (PC-3 + genistein); however, no such effects were observed for daidzein. While both genistein and daidzein reduced the proliferation of prostate cancer cells at their respective IC_{50} concentrations, during the 72 h of incubation only genistein provoked effects on the dynamic phenotype and decreased invasiveness. The effect was more evident in PC-3 cells compared to LNCaP cells. Our results imply that (1) invasive activity is at least partially dependent on membrane fluidity, (2) genistein may exert its antimetastatic effects by changing the mechanical properties of prostate cancer cells and (3) daidzein should be applied at higher concentrations than genistein in order to achieve pharmacological effects.

Keywords Prostate cancer · Metastasis · Membrane fluidity · Invasiveness · Genistein · Daidzein

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Introduction

Various cells may exhibit a complex, function-related phenotype, mostly fashioned by the biophysical events occurring in their membranes (Zimmerberg and Kozlov 2006). Each phenotype develops as a result of different (patho)physiological requirements (Scott and Stainier 2003), while higher fluidity is favored in migratory cell phenotypes (Swaminathan et al. 2011). Pertinent to this, the capability of cancer cells to metastasize and the alterations of their membrane fluidity represent phenomena that were linked long ago (Deliconstantinos 1987). Some respectable data suggest that higher membrane fluidity enhances the malignancy of cancer cells in vitro (Zeisig et al. 2007). Also, it was found that the membranes of cancer cells with higher metastatic potential have a lower cholesterol/phospholipid ratio but greater unsaturated phospholipid content (Sherbet 1989).

The loss of epithelial cell polarity and adhesive contacts, followed by increased cell motility constitute a complex transformation, the epithelial–mesenchymal transition (Guarino et al. 2007). In this process of invasive cell

phenotype acquisition, membrane mechanics and protrusion formation play an important role (Yamaguchi et al. 2005; Maxfield and Tabas 2005; Sahai 2007; Gonda et al. 2010). All of these changes are critical for cancer metastasis and intertwined with their dependence on membrane fluidity. Gonda et al. (2010) showed in metastatic cancer cells that membrane fluidity increases during intravasation, reaching a peak in the blood vessels. In addition, increased membrane fluidity promotes the diffusion speed of membrane proteins, which accelerates the reaction rate between receptors and their ligands or adhesion proteins and their extracellular targets (Maxfield and Tabas 2005).

Prostate cancer (PC) is a malignancy with increased incidence in advanced age, which appears as an entity composed of androgen-dependent as well as androgen-independent cells at the time of clinical diagnosis (Heinlein and Chang 2004; Chang et al. 2009). Initially treatable when localized, PC is known to progress to a metastatic disease of the lymph nodes, bones and lungs when no effective treatment is presented (Albertsen et al. 2005). PC incidence and mortality rates vary largely worldwide, the widest margin being observed between Western countries and Asia (up to 25- and 6-fold, respectively) (Jemal et al. 2011). Such a large discrepancy has been attributed to the soy-rich diet characteristic of the Asian population. Namely, a meta-analysis showed that consumption of tofu (>34.5 g/day), combined soy food (>111.8 g/day) as well as the most abundant dietary soy isoflavones, genistein (>62 mg/day) and daidzein (>36.3 mg/day), is associated with a 40–50 % decrease in risk for PC development in Chinese men (Lee et al. 2003). However, data show that soy isoflavones may promote some estrogen-sensitive cancers (Taylor et al. 2009) and that soy milk or miso consumption may not significantly reduce the risk of PC (Hwang et al. 2009). Taking into account that genetic influences are also involved in the etiology of PC, it seems that these may determine some individualized diet recommendations (Stacewicz-Sapuntzakis et al. 2008).

The antimetastatic activity of genistein has been related to the prevention of PC cell detachment (Bergan et al. 1996) and to inhibition of the expression of matrix metalloproteinases 2 and 9 (MMP-2 and -9), the key enzymes for extracellular matrix degradation (Huang et al. 2005; Li et al. 2006; Xu et al. 2009). Daidzein has been reported not to reduce metastasis in a PC rodent model (Singh-Gupta et al. 2010), and to the best of our knowledge, no other data on metastasis-related effects of daidzein are available. It is noteworthy that some compounds seem to inhibit cancer cell invasiveness by decreasing membrane fluidity (Kido et al. 1991; McDonnel et al. 2003). Therefore, the ability of genistein and daidzein to affect the membrane fluidity and invasive activity of PC cells may be crucial for decreasing their metastatic potential.

In the present study, we examined the effects of genistein and daidzein on near-the-surface membrane fluidity (important for adhesiveness) as well as on the proliferation, invasiveness and dynamic phenotype in the extracellular matrix (Matrigel) of LNCaP (low metastatic potential, androgen-dependent) and PC-3 (high metastatic potential, androgen-independent) PC cells in order to elucidate the specific antimetastatic properties of soy isoflavones. The IC_{50} concentrations of genistein and daidzein (12.5 and 25 μg/ml, respectively) used in the experiments are about 50 % higher in relation to the ones that are bioavailable after ingestion and absorption (Birt et al. 2001; Lee et al. 2003; Jefferson et al. 2007).

Materials and Methods

Reagents and Cells

Fetal calf serum (FCS), RPMI-1640, phosphate-buffered saline (PBS), 3-(4,5-dimethyl-2-thizolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), carboxyfluorescein diacetate succinimidyl ester (CFSE) and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO). Human androgen-dependent prostate cancer LNCaP and human androgen-independent PC-3 cell lines were a kind gift from Prof. Ferdinando Nicoletti (Department of Biomedical Sciences, University of Catania, Italy). Cells were regularly maintained in HEPES-buffered RPMI-1640 medium supplemented with 10 % FCS, 2 mM L-glutamine, 0.01 % sodium pyruvate and antibiotics (culture medium) at 37 °C in a humidified atmosphere with 5 % CO_2. After standard trypsinization, cells were seeded to the desired density for viability, invasiveness and Matrigel growth determination.

Viability Test

The number of adherent viable cells was assessed using MTT and crystal violet (CV) assays. Cells (3.5×10^3/well) were exposed to a wide range of doses of genistein and daidzein (6–75 μg/ml) for 72 h, and viability was estimated. Tests were performed as described elsewhere (Mijatovic et al. 2005), while the identified IC_{50} concentration for genistein was 12.5 μg/ml and that for daidzein was 25 μg/ml.

Membrane Order Parameter Measurements

LNCaP and PC-3 cells were harvested and resuspended in 5 % FBS–RPMI-1640, adjusting the final cell density to 0.7×10^6/ml. Samples were free of cell aggregations. Aliquots of genistein and daidzein (LC Laboratories, Woburn, MA), dissolved in DMSO, were added to LNCaP

and PC-3 cells to obtain the final concentrations of 12.5 μg/ml of genistein and 25 μg/ml of daidzein. The specified IC_{50} concentrations of soy isoflavones were selected on the basis of the viability test results. The final proportion of DMSO was 0.5 % in all samples. Cells were incubated for 10 min at room temperature and labeled with membrane spin-probe 5-doxyl stearic acid (5-DS; 2-[3-carboxypentyl]-2-tridecyl-4,4-dimethyloxazolidine-3-oxyl; Molecular Probes, Junction City, OR) as described previously (Ajdžanović et al. 2010, 2011); and the electron paramagnetic resonance (EPR) spectra were recorded. The reason we did not use cells primarily cultivated with soy isoflavones lies in the previous finding that any remotely intensive cell manipulation (which harvesting certainly is) causes membrane fluidity changes (Ajdžanović et al. 2012). Samples were placed in Teflon tubes with a wall thickness of 0.025 mm and an internal diameter of 0.6 mm (Zeus Industries, Raritan, NJ) and inserted into quartz capillaries. The incubation and EPR measurements were performed in air. EPR spectra were recorded using a Varian (Palo Alto, CA) E104-A EPR spectrometer operating at X-band (9.1 GHz) and adjusted to the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW; scan range, 100 G; scan time, 4 min; time constant, 0.25 s. Temperature was controlled at 20 °C during the measurements. Spectra were recorded and analyzed using EW software (Scientific Software, Bloomington, IL). The order parameter (S), which is reciprocally proportional to fluidity, was calculated as described previously (Ajdžanović et al. 2010).

Matrigel Growth

Evaluation of growth on Matrigel® (10 mg/ml; BD Labware, Bedford, MA) was done as previously described (Krubasik et al. 2006). Cells were plated at 7×10^3/well on a reconstituted basement membrane (Matrigel) in RPMI–10 % FCS and after 4 h treated with IC_{50} doses of genistein and daidzein. Cells were incubated for 72 h and photographed by phase microscopy (Axiovert microscope; Zeiss, Oberkochen, Germany).

Transmigration Assay

To quantitatively evaluate the effect of genistein or daidzein on the in vitro invasion potential of PC-3 cells, the transmigration assay was used as previously described (Mojic et al. 2012). In brief, 1×10^5 PC-3 cells were exposed to 7.5 and 15 μg/ml of genistein or 12.5 and 25 μg/ml of daidzein for 48 h, when the number of invading cells was counted.

Cell Proliferation

The rate of cell proliferation was measured by flow-cytometric analysis of the cells labeled with CFSE exactly as described (Maksimovic-Ivanic et al. 2009).

Statistical Analysis

EPR data are presented as means ± standard deviation (SD) for at least five separate experiments. Significant differences were calculated by the nonparametric two-tailed Mann–Whitney test. Means were considered significantly different at $p < 0.05$.

Results

Both viability tests showed that genistein and daidzein significantly abrogated the growth of LNCaP and PC-3 cells with similar, highly reproducible IC_{50} concentrations (Fig. 1). Therefore, these IC_{50} concentrations (12.5 and 25 μg/ml of genistein and daidzein, respectively) were selected for further examination.

Figure 2 shows characteristic EPR spectra of LNCaP and PC-3 cells labeled with 5-DS. The values of the order parameter (calculated as presented in Fig. 2), which is reciprocally proportional to membrane fluidity, are presented in Table 1. A significant decrease in membrane fluidity was detected in both LNCaP and PC-3 cells after treatment with genistein. Genistein manifested a more pronounced effect on LNCaP cells. Daidzein did not provoke significant changes in membrane fluidity.

Figure 3 shows the effects of genistein and daidzein at IC_{50} on the dynamic phenotype of LNCaP and PC-3 cells in 2.5 D extracellular matrix, Matrigel. It can be observed that expansion and navigation through the "cracks" of degraded matrix are more pronounced in untreated PC-3 compared to LNCaP cells. Both genistein and daidzein inhibited invasive trends of PC-3 and LNCaP cells, while LNCaP treated with daidzein showed, to some extent, similar morphology to untreated cells. Morphological transformation of cells exposed to IC_{50} values of both compounds was accompanied by a loss of proliferative potential (Fig. 4a). Having in mind that PC-3 cells are highly metastatic, the influence of genistein or daidzein on their invasiveness was further evaluated by the transmigration assay. The results (Fig. 4b, c) clearly indicated that genistein, but not daidzein, strongly suppressed the invasive properties of PC-3 cells even in a twice lower dose than IC_{50}. In summary, the effects of genistein on invasive activity and the following morphology were more pronounced in comparison to those of daidzein, particularly when taking into account that daidzein was supplemented

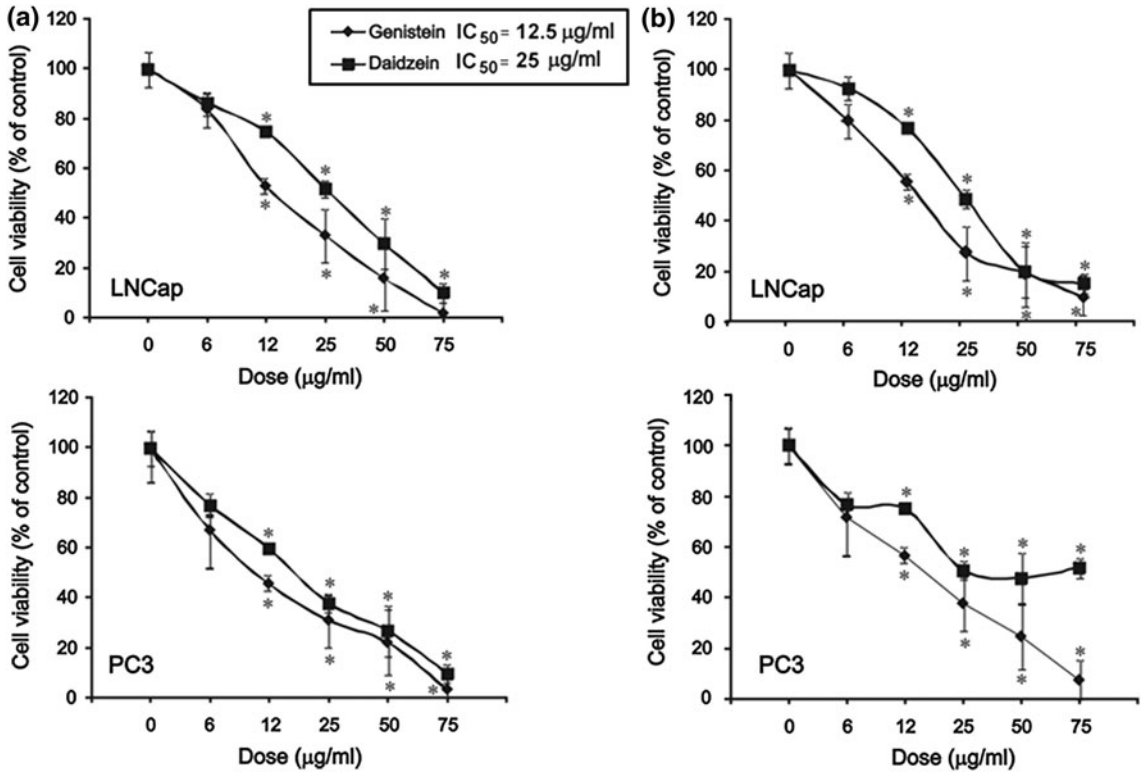

Fig. 1 Viability of LNCaP and PC-3 cells exposed to genistein or daidzein, determined by MTT (**a**) or CV (**b**) assay. * $p < 0.05$ refers to untreated cultures

at a higher nominal dose. The effects of genistein on the observed parameters were more pronounced in more aggressive PC-3 compared to LNCaP cells, while daidzein provoked similar levels of inhibition in both studied cell types.

Discussion

The invasive activity of PC cells plays a key role in metastasis occurrence and represents a major cause of treatment failure. This underlines the importance of knowing the properties that enable cancer cells to move from the prostate to other organs. Here, we showed that PC-3 cells exhibit a more invasive phenotype in comparison to LNCaP cells, which is in line with their higher metastatic potential. On the other hand, the membrane fluidity of the two cell types did not differ. This is in accordance with previous assertions that the membrane fluidity of cancer cells is not relevant until intravasation takes place (Gonda et al. 2010).

Present application of genistein, but not daidzein, resulted in a significant decrease in membrane fluidity near the surface in both LNCaP and PC-3 cells. Genistein was previously shown to exert rigidifying effects on a cancer cell model membrane (Tsuchiya et al. 2002). Our recent studies have demonstrated that genistein as well as isoflavone glucoside-rich soy extract decrease superficial membrane fluidity in erythrocytes (Ajdžanović et al. 2010, 2011). It seems that genistein dominantly intercalates into the lipid headgroup region, to some extent into the polar–apolar interface and only to a minimal degree into the hydrophobic core of the membrane (Kuzdzal et al. 2011). The genistein-provoked decrease in membrane fluidity may be coupled to increased adhesiveness (Gonda et al. 2010), lower deformability (Zicha et al. 1999) and restricted ability to navigate through the "cracks" in the extracellular matrix (Gonda et al. 2010). In addition, genistein may suppress general motility of cancer cells by making the formation of pseudopodial protrusions more energy-requiring (Gonda et al. 2010). The mechanism of such an action is intriguing. According to the structural formula, genistein contains a defined hydrophilic moiety composed of two hydroxyl groups placed close to each other on the same ring, while such a moiety is absent in daidzein. Because of this, genistein intercalates the membrane, positions itself near the surface and orients itself so that the hydrophilic moiety is immersed in the aqueous phase of the lipid bilayer, while daidzein enters deeper into the hydrophobic core of the membrane (Ajdžanović et al. 2010, 2011). Obviously, genistein decreases the entropy near the membrane surface, which results in lower membrane

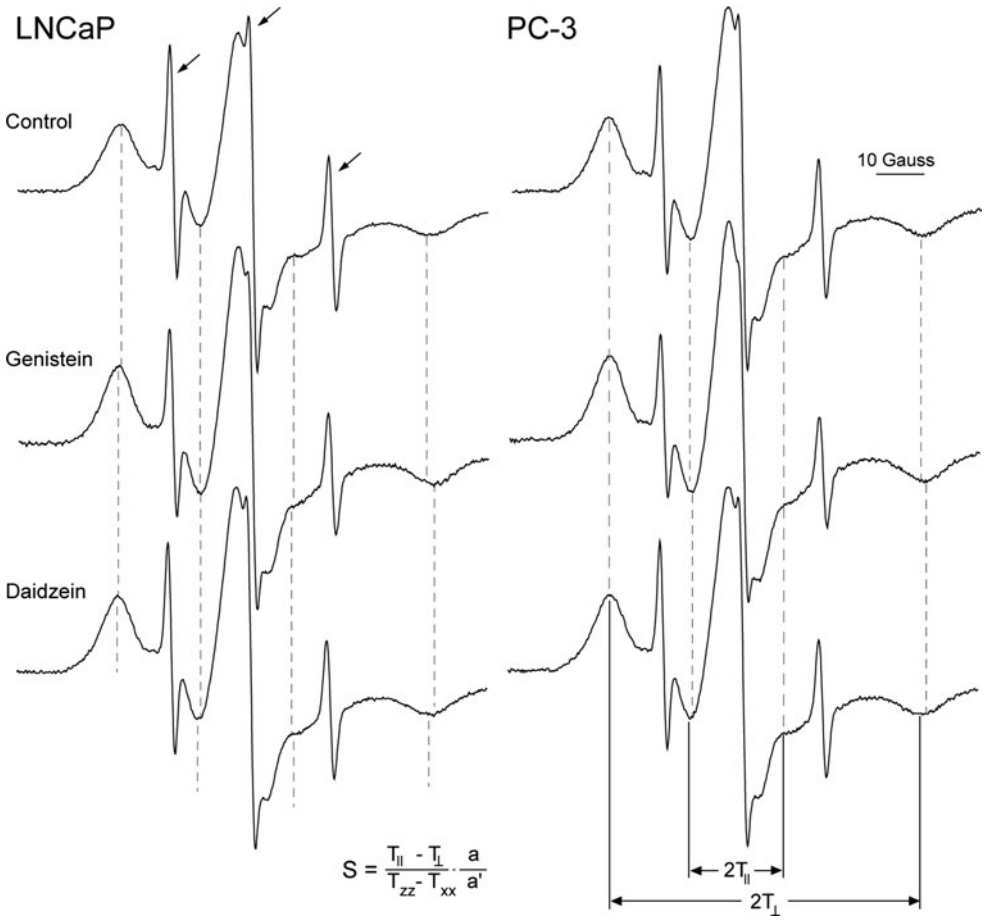

Fig. 2 Characteristic EPR spectra of metastatic LNCaP and PC-3 cells labeled with 5-DS: without the presence of soy isoflavones (control) and treated with genistein (12.5 μg/ml) or daidzein (25 μg/ml). *S* order parameter; $2T_{II}$ outer hyperfine splitting; $2T_{\perp}$ inner hyperfine splitting; *a* isotropic hyperfine coupling constant in crystal $[a = 1/3(T_{xx} + T_{yy} + T_{zz})]$; a' isotropic hyperfine coupling constant in membrane $[a' = 1/3(T_{II} + 2T_{\perp})]$; T_{xx}, T_{yy} and T_{zz}, hyperfine constants (for 5-DS they were taken to be $T_{xx} = T_{yy} = 6.1$ G, $T_{zz} = 32.4$ G) (Ajdžanović et al. 2010). *Three narrow lines* originate from the 5-DS in the solution (*arrows*)

Table 1 Order parameters (S), obtained using 5-DS, in metastatic PC cells, LNCaP and PC-3: without the presence of soy isoflavones (control) and treated with genistein or daidzein

	LNCaP (0.7×10^6 cells/ml)		PC-3 (0.7×10^6 cells/ml)	
	S	*p*	*S*	*p*
Control	0.722 ± 0.006	–	0.723 ± 0.007	–
Genistein [12.5 μg/ml (10 min)]	0.753 ± 0.010	0.046	0.741 ± 0.004	0.040
Daidzein [25 μg/ml (10 min)]	0.726 ± 0.004	n.s.	0.730 ± 0.013	n.s.

Data are shown as means ± SD. *S* values were taken to be significantly different relative to corresponding controls at $p < 0.05$. The effects of genistein on LNCaP and PC-3 cells were statistically significant ($p = 0.049$)

n.s. nonsignificant

fluidity, while the daidzein effects remain undetectable in this shallow region due to its deeper penetration. Androgen-dependent LNCaP cells seem to be more sensitive, according to the more pronounced decrease in membrane fluidity, so some effects may be receptor-mediated as well. Namely, it was suggested that LNCaP cells bear genistein-sensitive membrane androgen receptors, involved in short-term signaling and cytoskeletal rearrangements (Kampa et al. 2002; Oh et al. 2010), events that may affect membrane tension.

The effects we observed on PC cell invasiveness, even at subtoxic doses, and the dynamic phenotype in Matrigel were clearly more pronounced for genistein than daidzein. The applied (IC_{50}) concentration of genistein was more than twofold lower compared to daidzein, which makes the difference even more apparent. Considering that daidzein has been previously reported not to reduce metastasis (Singh-Gupta et al. 2010), we assume that it should be applied at higher doses to achieve clinically relevant effects. The genistein-provoked suppression of invasive activity and degradation of the extracellular matrix could be partially attributed to its effects on the expression of MMPs. The effects were more pronounced in PC-3 compared to LNCaP cells. It has been proposed that the high metastatic potential of PC-3 cells largely relies on the increased expression of MMP-9 (Aalinkeel et al. 2004), which is inhibited by genistein (Li et al. 2006). On the other hand, LNCaP cells generally show twofold lower

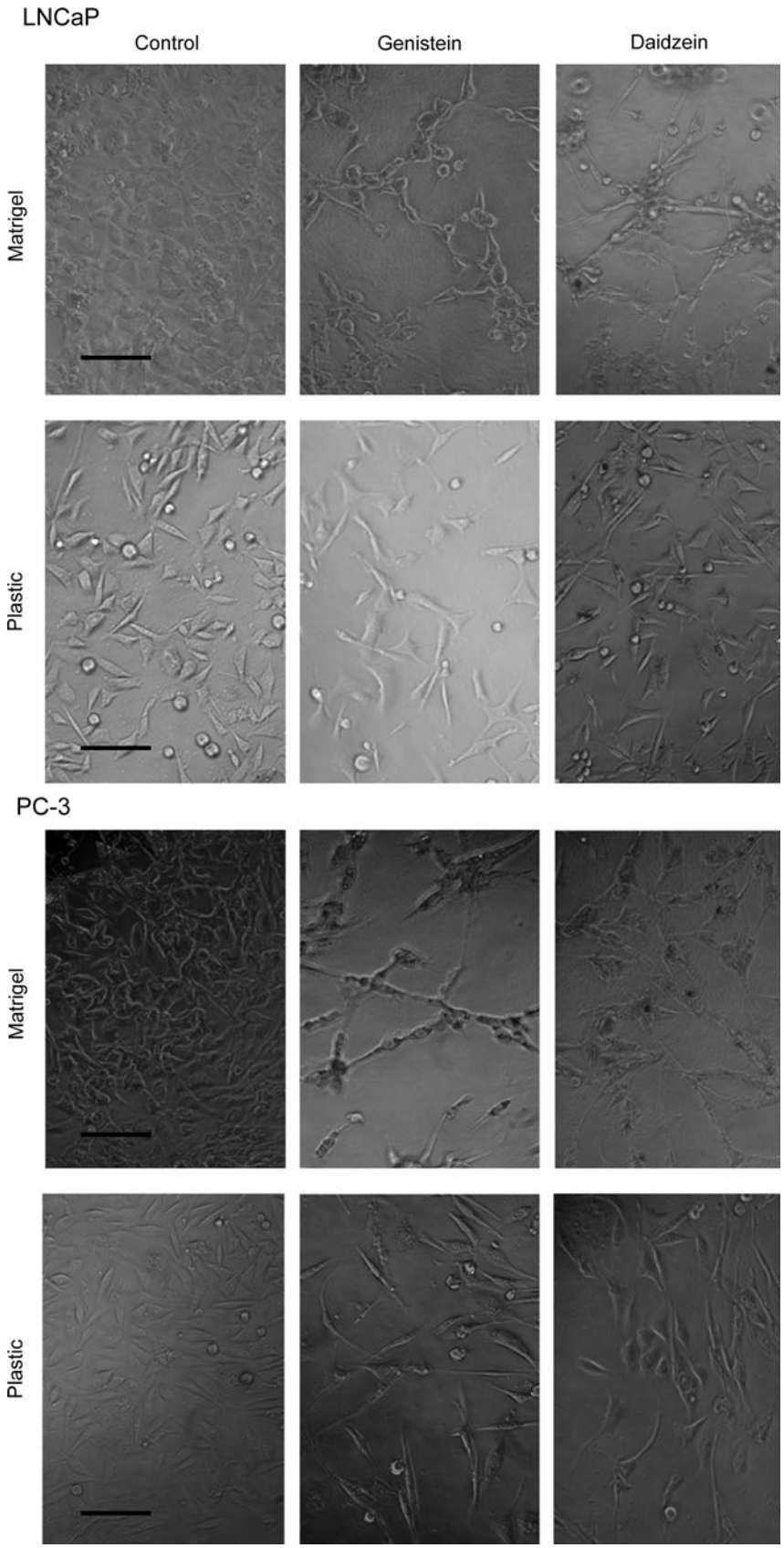

Fig. 3 Dynamic phenotype of metastatic LNCaP and PC-3 cells in 2.5 D extracellular matrix-Matrigel, after treatment with genistein or daidzein (objective magnification ×10, *bar* 50 μm)

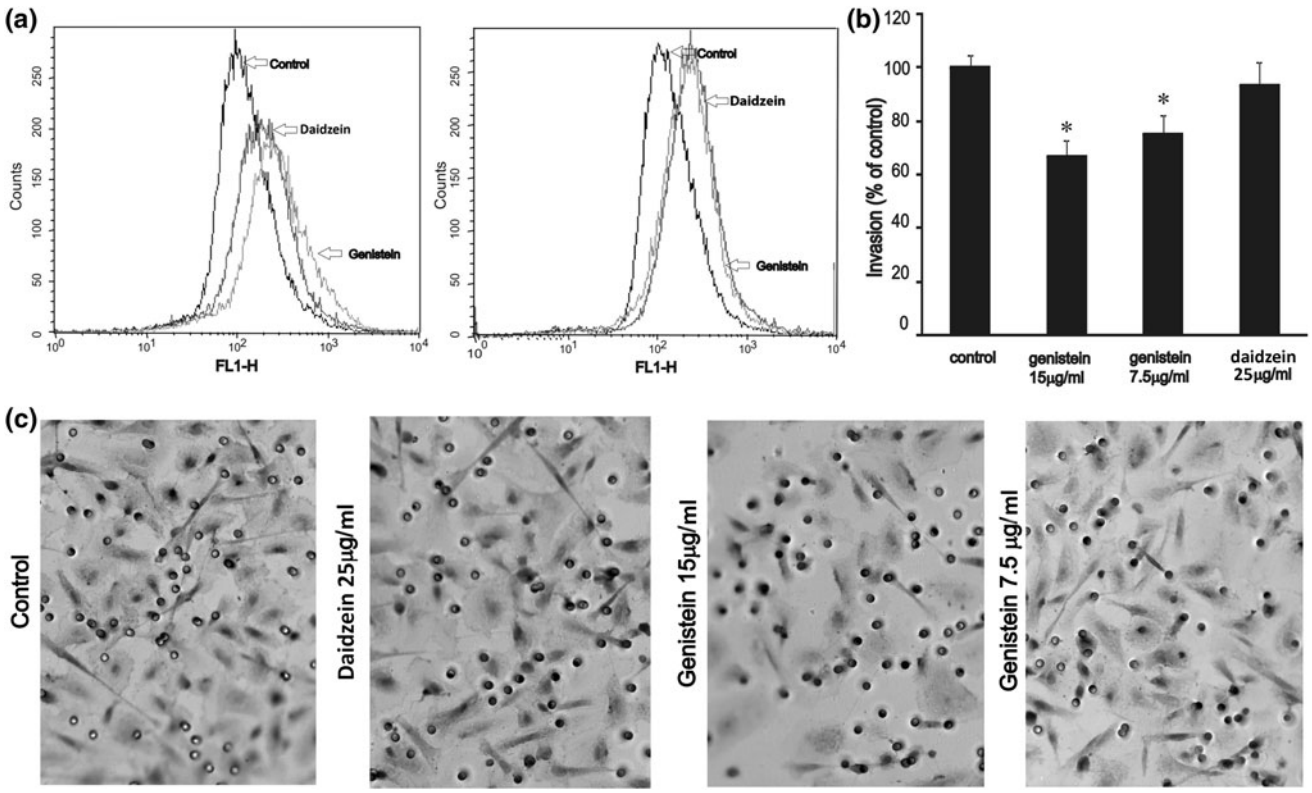

Fig. 4 Proliferation of LNCaP and PC-3 cells in the presence of an IC_{50} dose of genistein (12.5 μg/ml) or daidzein (25 μg/ml) (**a**). Invasion of PC-3 cells through a Matrigel-coated transwell exposed to genistein or daidzein (**b**, **c**)

expression of MMP-9 compared to PC-3 cells (Aalinkeel et al. 2004). In addition, genistein has been reported to inhibit the expression of MMP-2 (Huang et al. 2005; Xu et al. 2009), which is upregulated during PC cell progression (Upadhyay et al. 1999).

Conclusion

Summarizing our findings, it can be concluded that the invasive activity and dynamic phenotype of PC cells are at least partially dependent on membrane fluidity. We propose that the positive beneficial effects of genistein, and probably highly dosed daidzein, in PC treatment may be related to the suppression of metastatic potential via decreasing cancer cell membrane fluidity.

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Conflicts of Interest The authors declare that there are no conflicts of interest.

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