Invasive Cx43\textsuperscript{high} sub-line of human prostate DU145 cells displays increased nanomechanical deformability\textsuperscript{*}

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Connexin(Cx)43\textsuperscript{high} cells are preferentially recruited to the invasive front of prostate cancer in vitro and in vivo. To address the involvement of Cx43 in the regulation of human prostate cancer DU145 cell invasiveness, we have analysed the nanoelasticity of invasive Cx43\textsuperscript{high} sub-sets of DU145 cells by atomic force microscopy (AFM). The Cx43\textsuperscript{high} DU145 cells displayed considerably higher susceptibility to mechanical distortions than the wild type DU145 cells. Transient Cx43 silencing had no effect on their elastic properties. Our data confirm the relationship between the invasive potential, Cx43 expression and nanoeffectivity of the DU145 cells. However, they also show that Cx43 is not directly involved in the maintenance of DU145 invasive phenotype.

Key words: prostate cancer invasion, Cx43, cell elasticity, motility, AFM

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Abbreviations: AFM, atomic force microscopy; DMEM, Dulbecco’s-modified Eagle medium; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; GJIC, gap junctional intercellular communication; IgG, immunoglobulin G; kPa, kilopascals; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; S.E.M., standard error of the mean

INTRODUCTION

Metastatic cascade of prostate cancer is initiated by the expansion of invasive cell sub-populations(s) within a phenotypically heterogeneous tumour cell mass (Shibata & Shen, 2013; Sottroriva et al., 2010). These cells show the predilection to colonise distant organs, which results from their relatively high motility, susceptibility to chemotactic, haptotactic, parabaric and juxtanotic signals (Miekus et al., 2005; Gupta & Massague, 2006; Langley & Fidler, 2007; Blick et al., 2008; Friedl & Wolf, 2010), and from their increased nanoeffectivity (Kumar & Weaver, 2009; Suresh, 2007). Phenotypic switches regulating the recruitment of cancer cells to the “invasive front” of prostate tumour are determined by extrinsic “paracrine” and “parabaric” signals, and by the “by-stander” effects mediated by connxin-formed gap junctions (Mol et al., 2007; Czyz et al., 2012). However, the links between the function of connexins and the invasive potential of prostate cancer cells represent a still weakly explored aspect of prostate cancer progression.

The primary function of connexins is the formation of membrane channels that link cytoplasmatic compart-
sphere. Nanomechanical studies were performed using the Agilent 5500 atomic force microscope (AFM, Agilent Technologies, Austin, Texas, USA), equipped with a temperature/CO₂ chamber. Measurements were carried out in a fresh PBS containing Mg²⁺ and Ca²⁺ ions and glucose (1 mg/ml) at 37°C. Optical preview was used during the measurements to ensure that the analyses were performed on cells displaying a typical morphology. Mechanical analysis was performed in a force spectroscopy mode (Dufrene, 2003). Force measurements were collected using 2 types of standard silicon nitride cantilevers (Veeco Probes, USA) with a nominal tip radius of <50 nm and of 20 nm, respectively. Spring constant calibration of the probes was made before and after mechanical analysis of cells using the thermal tune procedure. Before each cell was measured, its topography was imaged using a tapping mode to precisely localize the central region of a cell. To prevent any cell damage and to reduce any substrate-induced effects, the measurements were collected in the force ranges resulting in shallow indentations of the cells (<500 nm). The half opening angle of the AFM tip was 25°, and the Poisson ratio of the cell was taken to be 0.5, which is typical for soft biological materials (Touhami et al., 2003). Curves from 10 to 20 randomly selected points were collected from the central region of the cell at a rate of 1 Hz. 10 force curves were measured at each point for statistical analysis. A total number of at least 20 cells was investigated for each DU145 cell population. The values of the Young’s modulus were estimated from the force curves by converting force-displacement curves into force-indentation curves and fitting them with the modified Hertz model (Radmacher et al., 1996). Young’s modulus data were expressed as means ± SEM. Statistical analysis was performed using two-sample independent Student’s t-test; *p≤0.05.

**Immunocytochemistry and immunoblotting.** For immunocytochemical visualization of Cx43, cells were fixed with methanol:acetone (7:3, –20°C), labeled with rabbit anti-Cx43 IgG (Sigma), Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) and counterstained by 0.5 µg/ml bis-benzimide (Hoechst) (Baran et al., 2009). Visualization of Cx43-positive plaques was performed with a Leica DMIRE2 microscope. For Western blot analyses, the DU145 cell cultures were dissolved in lysis buffer and cellular proteins were applied to 15% SDS-polyacrylamide gels, followed by their transfer to nitrocellulose membranes. Blots were exposed to primary rabbit polyclonal anti-Cx43 and mouse monoclonal anti-α-tubulin antibody (both from Sigma) followed by detection of the antibodies using HRP-labelled secondary antibodies (Invitrogen) and a SuperSignal West Pico Substrate (Pierce, Rockford, IL) (Daniel-Wojcik et al., 2008).

siRNA inhibition of Cx43 expression. DU145 cells were seeded at a density of 7 × 10⁴ cells per well in a 12-well plate in an antibiotic-free DMEM-F12 HAM medium supplemented with 10% FBS and grown overnight at 37°C. MISSION®esiRNA GJA1 (114 pmol, Sigma) and Lipofectamine™2000 (Invitrogen) were used for transient Cx43 silencing in the DU145_48 cells according to the manufacturer's protocol (Piwowarczyk et al., 2015). The efficiency of the inhibition of Cx43 expression was subsequently analysed by using immunoblotting. Endpoint analyses were performed 48 hours after transfection.

**RESULTS AND DISCUSSION**

**Invasive Cx43<sup>high</sup> DU145 cells display relatively high deformability.**

We have previously described the heterogeneity of the basic DU145 traits crucial for their invasiveness, including the motility and Cx43 expression (Szpak et al., 2011). The existence of invasive subpopulations within the DU145 cell line prompted us to perform single-cell analyses of long-term relationship between the Cx43

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**Figure 1. Efficiency of the DU145 cell transmigration correlates with a relatively high nanomechanical elasticity of the DU145 cells.**

(A) DU145 cells were seeded onto microporous membranes (pore diameter-8 μm) at the density of 300 cells per mm². The cells were allowed to transmigrate across the pores and the chambers were placed in another well 48 h thereafter (B). After the next 24 h, the inserts were moved to another well and allowed to transmigrate for 24 h (C). The cells in each well were propagated to obtain the DU145_48 and DU145_72 subset. (D) AFM analyses revealed a significantly increased fraction of deformable cells within the DU145_48 subset (middle) and a slight enrichment of the DU145_72 subset in deformable cells (right), when compared to the “wild type” DU145 cells (left). Pictures represent a scan size equal to 45x45 µm (scale bar = 5 µm). (E) The average values of Young’s modulus estimated for each population and given in kilopascals (kPa). Results are representative of 3 independent experiments. *p<0.05 (two-sample independent Student’s t-test).
A similar interrelation between these cells calculated for each population (Betz et al., 2011). Therefore, Cx43 down-regulation with a persistence of cellular invasive phenotype through multiple cell division cycles is mandatory for cancer invasion. Phenotypic persistence of the DU145 cells is a primary determinant of their transmigration potential (Friedl & Wolf, 2010; Kumar & Weaver, 2009)

Figure 2. Cx43 silencing does not affect the nanomechanical elasticity of the DU145_48 cells. (A) Cx43-positive plaques were more abundant in the DU145_48 and DU145_72 subset than in the wild type DU145 populations. Bar – 25 μm. Transient down-regulation of Cx43 in the DU145_48 cells (B) did not affect their elasticity (C). Values of E represent the Young’s modulus given in kilopascals (kPa). (D) The average values of Young’s modulus estimated for the DU145_48 and DU145_72 cells (in comparison to the DU145_WT cells; open bars) and of the DU145_48 cells after Cx43 silencing (DU145_Cx43si) compared to the control DU145_48 cells (filled bar). Results are representative of 3 independent experiments. Error bars represent S.E.M. *p<0.05 (two-sample independent Student’s t-test).

Invasive DU145 sub-sets were analysed between the 5th and 15th passage after transmigration. Therefore, these data show that single DU145 cells can give rise to progeny characterised by heritably increased deformability, which may potentially facilitate their invasion in vivo. They also confirm that microevolutionary processes that are responsible for the in vivo formation of prostate cancer invasive front may be recapitulated in vitro. Previously, no differences in the motile activity, morphology and the architecture of actin cytoskeleton were observed between DU145_48, DU145_72 and “native” DU145 cells (Szpäk et al., 2011). Thus, the nanomechanical elasticity of the DU145 cells is a primary determinant of their transmigration potential (Friedl & Wolf, 2010; Kumar & Weaver, 2009). A similar interrelation between increased susceptibility to mechanical distortions and the invasiveness was observed in breast cancer cells (Li et al., 2008). Persistence of cellular invasive phenotype through multiple cell division cycles is mandatory for cancer invasion. Phenotypic persistence of the DU145 sub-sets demonstrates that the observed differences in cell elasticity result from permanent cell reprogramming rather than from “secular” changes of cell shape and adhesion (Gupta & Massague, 2006; Langley & Fidler, 2007; Friedl & Wolf, 2010; Ryszaw et al., 2014).

We have previously shown the involvement of Cx43 in regulation of the nanoelasticity of the rat prostate carcinoma AT-2 cells (Ryszaw et al., 2014). These cells reacted to ectopic Cx43 down-regulation with a considerable increase of their mechanical stiffness. Immunofluorescence and immunoblot analyses confirmed that the function and the nanoelasticity of those cells. For this purpose, sub-lines of the DU145 cells were propagated from single cells that most readily transmigrated microporous membranes under isotropic conditions, i.e. in the absence of chemotaxant gradients (Fig. 1A). We have established two invasive sub-lines of the DU145 cells: the DU145_48 cells represented the progeny of a minute (ca. 2%) transmigrating cell population (Fig. 1B). The DU145_72 sub-set were propagated from the “second wave” of transmigrating DU145 cells (Fig. 1C). As shown previously, such DU145 subsets are characterised by similar motile activity (Szpäk et al., 2011). Therefore, we further analysed elastic properties of both DU145 subsets with atomic force microscopy (AFM) to estimate the role of the susceptibility to mechanical distortions in regulation of the DU145 invasive potential.

Values of Young’s modulus (E) estimated for individual “wild type” DU145 cells were almost equally distributed between 1.0 to 3.0 kPa (Fig. 1D). However, a considerable (roughly 25%) fraction of cells characterized by E>3 kPa could be also discriminated, whilst only a minute set of wild type DU145 cells displayed E=0.5–1.0 kPa. Importantly, the DU145_48 cell sub-line was considerably enriched in “elastic” cells characterized by E<1 kPa (30%). The fraction of cells displaying E values between 1.0 to 3.0 kPa (which were dominant in “native” DU145 populations) was less numerous in the DU145_48 sub-set. In these analyses, we did not observe the events of E>3 kPa. The cells that comprised the “second wave” of DU145 transmigration gave a progeny (DU145_72), which was characterised by E values distribution more similar to that observed for the wild type DU145 populations. It ranged between 0.5 to 4.2 kPa, with several local maxima (1.5 kPa, 2.4 kPa and 3.3 kPa). Moreover, some events of E>3 kPa (absent in the DU145_48 sub-line) were seen in the DU145_72 populations. The difference in the distribution of E values observed between the analyzed DU145 populations is also illustrated by the difference in the averaged values of Young’s modulus (E) calculated for each population (Fig. 1E).
DU145_48 population is characterized by elevated levels of Cx43 in comparison to the “native” DU145 and DU145_72 cells (Fig. 2A). This observation confirms that the nanoelectivity Cx43<sup>high</sup> DU145 cells is somehow linked to the Cx43 function. To elucidate whether Cx43 directly participates in regulation of the nanomechanical properties of the DU145 cells, we transiently silenced Cx43 in the DU145_48 cells (Fig. 2B) and analysed their elasticity by AFM. For this purpose, we used more precise (20 nm tip) cantilevers to increase the resolution of the technique. Ectopic Cx43 down-regulation did not increase the mechanical stiffness of the DU145_48 cells (Fig. 2C; summarized in Fig. 2D). A similar distribution of E values was detected by AFM analyses of the control and Cx43siRNA-transfected DU145_48si cells. These data stay in agreement with our previous observations showing that Cx43 silencing and chemical inhibition of GJIC does not affect the relative abundance of Cx43<sup>high</sup> DU145 cells in the invasive front (Szpak et al., 2011). However, they contradict the suggestions on the Cx43 regulatory role in the regulation of cancer cells’ susceptibility to mechanical distortions (Cronier et al., 2009). Even though Cx43 has been implicated in the regulation of AT-2 cell elasticity (Ryszawy et al., 2014), apparently it is not involved in the determination of DU145 elasticity. Collectively, Cx43 may accompany the relatively high deformability of individual DU145 cells, which decreases their sensitivity to adverse mechanical stress factors during the invasion process. However, it is not directly involved in its regulation.

CONCLUSION

Microevolution of “invasive” cell subpopulations characterised by increased susceptibility to mechanical distortions is crucial for the “metastatic cascade” of numerous cancers (Cross et al., 2007; Gupta & Massague, 2006; Suresh, 2007; Cai et al., 2010). Cx43 is involved in the evolutionary processes which are crucial for the metastatic cascade of prostate cancer (Watanabe et al., 2002; Mickus et al., 2005; Langley & Fidler, 2007; B lick et al., 2008). A selective transmigration of Cx43<sup>high</sup> prostate cancer DU145 cell sub-populations under the conditions mimicking early cancer invasion confirmed our earlier reports on such microevolution in the prostate cancer cell populations in vitro. However, an apparently coincidental nature of the correlation between Cx43<sup>high</sup> phenotype of the DU145 cells and their susceptibility to mechanical distortions remains in contrast to our previous observations on mechanistic involvement of Cx43 in the process of epithelial-mesenchymal transition (EMT) of the AT-2 cells (Ryszawy et al., 2014). These contrasting data show the complexity and tissue-specificity of the relationship between cell invasiveness, Cx43 expression and the nanomechanical elasticity of cancer cells, which govern the metastatic cascade of prostate cancer. They expand our knowledge on the subtlety of interactions between Cx43 and cytoskeleton (Prochnow & Dermietzel, 2008; Cronier et al., 2009; Olk et al., 2009) in the regulation of cancer cell adhesion, directed motility, susceptibility to mechanical distortions and sensitivity to adverse mechanical stress during invasion (Suresh, 2007; Kumar & Weaver, 2009; Shibata & Shen, 2013). A more comprehensive study on Cx43 involvement in the regulation of the nanoelectivity of prostate cancer cells, performed on an array of prostate cancer cell lineages derived from biopsies, is necessary to fully understand the differences in the relationship between Cx43 levels and invasiveness, observed between the AT-2 and DU145 lineages. This should help to fully assess specificity and heterogeneity of Cx43 functions in the regulation of the metastatic potential of prostate cancer cells.

Conflicts of interest

The authors declare no conflict of interest.

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