Original Article

Towards Dissecting Molecular Routes of Intercellular Communication in the Tumour Microenvironment: Phenotypic Plasticity of Stem Cell-Associated Markers in Co-culture (Carcinoma Cell/Fibroblast) Systems

(cancer-associated fibroblasts / cancer stem cell / CD44 / galectin / side population / tumour stroma)

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Abbreviations: ABC – ATP-binding cassette, CAFs – cancer-associated fibroblasts, CCD – charge-coupled device, CD133 (29, 44) – cluster of differentiation 133 (29, 44), CSCs – cancer stem cells, DAPI – 4, 6-diaminidino-2-phenylindole dilactate, D-MEM – Dulbecco's modified Eagle's medium, EMEM – minimum essential medium with Earle's salt, EMT – epithelial-mesenchymal transition, FACS – fluorescence-activated cell scanning, FaDu – hypopharyngeal carcinoma cell line, FBS – foetal bovine serum, FITC – fluorescein isothiocyanate, Gal-1-BS – galectin-1-binding sites, HF – human fibroblast, HNSCC – head and neck squamous cell carcinoma, K8 (10, 19) – keratin 8 (10, 19), MDR1 – multidrug resistance receptor 1, PBS – phosphate-buffered saline, SCCF – squamous cell cancer fibroblast, SP – side population, Swam – swine anti-mouse, Swar – swine anti-rabbit, TRITC – tetramethyl rhodamine isothiocyanate.

Abstract. Increasing evidence attributes tumour fates to a small population of cells (cancer stem cells) capable of surviving therapeutic interventions. Investigation of their characteristics, especially in cross-talk with other cell types of the tumour microenvironment, can pave the way to innovative therapeutic concepts. The central issue of this study was to evaluate the impact of stroma on tumour cells with stem cell-like features in a squamous cell carcinoma model (FaDu). Six different types of experimental conditions were tested using distinct compositions of the culture system, and both morphologic and molecular features of the tumour cells were analysed. In detail, FaDu cells alone were used as a control, compared to tumour cells from co-culture, with squamous cell cancer-derived stromal fibroblasts or normal skin human fibroblasts, both in the direct and indirect (insert) systems, adding analysis of side population cells of FaDu culture. Measurements were taken on days 2, 7 and 9 of culture and immediately after preparation in the case of the side population. A panel of antibodies against keratins 8, 10, 19, stem cell markers CD29, CD44, CD133, as well as biotinylated adhesion/growth-regulatory galectin 1 served as a toolbox for phenotypic characterization. Co-culture with fibroblasts prepared from tumour stroma and with dermal fibroblasts affected marker presentation, maintaining an undifferentiated stage phenotypically related to stem cells. Side-population cells showed close relationship to cancer stem cells in

these characteristics. In conclusion, normal and tumour stromal fibroblasts are capable of shifting the marker expression profile of FaDu cells to a stem-cell-like phenotypic pattern in co-culture.

Introduction

Head and neck squamous cell carcinomas (HNSCC) have initially been solely considered as malignant tumours stemming from transformed squamous epithelial cells (Nagai, 1999). However, increasing attention is being paid to the mutual interplay with the other cell types in the tumour microenvironment (e.g. fibroblasts, endothelial cells, immune cells and bone marrow-derived mesenchymal stem cells) and constituents of the extracellular matrix (Wels et al., 2008; Borovski et al., 2011). In fact, all these components shape the specific conditions of the individual tumour. Despite remarkable progress in understanding the key genetic alterations and their role in establishing malignancy of squamous cell epithelia, the contribution of non-epithelial tumour components to both tumour origin and progression poses a current challenge of high biomedical relevance.

Stem cells are centrally responsible for maintaining proper viability of the squamous epithelium by enabling repair and renewal after damage or loss. By appearance, the stem cell is small and round in shape (Amit and Itskovitz-Eldor, 2012), and it is notably able to divide asymmetrically, keeping a small pluripotent subpopulation in relation to the bulk of tissue-forming cells. Two types of stem cells are recognized in the human body during ontogenesis: embryonic and adult stem cells, the latter induced to become specialized mature cells under the distinct conditions (Knoblich, 2008; Vezzoni and Parmiani, 2008; Bhattacharyya and Khanduja, 2010).

It is a reasonable assumption that tumour formation may be associated with genetic alterations of the stem cells. The principles of the cancer stem cell (CSC) theory were already shaped several decades ago. Since then, several markers have been supposed to indicate stem properties in HNSCC, among them CD44, keratin 8 (K8), keratin 19 (K19), CD29 (β_1-integrin), presence of nuclear binding sites for an endogenous adhesion/growth-regulatory lectin, i.e. galectin 1 (Gal-1; for details on this effector class see Kaltner and Gabius, 2012; Smetana et al., 2013) and aldehyde dehydrogenase activity (Chovanec et al., 2004; Smetana et al., 2006; Harper et al., 2007; Prince et al., 2007; Chen et al., 2009; Facompre et al., 2012; Yu et al., 2013). Proper functioning of adult stem cells appears to be regulated by the microenvironment, called „niche“, as assumed for tumour-stroma cross-talk. Dysregulation at this level may affect stem cell behaviour, leading to tumorigenesis and tumour progression (Sneddon and Werb, 2007; Strnad et al., 2010). As noted above, the stem cell niche can also be regarded as a complex of different cell types including microvessels and also extracellular matrix components such as proteoglycans and adhesion molecules like fibronectin, all bearing glycans which serve as signals for cellular communication (for details on the concept of the sugar code see Gabius et al., 2011; for model studies on orchestration of glycan/lectin expression for tumour or immune growth regulation see André et al., 2007; Wu et al., 2011; Amano et al., 2012). Obviously, efforts to delineate rules of this interplay hold promise for opening the way to new therapeutic options. Within this long-term project line revealing particular properties and capabilities of stromal fibroblasts of tumours, which may not simply be bystanders, is one aim.

Thus, this study was designed to evaluate the impact of tumour stromal fibroblasts on the stem cell phenotype of a representative squamous cell cancer line *in vitro* and to compare the results with the effect of normal fibroblasts. In detail, the two mentioned types of fibroblasts were co-cultured with the cells of the hypopharyngeal FaDu line previously characterized especially with respect to galectin binding (Smetana et al., 2006). In addition, the side population was isolated from the FaDu cell line, and these cells were also studied for the presence of selected markers.

Material and Methods

Cells and their cultivation

Three types of cells were used in this experiment, i.e. the FaDu cell line of epithelial cells originating from a tumour of the hypopharynx (ATCC® HTB-43™) (Rangan, 1972). Squamous cell cancer-associated fibroblasts (SCCF) were isolated from the stroma of an oral squamous cell carcinoma surgically removed at the Department of Oral and Maxillofacial Surgery, First Faculty of Medicine, Charles University in Prague, following routine procedures (Lacina et al., 2007; Dvorankova et al., 2012). Normal human dermal fibroblasts (HF) were obtained from skin samples of a non-tumour patient who had undergone aesthetic surgery at the Department of Plastic Surgery, Third Faculty of Medicine, Charles University in Prague, using a standard protocol (Lacina et al., 2007; Kodet et al., 2011; Kolar et al., 2012). In all cases, tissue specimens were obtained with the patients' informed consent according to the Declaration of Helsinki and after the local Ethical Committee approval. FaDu cells were routinely cultured in minimum essential medium with Earle's salt (EMEM) supplemented with 10% foetal bovine serum (FBS), sodium pyruvate and antibiotics (Biochrom, Berlin, Germany) at 37 °C and 5% CO_2. The cells from 5th subculture were seeded at a density of 2×10^3 cells/cm^2 on coverslips placed in 6-well dishes (Corning, New York, NY) and cultured for 2, 7 and 9 days.

In the second part of the experiment the FaDu cells were co-cultured with SCCF or HF in a direct system enabling intercellular contacts. Initially, both SCCF and HF cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) containing 10% foetal bovine serum and antibiotics (Biochrom) at 37 °C and 5% CO_2. For co-culture, HF from the 9th subculture or SCCF from the

7th subculture were seeded on coverslips (density 500 cells/cm^2) together with FaDu cells (density 2 × 10^3/cm^2). The resulting preparations were co-cultured in EMEM with 10% FBS for 2, 7 and 9 days.

Part 3 of our study applied an indirect (insert) transwell system using a microporous membrane (Corning), which precluded direct intercellular contacts between the epithelial cells and either SCCF or HF. Experimentally, FaDu cells (5th subculture) were seeded on coverslips at a density of 5 × 10^3 cells /cm^2, then inserts were placed in the wells and SCCF or HF (density 1 × 10^3/cm^2) were positioned on them. Similar to the previous experiments, the period of time of co-culture was 2, 7 and 9 days. After each period, adherent cancer cells were carefully washed three times with Dulbecco's PBS (Biochrom), the coverslips were dried and stored at -20 °C prior to immunocytochemical processing.

Finally, a side population was isolated from the culture of FaDu cells in the exponential growth phase (after 72 h of incubation under standard conditions). Cell suspensions were centrifuged for 5 min at 1200 rpm (300 *g*), the pellets were then washed twice with PBS and resuspended in 2 ml of Eagle's medium containing 2% bovine serum albumin, then the solutions were pipetted to test tubes at the final concentration of 1 × 10^6 cell/ml and stained with 5 µg/ml of Hoechst 33342 (Sigma-Aldrich, Prague, Czech Republic) at 37 °C for 120 min. After this incubation period, the cells were washed with cold Hanks' balanced salt solution. Thereafter, all test tubes with cells were maintained on ice and all solutions added were adjusted to this temperature. Five minutes before FACScan analyses (FACSvantage SE, BD Biosciences, Heidelberg, Germany), 2 µg/ml of propidium iodide was added for dead-cell identification. The obtained survived cells were seeded onto coverslips, immediately washed with PBS, coverslips were then dried and kept frozen at -20 °C. No experiment focusing on the interaction between the side population and fibroblasts was made.

Characterization of cell phenotype

To enable the probes to reach their intracellular targets during immuno- and galectin cytochemical characterization, cells were treated with Triton X-100 (Sigma-Aldrich). After fixation using 2% paraformaldehyde in PBS (pH 7.2), non-specific protein binding of the probes was precluded by applying bovine serum albumin for saturating non-specific protein-binding sites (Sigma-Aldrich). Cell phenotype was evaluated in technical triplicates for each evaluated marker.

Home-made biotinylated galectin 1 used as a probe to visualize the presence of accessible binding sites (Gal-1-BS) was prepared and tested for maintained activity as described (Purkrabkova et al., 2003). A panel of mouse monoclonal antibodies as first-step reagents to detect keratin 8 (K8), keratin 10 (K10), keratin 19 (K19) (all from DAKO, Glostrup, Denmark), CD133 and CD44 (all from Abcam, Cambridge, UK), CD29 (Immunotech, Marseille, France) was used. Fluorescein isothiocyanate (FITC)-labelled swine anti-mouse (Swam-FITC) and anti-rabbit (Swar-FITC) immunoglobulin fractions (AlSeVa, Prague, Czech Republic; DAKO) and tetramethyl rhodamine isothiocyanate-labelled ExtrAvidin – ExtrAvidin-TRITC (Sigma-Aldrich, Prague, Czech Republic) – were used as second-step reagents for visualization of bound probes. Cell nuclei were counterstained with 4,6-diaminidino-2-phenylindol dilactate (DAPI; Sigma-Aldrich); thereafter, specimens were mounted in Vectashield (Vector Laboratories, Peterborough, UK). The used reagents are summarized in Table 1.

Table 1. Markers used in the experiment

First-step reagents	
K8	DAKO, Glostrup, Denmark
K10	DAKO, Glostrup, Denmark
K19	DAKO, Glostrup, Denmark
CD29	Immunotech, Marseille, France
CD44	Abcam, Cambridge, United Kingdom
CD133	Abcam, Cambridge, United Kingdom
Gal-1-BS	Institute of Physiological Chemistry, Faculty of Veterinary Medicine, Ludwig-Maximilians University, Munich, Germany
Second-step reagents	
Swam-FITC	AlSeVa, Prague, Czech Republic; DAKO
Swar-FITC	AlSeVa, Prague, Czech Republic; DAKO
ExtrAvidin-TRITC	Sigma-Aldrich, Prague, Czech Republic
Other	
DAPI	Sigma-Aldrich, Prague, Czech Republic
Hoechst 33342	Sigma-Aldrich, Prague, Czech Republic

Controls of the specificity included assessment of antigen-independent staining with isotype-matched antibodies or by omission of the first-step reagents as well as demonstration of sugar-dependent inhibition of galectin binding. The specimens were monitored at the light microscopic level using an Eclipse fluorescence microscope (Nikon, Prague, Czech Republic) equipped with a Cool-1300Q CCD camera (Vosskühler, Osnabrück, Germany). Data were analysed with a LUCIA 5.1 computer-assisted image analysis system (Laboratory Imaging, Prague, Czech Republic).

Results

Cell morphology

At the early stage of FaDu culture (day 2 of subculture), small colonies of cells were rarely observed (Fig. 1/A). Formation of contacts between cells with typical epithelial morphology spread in the monolayer was a prominent feature on day 7 of culture. At this stage, cells with fibroblast-like morphology were observed on the periphery of cell colonies. Multilayered cell presenta-

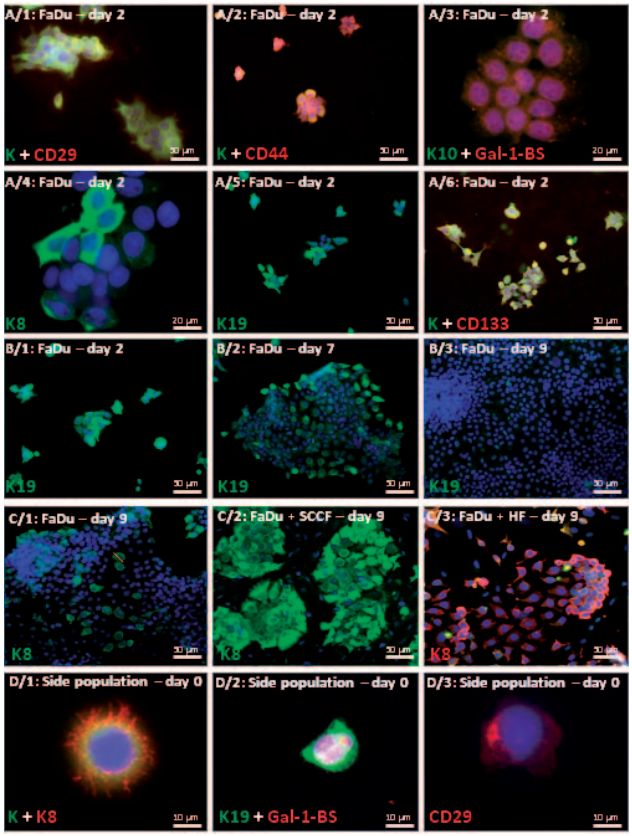

Fig. 1. **A/1-6**: Monitoring of FaDu cells in the course of 2nd day culture. The cells are positive for stem cell markers (CD29, CD44, Gal-1-BS in the nucleus, K8, K19, CD133). There is no positivity for K10, marker of differentiated cells. K – pankeratin.
B/1-3: Monitoring of FaDu cells on day 2 (B/1, subconfluent growth), day 7 (B/2, growth as a monolayer) and day 9 (B/3, multilayered growth) when K19 was detected. The panels illustrate the reduction of signal intensity in the course of cell culture down to negativity in cells forming multilayered colonies.
C/1-3: Effect of adding normal (HF) and cancer stromal (SCCF) fibroblasts to cultures of FaDu cells, significantly stimulating expression of K8 in the cancer epithelium.
D/1-3: Cells of the side population on day 0 of cell culture. Profiles and intensities of signals for K8, K19, Gal-1-BS in the nucleus and for CD29.

tion was reached on the 9th day of subculture. Predominantly large spread cells were observed in this growth phase, with a few regions of small cells being identified (Fig. 1/B). In the case of direct co-cultivation (with both SCCF and HF), we observed larger colonies of subconfluent cells in the vicinity of fibroblasts on the 2nd day of the subcultures. Evidently, this feature was more prominent in co-culture with SCCF than with HF (Fig. 1/C). Cell appearance was similar in cultures with insert and direct contacts. As seen for FaDu cell culture alone, formation of intercellular contacts (day 7) and multilayered growth (day 9) was observed in the co-cultures. Of note, a population of small cells with fibroblast-like morphology on the periphery of colonies in contact with fibroblasts (either SCCF or HF) was seen up to the 9th day of culture. These observations indicated a slight difference when using SCCF and HF as stromal component, the tumour-associated fibroblasts being more potent as stromal activators than normal fibroblasts (Fig. 1/C).

Cell phenotype

During the 2nd day of culture of FaDu cells alone, there was strong expression of CD29, CD44, CD133, K8, K19 and Gal-1-BS in the nucleus (Fig. 1/A). CD29, CD44, CD133, K8 and K19 were still present on day 7, with a tendency for intensity decrease (Table 2). On day 9, only few cells were found to be positive for CD44, CD29 and K8. The level of presence of CD133 was strongly decreased, while no staining for K19 was observed (Table 2). During all stages, positive cells were present, either in the centre of the colonies or in their periphery, or both.

Regarding marker expression in the samples of co-culture, staining for K8, K19 and CD29 was present in cells on the periphery of the colonies on day 2. The staining intensity with an increase of the pool of positive cells, especially in the cases of K8, K19 and CD29 as well as for the nuclear presence of Gal-1-BS, was maintained to the 7th day of culture. Positivity for K8 and K19 was mainly seen at the colonies' margins, while CD29 was detected also centrally. Gal-1-BS were observed in the cytoplasm of almost all cells. The cells on the periphery of colonies were consistently positive for K19 and K8 on day 9. In terms of staining intensity, the

Table 2. Semi-quantitative assessment of intensity of marker-specific signals in FaDu cells

	FaDu			FaDu+SCCF/HF			Side population
	2nd day	7th day	9th day	2nd day	7th day	9th day	1st day
CD29	***	**	*	***	***	***	***
K8	***	**	x	***	***	***	***
K19	***	*	x	***	***	***	***
Gal-1-BS N	***	x	x	***	***	**	***
Gal-1-BS C	***	***	***	***	***	***	**

Scale: X – negative signal, * – weak but specific signal intensity, ** – medium signal intensity, *** – strong signal intensity
Sites of localization of Gal-1-binding sites: N – nuclear, C – cytoplasmic

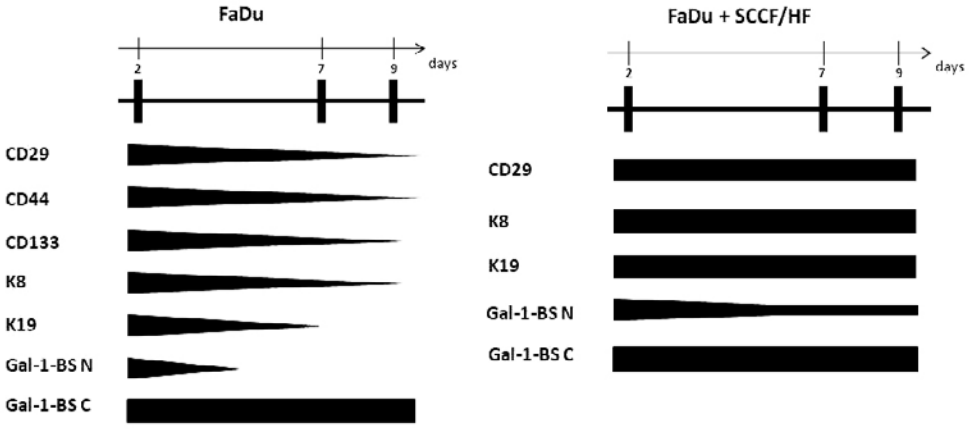

Fig. 2. Comparison of the level of expression of the selected markers in FaDu cells in relation to the type of culture conditions

signal for Gal-1-BS in the nucleus was decreased and limited to particular cells without regional preference (Fig. 2).

Indirect FaDu + SCCF/HF co-culture did not reveal any differences in the size of cell populations compared to direct co-culture (not shown). Under these conditions, strong positivity for K19 and K8 expression was seen on the periphery of colonies, with a few positive cells in the centre during the day 2 of culture, Gal-1-BS were observed mainly in the cytoplasm of cells, and only a very few of them expressed Gal-1-BS in the nucleus. When monitoring the day 7 co-cultures, the pool of K19/K8-positive cells was more numerous compared to isolated FaDu culture, seen either at the periphery or in the centre of the colonies. The cytoplasmic presence of Gal-1-BS was observed invariably. In the day 9 cultures, the number of cells in the colony increased, but the proportion of K19/K8-positive cells was maintained. Interestingly, the nuclear presence of Gal-1-BS was detected.

The side-population (SP) cells were examined after their isolation. These cells were small in size, round, and formed small colonies. K8, K19, CD29 and nuclear Gal-1-BS were consistently present in these cells (Fig. 1/D).

Discussion

The obtained results revealed a dependence of marker expression on the conditions of culture. Fibroblasts from cancer (SCCF) and also normal cells (HF) were capable of maintaining rather a high level of the marker presence. These observations extend our previous analysis of the effects of length of period in culture (Smetana et al., 2006).

The cancer stem cell theory has been adopted from blood malignancies to solid tumours of different types (Singh et al., 2003; Grichnik, 2006; Wang et al., 2006; Zhang et al., 2006; O'Brien et al., 2007; Prince et al., 2007; Eramo et al., 2008). Several proteins are supposed to be markers of stem cells in HNSCC and other solid tumours, among them CD44, a receptor for hyaluronan, as one of the most promising candidates (Cichy and Pure, 2003). Expression of intermediate filaments, especially keratins, is an indicator of the status of epithelial cell differentiation. Unfavourable prognosis of HNSCC negatively correlates with the expression of K8 and K19 (Fillies et al., 2005). Normal oral mucosa of patients with tongue carcinoma harbours a high level of K19 in comparison to patients without cancer (Copper et al., 1993). CD29 (β_1-integrin) is strongly present in normal basal cells of the squamous epithelium and also in the colonies of small cells with marked proliferative activity (Harper et al., 2007). Its presence is associated with a low differentiation status of cancer epithelium, likely reflecting an early stage of tumour development, eventually to CSCs (Geng et al., 2010). Additionally, β_1-integrin expression is seen in mesenchymal stem cells (Semon et al., 2010). Co-expression of $CD44^+/\alpha_2\beta_1$-integrin$^+$/$CD133^+$ in prostate cancer is supposed to be characteristic of these cancer stem cells (Collins et al., 2005). CD133 therefore also appears as a candidate for a cancer stem cell marker in tumours of various origin (Gharagozloo et al., 2012; Zaidi et al., 2009). In addition, the nuclear presence of Gal-1-BS served as a marker for poorly differentiated cells, in characteristics close to stem cells, in different cell types including cells from hair follicle and interfollicular epidermis (Chovanec et al., 2004; Kodet et al., 2011).

CSCs have an ability to escape cell death during radio- or chemotherapy (Tan et al., 2006). Appearing to mimic this ability, a part of the cancer cells, called the side population, are able to survive the contact with cytotoxic dye Hoechst 33342 under *in vitro* conditions. This ability to exclude the Hoechst dye is due to the activity of multidrug resistance transporter 1 (MDR1), a member of the ABC transporter-transmembrane proteins, which are discussed to account for the resistance to chemotherapy (Hadnagy et al., 2006). At present, the properties of CSCs and side population cells appear correlated in different kinds of cancer, namely gallbladder carcinoma, urinary bladder carcinoma and human oral cancer (Yanamoto et al., 2011; Li et al., 2012; Zhang et al., 2012). Here, the immunocytochemical characteristics of stem cells were detected in the side population.

The crucial point of this study was to observe tumour stroma influencing and preserving the cancer stem cell phenotype in a squamous cell carcinoma line (FaDu). Together with this finding, intercellular contacts between cancer and stromal cells didn't seem to be limiting for tumour growth. Logical implication shows the tumour stroma as responsible for cancer tolerance to systemic therapy and irradiation as well as a possible regulator for the cancer cell behaviour at the distant sites during metastasis (Valastyan and Weinberg, 2011). Cancer-associated fibroblasts (CAFs) as part of bioactive cancer microenvironment influence cancer biological properties (Valach et al., 2012). The presented findings underscore the activity of stromal elements such as SCCF or even normal fibroblasts in the culture model. Hence, regarding the paper by Costea et al. (2006b), the therapeutic strategy cannot focus on the cancer cells only, but should impact their surrounding environment as well.

Improving our understanding of CSC biology and EMT along with taking a step towards establishing a close-to-physiology model for side population research are expected to advance research with clinical perspective. Two approaches are suggested to be instrumental in this context: elimination of CSCs and influencing the tumour stroma to bring down epithelial-mesenchymal signals responsible for CSC renewal and promotion of the metastatic process (Costea et al., 2006; Malanchi et al., 2012). In conclusion, the tested culture conditions, with fibroblasts as a regular element of the tumour stroma introduced to the *in vitro* system, significantly influenced the phenotypic pattern of FaDu cells. These data encourage further studies on co-culture systems for *in vitro* modelling of intercellular communication in cancer stem cell research.

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