

Establishment of a 3D co–Culture Model to Investigate the Role of Primary Fibroblasts in DCIS of the Breast.

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Abstract

Purpose

Ductal carcinoma in situ (DCIS) is a preform of breast cancer. 13-50% of these lesions will progress to invasive breast cancer (IBC), but the individual progression risk cannot be estimated. Therefore, all patients receive the same therapy, resulting in potential overtreatment of a large proportion of patients. The role of the tumor microenvironment (TME) and especially of fibroblasts appears to be critical in DCIS development and a better understanding of its role may aid individualized treatment.

Methods

Primary fibroblasts isolated from benign or malignant punch biopsies of the breast and MCF10DCIS.com cells were seeded in a 3D cell culture system. The fibroblasts were cultured in a type I collagen layer beneath a Matrigel layer with MCF10DCIS.com cells. Dye-quenched (DQ) fluorescent collagen was used in both layers to demonstrate proteolysis. Confocal microscopy was performed on day 2, 7 and 14 to reveal morphological changes, which could indicate the transition to an invasive phenotype.

Results

MCF10DCIS.com cells form smooth, round spheroids in co-culture with healthy fibroblasts, but show an irregular shape with spikes in co-culture with tumor-associated fibroblasts (TAFs). These morphological changes could represent the progression to an invasive phenotype. In addition, TAFs show a higher proteolytic activity compared to healthy fibroblasts. The distance between DCIS cells and fibroblasts decreases over time.

Conclusion

TAFs seem to play an important role in the progression of DCIS to IBC. The better characterization of the TME could lead to the identification of DCIS lesions with high or low risk of progression. This could enable personalized oncological therapy, prevention of overtreatment and individualized hormone replacement therapy after DCIS.

Introduction

Breast cancer is the most common cancer type in women divided into an invasive and non-invasive histological type(s) according to the WHO classification [1]. The risk of progression to an invasive form varies among pre-invasive lesions. The highest risk for transition to an invasive breast cancer and thus to a potentially life-threatening disease is seen in DCIS with 13-50% of the cases [2]. So far, it is not possible to predict which DCIS lesion will become invasive and therefore all patients with a DCIS are treated equally resulting in over-treatment of some patients.

Therapy of DCIS includes surgical treatment either breast-conserving or a mastectomy depending on the tumor-size and breast relation. The possibility of radiotherapy after breast-conserving surgery and/or endocrine therapy should be discussed with the patient individually based on a risk-benefit assessment, considering potential adverse effects [3,4,5].

Possible therapy associated morbidity through radiation or surgery can affect the lifestyle of the patient and reduce the quality of life [6]. Therefore, DCIS over-treatment leads to potential adverse effects regarding patients' health and constitutes a burden on the health system [7]. Moreover, there is no data regarding the use of hormone replacement therapy after DCIS, which is usually avoided due to the fear this could trigger an invasive transformation/ reformation [8].

Prognostic parameters are needed to assess the patient's individual risk. In case of a low risk, interventional therapy could be replaced by regular follow up [9].

DCIS is considered a non-obligatory precursor to invasive breast cancer [10] and there are two progression theories; the 'genetical' and the 'non-genetical' theory.

The genetical theory is supported by studies that have revealed the genetic similarity and the likely common origin of DCIS and invasive carcinoma. [11-13] Clinical observations have also shown that the two entities are often located at the same anatomical site or directly next to each other, which suggests an evolutionary continuum. Gene expression analysis have been conducted and provide evidence of a common genetic background [14]. Other studies have shown a significantly different expression pattern for distinct genes, which could indicate driver pathways that play an important role in the progression from DCIS to IBC [15-17]. However, a single mutation as a cause for the progression to IBC seems unlikely

So far, no biomarker could be found to clinically predict the progression from DCIS to IBC.

The non-genetical theory suggests the tumor microenvironment (TME) to play a decisive role in the progression of DCIS to IDC [18]. DCIS is surrounded by an outer layer of myoepithelial cells and an intact basement membrane. The layer of normal myoepithelial cells acts as a "gatekeeper" and has tumor suppressive effects on the in situ lesion [19]. Various studies have shown that the loss of this suppressive effect leads to the progression to IBC [19, 20]. Except from a physical barrier against invasion, myoepithelial cells also secrete various extracellular matrix components (ECM) which inhibit the invasive capacity of DCIS in a paracrine way [21]. Increased stromal cell expression of ECM-modifying enzymes [22,23] as well as glucocorticoids seem to favor the progression of DCIS to IDC in vitro and in vivo [24]. Stromal fibroblasts as well as tumor infiltrating lymphocytes (TILs) and their T- to B-cell ratio seems to play a role in the progression of DCIS to IDC [22,25].

Altogether, the current literature leads to the conclusion that the transition from DCIS to IBC is largely not dependent on intrinsic mutations. The invasive potential appears to be rather due to extrinsic factors or the TME [26]. These include the ECM, the myoepithelial cell layer, immune cells and fibroblasts. Therefore, there is a need to establish basic novel experimental models of DCIS that take the TME into account. In

this study, we describe a novel 3D co-culture model of DCIS and primary fibroblasts as a major component of the TME that can be used to study the progression of DCIS in a convenient laboratory setting. This work primarily focuses on morphological changes potentially representing an invasive phenotype in this model.

Materials And Methods

Materials

All supplies and chemicals were from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) unless otherwise stated.

Cell culture

The human DCIS model cell line MCF10DCIS.com, cloned from a cell culture initiated from a xenograft lesion [27] was obtained from Karmanos Cancer Institute (Detroit, MI, USA) and cultured in DMEM/F12 containing 20 % Calf Serum und 10% Autoclaved DMSO. The human triple-negative breast cancer cell line MDA-MB-231 was obtained from ATCC/LGC Promochem (Wesel, Germany) and maintained in DMEM containing 1% glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. Primary fibroblasts were isolated from malignant or benign punch biopsies of the breast. The study was carried out following the Declaration of Helsinki and approved by the local ethics commission (Ethikkommission der Ärztekammer Westfalen-Lippe und der Medizinischen Fakultät der WWU; approval no.1 IX Greb 1 from 19 September 2001, updated 2012). The participants gave written informed consent. Tumor associated fibroblasts (TAFs) used derived from a moderately differentiated breast cancer (NST), ER 95%, PR 80%, Her2 neu neg, Ki 67 20% of a 62 years old patient (type 1) or from a highly differentiated mucinous breast cancer, ER 90%, PR 90%, Her2 neu neg, Ki 67 5% of a 82 years old patient (type 2). The non-TAFs were derived either from breast tissue of a 50 years old patient with microcalcifications but no indication of malignancy or from a fibroadenoma of a 19 years old patient and were cultured in RPMI Medium containing 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere of 7% CO₂ at 37 °C .

3D co-culture model

The co-culture of DCIS cells and primary fibroblasts was based on the MAME model (mammary architecture and microenvironment engineering) of Sameni et al. and was modified to meet the needs of primary cells [28]. Primary fibroblasts were isolated from the punch biopsies and were then cultured in a Type I collagen layer (Discovery Labware, Inc. by Corning, Bedford, MA, USA) under a Matrigel layer (Discovery Labware, Inc. by Corning, Bedford, MA, USA) with MCF10DCIS.com cells in a 6 well plate using the culture media described above. Dye- quenched fluorescent collagen (Invitrogen by Thermo Fisher Scientific, Eugene, USA) was used in both layers to demonstrate proteolysis (DQ-collagen I in collagen I and DQ-collagen IV in Matrigel). The 3D co-culture was then examined under the confocal microscope after 2, 7 and 14 days to reveal morphological changes that could indicate the transition to an invasive

phenotype. Cells were marked with cell tracker red, whereas dye-quenched DQ-collagen showed a green signal. The control with only DCIS was compared to DCIS in co-culture with either TAFs or 'healthy' fibroblasts.

The co-culture consists of two dye-quenched (DQ) substrates and three layers (Figure 1). For the preparation of the reconstituted basement membrane (rBM) substrate, Matrigel ((Discovery Labware, Inc. by Corning, Bedford, MA, USA) was thawed the day before at 4 ° C and then diluted dye-quenched (DQ) collagen IV (Invitrogen by Thermo Fisher Scientific, Eugene, USA) in a pre-cooled tube was added to a final concentration of 25 µg/ml. The collagen I solution was prepared on ice. Collagen I (Discovery Labware, Inc. by Corning, Bedford, MA, USA) was mixed with PBS in a ratio of 1:8. The pH was adjusted to 7,2-7,6 with 1 part of 0,16 M NaOH and checked with pH strips. DQ-collagen I (Invitrogen by Thermo Fisher Scientific, Eugene, USA) was added to a final concentration of 25µg/ml. The desired number of fibroblasts (10^3) in 10 µl medium was added to 60 µl of collagen I and DQ-collagen I mixture and constituted the bottom layer of the co-culture. The culture dish was then incubated to solidify for 30 minutes without CO₂ at 37 ° C, followed by 10 minutes with 5% CO₂ at 37° C and then brought to room temperature. On top of that, a layer of 60µl Matrigel mixed with DQ-collagen IV was placed. The culture dish was then incubated for 10 min at 37 ° C and 5% CO₂ to solidify. A top layer of 50µl cell suspension including the desired number of MCF10DCIS.com cells (5×10^3) with 2% Matrigel was added and the culture dish was incubated for 40-60 minutes with 5% CO₂ at 37° C to allow the cells to adhere. The co-culture was then covered with 2 ml of medium with 2% Matrigel and incubated for the desired time of 14 days at 37 ° C and 5% CO₂. The medium with 1% Matrigel was changed every 3-4 days.

The two types of DQ-collagen, fluorescently labeled collagen that generates fluorescent fragments upon proteolytic degradation, were used to display proteolysis. Optical sections were captured, processed and reconstructed in 3D.

Confocal microscopy and data analysis

The co-culture was visualized on day 2, 7 and 14 under the confocal microscope (LSM 880 Carl Zeiss Microscopy GmbH, Jena, Germany) using two channels. The HeNe543 laser has an emission wavelength of 543 nm, thus stimulating the Celltracker fluorescent dye (541/ 565 nm) and enabling visualization of coloured cells. The argon diode 405-30 with an emission wavelength of 488 nm catches the DQ-collagen signal. The power of HeNe543 laser has been set to 10% and of the argon diode 405-30 to 20%. The pinhole was set to 1 AU (airy unit) so that the signal-to-noise ratio was as low as possible. All images were taken with an identical setting and reconstructed and processed in 3D using the Zen 2 black imaging software (Carl Zeiss Microscopy GmbH, Jena, Germany). In order to be able to present the possible morphological differences between the spheroids in the control and co-culture, a small measuring distance of 8µm was chosen. Accordingly, a measuring distance of 3.5µm was selected for the significantly smaller fibroblasts, so that even small differences could be detected. For the measurement of the distance between fibroblasts and MCF10DCIS.com cells, images were taken at a distance of 15µm.

Statistical analysis

For statistical analysis a two tailed t – test was performed. The significance level was set equal to 0,05.

Results

Establishment of a DCIS – fibroblast co-culture model

To establish a co-culture model of MCF10DCIS.com cells and fibroblasts, primary fibroblasts were isolated from a healthy donor, and two types of TAFs, one from a patient biopsy with moderately differentiated breast cancer (type 1) and one with highly differentiated mucinous breast cancer (type 2). Approximately 14 days after isolation, sufficient actively proliferating fibroblasts were available for 3D co-culture experiments.

Over the course of 14 days the DCIS cells proliferate and coalesce into grouped round structures forming spheroids, which in co-culture with TAFs show an irregular surface structure. Fluorescent proteolytic fragments of the collagen are found in association with the surface of DCIS spheroids and of the fibroblasts.

Immediately after seeding, both cell types show a round morphology when observed by phase contrast light microscopy, while after 48 hours they appeared morphologically different. The fibroblasts in the lower layer were elongated and individually located, while the MCF10DCIS.com cells formed spheroids (Figure 1). Over time these spheroids grow bigger. After 14 days in co-culture, their shadow formation can be clearly seen when focusing on the collagen layer indicating that the distance between the cells reduced over time. There was no statistically significant difference in the mean diameter of the control spheroids, in co-culture with healthy fibroblasts or with TAFs respectively (day 7: 9,26 A.U. vs. 9,36 A.U. ($p=0,85$), 9,26 A.U. vs. 9,2 A.U. ($p=0,94$), day 14: 11,53 A.U. vs 11,76 A.U. ($p=0,81$), 11,52 A.U. vs 11,19 A.U. ($p=0,71$)). Comparing healthy fibroblasts with TAFs there was also no statistically significant difference in the change of the mean diameter (day 7: 9,36 A.U. vs. 9,26 ($p=0,81$), day 14: 11,76 A.U. vs 11,19 ($p= 0,58$)).

Evaluation of proteolysis and cell morphology of DCIS cells by confocal microscopy

Next, we studied the morphology and proteolytic activity of cell tracker-labelled MCF10DCIS.com cells and fibroblasts with confocal fluorescence microscopy. On day 2 of the 3D-culture the spheroids looked small, smooth-edged and round. Proteolysis was already ongoing during the formation of the spheroids, as indicated by the green signal caused by the degradation of DQ-collagen IV (Figure 2). At this point, there were no morphological differences regardless of the type of TAFs (Figure 2). On day 7, the control spheroids remained smooth and round. Otherwise, the spheroids that were co-cultured with TAFs of type 1 or type 2 had an uneven appearance with the cells leaving the formation of the spheroid (Figure 3). The spheroids co-cultured with healthy fibroblasts had a morphology similar to the spheroids in the control. Spheroids retained their acquired morphology over time, so that on day 14 the spheroids in co-culture with TAFs of type 1 or 2 had a phenotype suggesting invasiveness compared to the spheroids co-cultured

with healthy fibroblasts (Figure 4). Spheroids in co-culture with healthy fibroblasts showed the same phenotype as the control spheroids. Proteolysis continues on day 7 and 12 in all wells, as evidenced by the persisting green signal caused by the degradation of DQ-collagen IV (Fig. 3, 4).

Assessment of an invasive phenotype and migratory activity in the 3D DCIS co-culture model

The spheroid morphology of an invasive cell line was also examined to better assess the significance of the morphological differences. The triple-negative, invasive breast cancer cell line MDA-MB-231 forms spheroids that are uneven and irregular in comparison to the MCF10DCIS.com cells in the control and similar to the MCF10DCIS.com cells in co-culture with TAFs (Figure 5). We therefore conclude that the morphological differences in the spheroids co-cultured with TAFs of type 1 and 2 correspond to the development of an invasive phenotype.

On day 2 as well as on day 7 and 14, a stronger intensity of the DQ-collagen I signal of the TAFs of type 1 and 2 could be seen compared to the healthy fibroblasts (Figure 6).

The intensity of the DQ-collagen I signal was quantified with the help of the ImageJ software. The mean fluorescence intensity per cell is shown in figure 6. The TAFs showed a higher proteolytic activity compared to the healthy fibroblasts on day 2 as well as on day 7 and 14.

Taken together the collagen and Matrigel layer were approximately 0.7 - 1 mm thick and so was the distance between fibroblasts and MCF10DCIS.com cells on day 2. On day 7 and 14 the distance became smaller. This applies to both TAFs and healthy fibroblasts. The cells migrate towards each other as observed under the light microscope, too (Figure 1, Figure 7). In the experiment shown, the distance between MCF10DCIS.com cells and healthy fibroblasts or TAFs is 890 μm and 700 μm on day 2, 760 μm and 560 μm on day 7 and 600 μm and 510 μm on day 14, respectively. This tendency has been observed in several experiments (n=9). However, there was no significant difference in the mean distance reduction between healthy fibroblasts and TAFs (day 2 to day 7: 120 ± 10 vs 115 ± 15 , $p = 0,81$, day 7 to 14: 130 ± 20 vs 35 ± 15 , $p = 0,14$) (Figure 7).

In summary, we show that MCF10DCIS.com cells form spheroids in Matrigel. The spheroid formation alone causes proteolysis indicated by the green DQ-collagen signal. The co-culture of MCF10DCIS.com cells with TAFs leads to the formation of irregular and uneven spheroids. This morphology of the spheroids corresponds to the morphology of the spheroids of invasive cells (MDA-MB-231). The TAFs also show an increased proteolytic activity compared to the healthy fibroblasts, as evidenced by the stronger DQ-collagen I signal. We also show that the cells apparently interact and migrate towards one another.

Discussion

Compared to the other precursors, DCIS lesions have the highest risk of progressing to IBC (15-30%) [2]. The driving force behind the development of IBC is not fully understood and clinical parameters to

estimate the further course of the in situ lesion are lacking. Current research suggests that the TME plays a critical role in this transition [18-26]. Further research into the causative factors for an invasion could enable personalized treatment. The aim of this work was to better characterize the role of fibroblasts in the conversion of DCIS to IDC. For this purpose, based on the published MAME model (28), a 3D co-culture model with fibroblasts primarily isolated from punch biopsies of the breast was established in order to initially look for morphological differences with confocal microscopy that indicate functional changes.

MCF10DCIS.com cells co-cultured with healthy fibroblasts form round and smooth spheroids in contrast to co-culture with TAFs: here the spheroids are uneven and resemble the morphology of the spheroids of invasive cells. We interpret these morphological differences between the spheroids as an indication that a reprogramming of fibroblasts into tumor associated fibroblasts plays a role in the conversion of DCIS lesions to an IBC. This coincides with the role of fibroblasts in the development of an in-situ to an invasive phenotype in current data [28, 30-33].

There is currently an intense debate on TAFs playing a crucial role in the development of DCIS into IDC and that these could represent a new therapeutic target. The biology and physiology of the TAFs as well as the full activation mechanism is still subject of research. Various studies have described that TAFs can arise from several cell types; resident tissue fibroblasts, bone marrow-derived mesenchymal stem cells, hematopoietic stem cells, epithelial cells (through epithelial-mesenchymal transition/ EMT) or endothelial cells (through endothelial-mesenchymal transition/ EndMT) [34,35].

The aim of systemic therapy, e.g. chemotherapy or endocrine therapy, is to destroy tumor cells. In most cases this is very successful in massively reducing the tumor mass. However, once the microenvironment has adopted a tumor-promoting phenotype, restoration of tumor growth of a few cancer cells that may potentially escape first-line therapy is very likely. Over 100 years ago, Paget et al. already suggested the importance of the TME with the "seed and soil" theory; the TME plays a critical role in the survival of tumor cells [36]. Obviously, the microenvironment can lead to renewed tumor growth. The targeted treatment of the tumor-promoting activities of TAFs is therefore an important therapeutic strategy [37].

Primary human breast fibroblasts (HMF) stimulated with TGF β 1 have similar characteristics to breast cancer-associated fibroblasts in vivo [38]. It has also been proven that certain miRNAs promote the conversion of resident fibroblasts into TAFs [39]. The elucidation of mechanisms that lead to the reprogramming of TAFs into normal fibroblasts could represent a new, promising therapeutic approach [40]. From this perspective, experimental strategies like our 3D co-culture system that address such issues are of high importance. Evaluating how current treatment strategies may affect cancer-associated fibroblasts, or identifying effective drugs targeting these cancer-associated cells may be the key in prohibiting or forecasting the progression of DCIS to invasive breast cancer.

The morphological differences between the spheroids presented in our work confirm that the TAFs are a critical entity in the development of an invasive lesion. The above-mentioned invasive phenotype of the spheroids is associated with increased proteolysis in the TAFs. The fluorescence signal from DQ-collagen

I was stronger in the TAFs, which indicates the greater invasive potential of such cells compared to the healthy fibroblasts. In addition, it was shown that co-cultured MCF10DCIS.com cells and fibroblasts migrate towards one another (Figure 7). Sameni et al. [28] have also observed that the distance between MCF10DCIS.com cells and TAFs (WS-12Ti) becomes smaller over time. If more cells migrate or these cells migrate faster towards each other when TAFs are involved still needs to be investigated.

The innovation of our work compared to the MAME method published by Sameni et al [28] is mainly the fact that the co-culture was partly carried out with primary cells. This enables a better simulation of the in vivo situation compared to commercially available cell lines. Although primary cells better represent the tumor biology in vivo, their life span is limited compared to immortalized cell lines and genetic changes may occur with every cell cycle compared to stable, immortalized cells. Cell lines provide an infinite supply of a relatively homogeneous cell population, so it can be ensured that consecutive experiments have a largely identical cell population. A generalization of results with primary cells should be avoided.

Our work has some limitations. The experimental approaches/attempts are not completely identical because of the lack of influence on the number and size of the spheroids formed, although the experimental conditions were as identical as possible, and the number of cells seeded was kept the same. Under the general assumption that any experiment is never completely identical due to technical limitations, from our point of view the experiments carried out are well comparable with each other and allow not only morphological analysis, but also functional investigations planned in the future. Overall, our results regarding the morphology of the spheroids, the DQ-collagen I signal and the distance between the cells were reproducible.

A weakness of in vitro cell cultures is that they are grown in the absence of their in vivo environment. In order to further investigate the development of an IDC from a DCIS, other cell types e.g. cells of the immune system, could be included. The 3D-culture is a design that better depicts the in-vivo situation than 2D cultures. Nevertheless, results from the 3D-culture should also be validated in vivo.

In summary, TAFs appear to play a crucial role in the development of DCIS lesions to IBC. MCF10DCIS.com cells develop an invasive phenotype in co-culture with TAFs which have a greater proteolytic activity compared to healthy fibroblasts. Our study provides the groundwork for follow-up studies to further investigate the role of fibroblasts including the biological behavior of fibroblasts deriving from tumors with different tumor biology (e.g. luminal G3, Her2-positive or triple-negative carcinoma). In addition, we suggest that the morphological differences seen reflect genetical changes in proliferation and / or invasion markers. Our novel 3D-culture model offers a simple in vitro model for the future study of molecular mechanisms underlying the communication of DCIS cells with their TME.

Declarations

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Figures

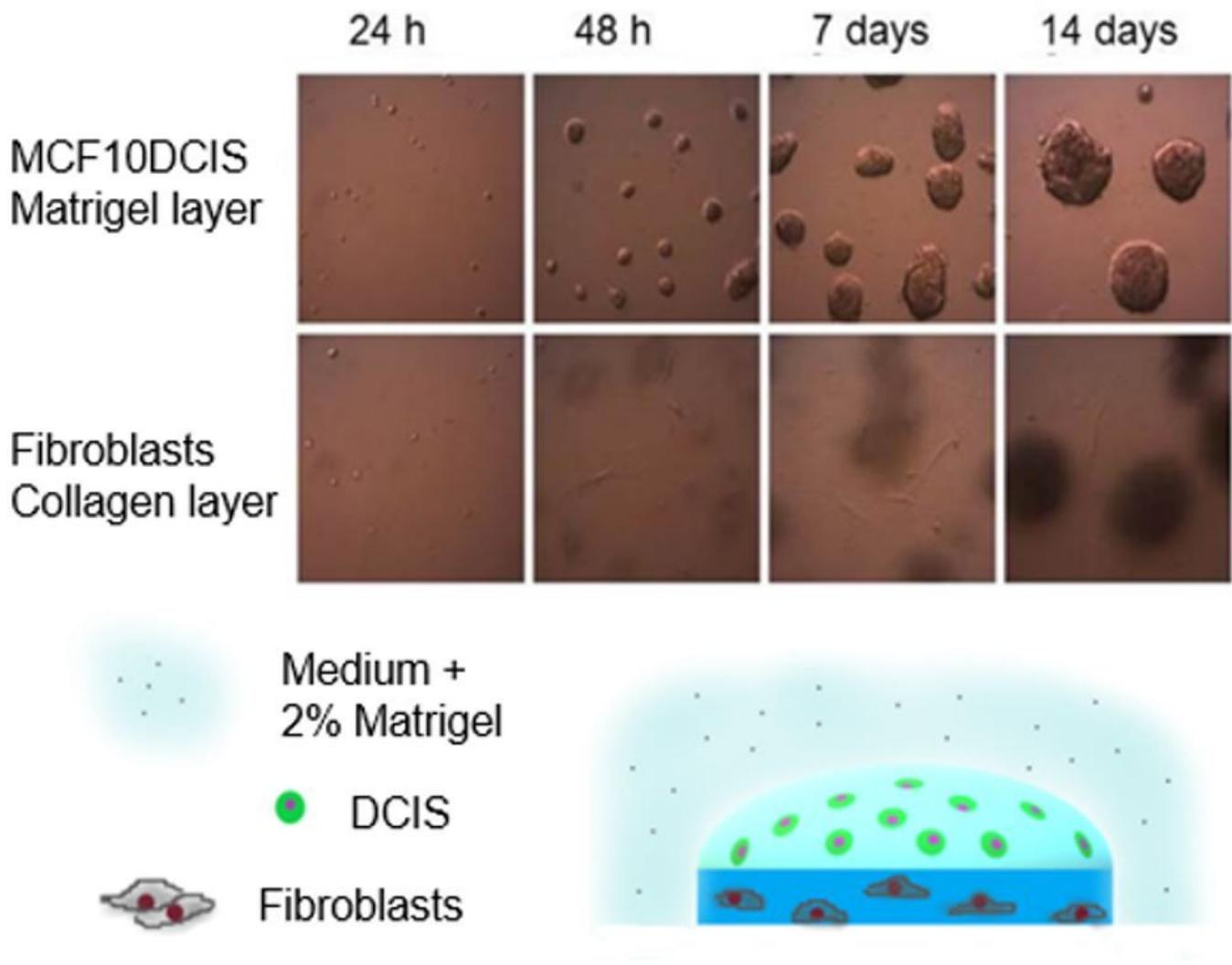


Figure 1

Composition of the 3D co-culture system and morphology of MCF10A.DCIS.com cells in Matrigel and of fibroblasts in collagen after 24h, 48h, 7 days and 14 days. The DCIS cells form spheroids, which grow over time. The distance between the two cell types decreases. The shadow of the spheroids appears in the collagen layer. Phase contrast microscopy. Magnification = 10x-fold.

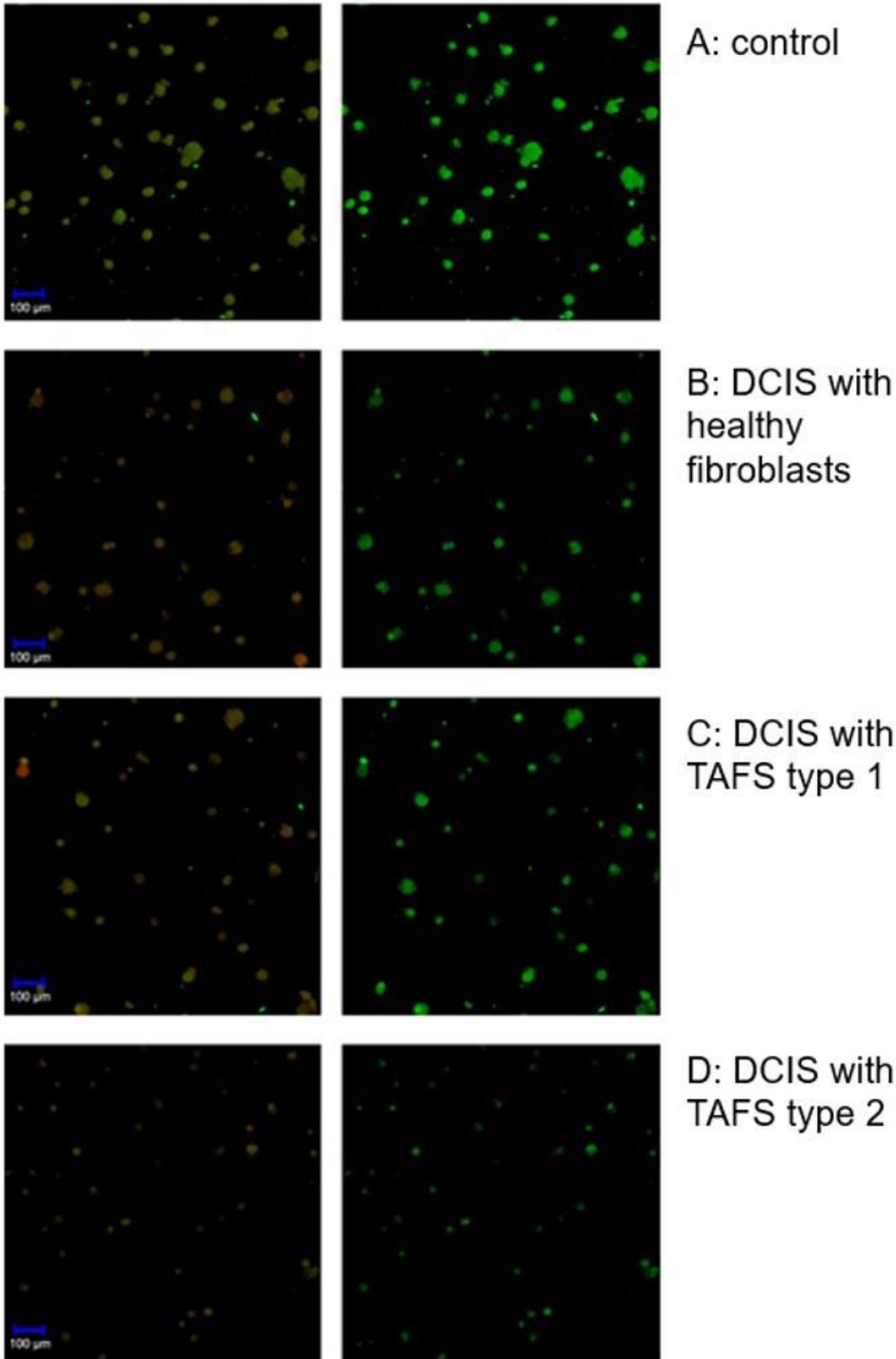


Figure 2

Evaluation of the 3D co-culture system by confocal microscopy. Spheroids on day 2 with DCIS control (A), DCIS with healthy fibroblasts (B) and with tumor-associated fibroblasts of type 1 (C) or type 2 (D). The left panels show the formation of the spheroids using the two channels (HeNe543, argon diode 405-30), while the right panels show the green signal with the argon diode 405-30 only caused by degradation of

DQ-collagen IV. Proteolysis takes place in all wells. At this time point there are no differences in the morphology of the spheroids.

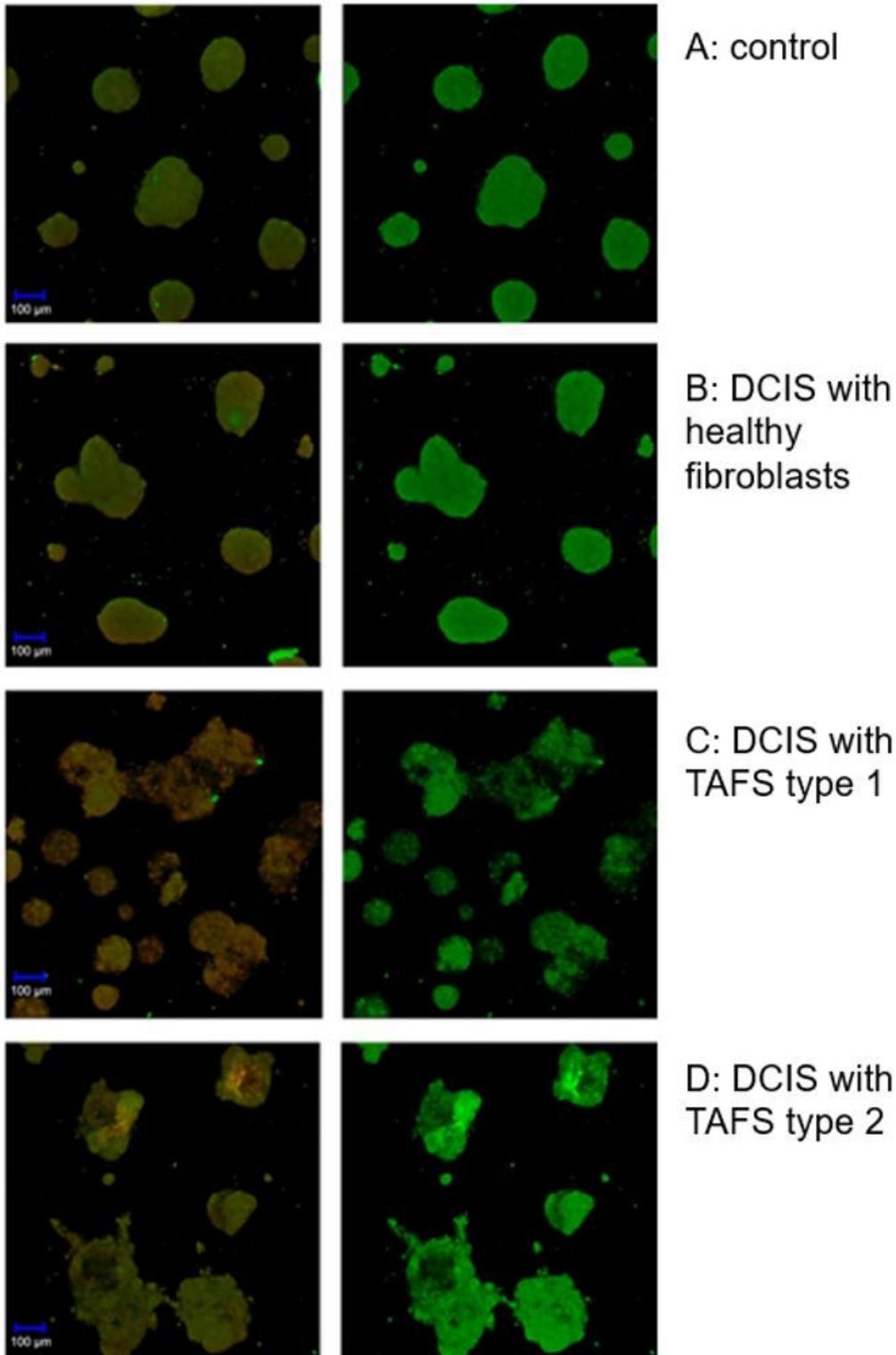
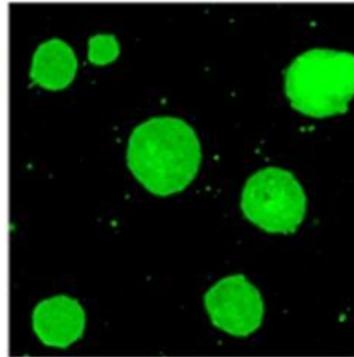
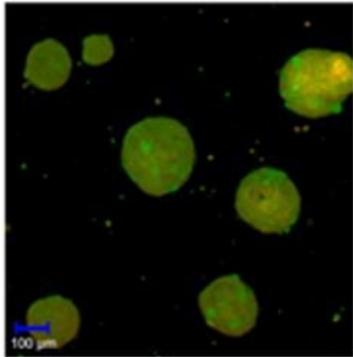


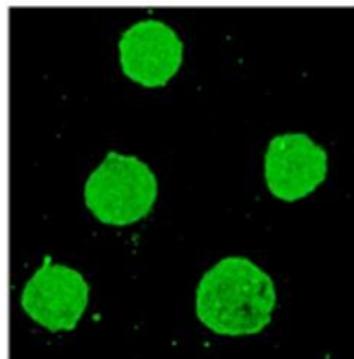
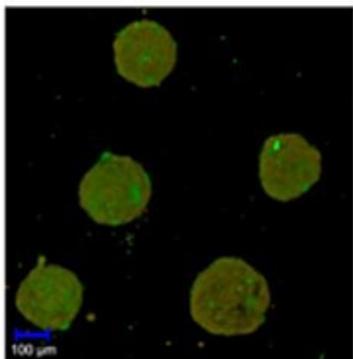
Figure 3

Formation of invasive structures in DCIS-TAF co-culture (day 7). Spheroids on day 7 with DCIS control (A), DCIS with healthy fibroblasts (B) and with tumor-associated fibroblasts of type 1(C) or type 2 (D). Spheroids in co-culture with tumor associated fibroblasts of type 1 (C) or 2 (D) have an uneven surface

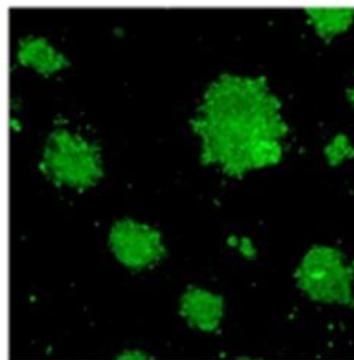
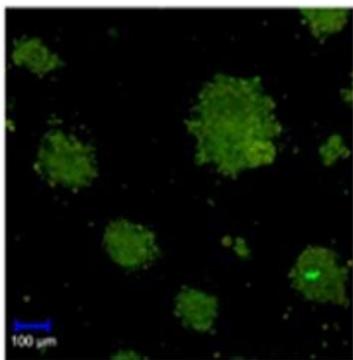
compared to the control (A) and to spheroids in co-culture with healthy fibroblasts (B). The left panels show the formation of the spheroids using the two channels (HeNe543, argon diode 405-30), while the right panels show the green signal with the argon diode 405-30 only caused by degradation of DQ-collagen IV.



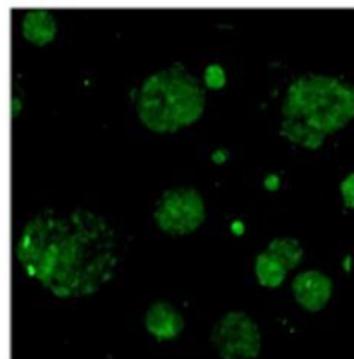
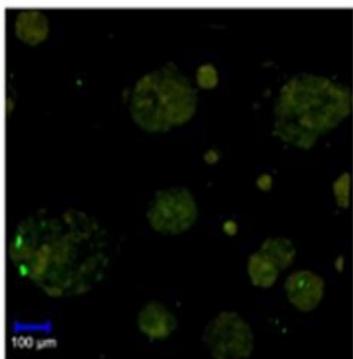
A: control



B: DCIS with healthy fibroblasts



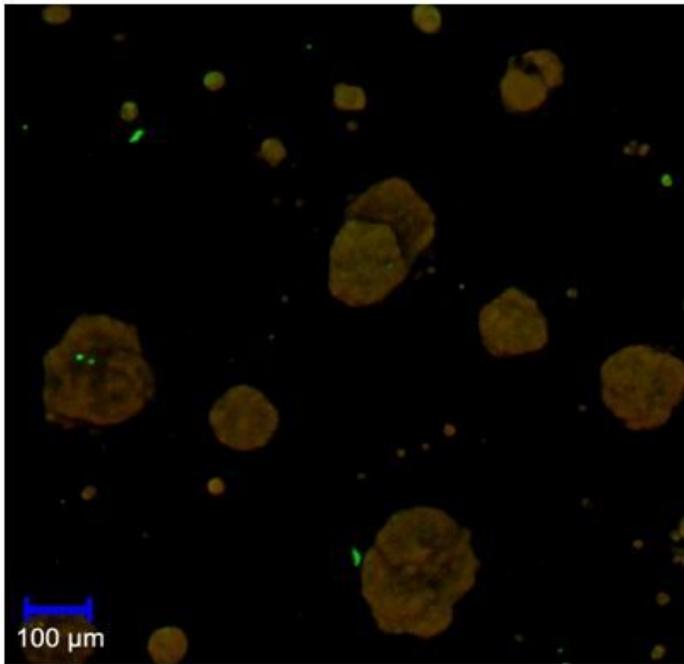
C: DCIS with TAFS type 1



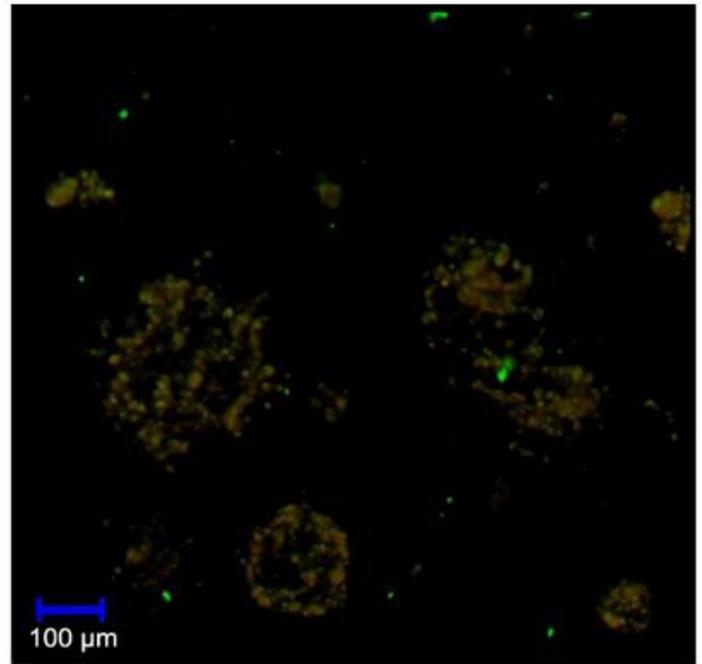
D: DCIS with TAFS type 2

Figure 4

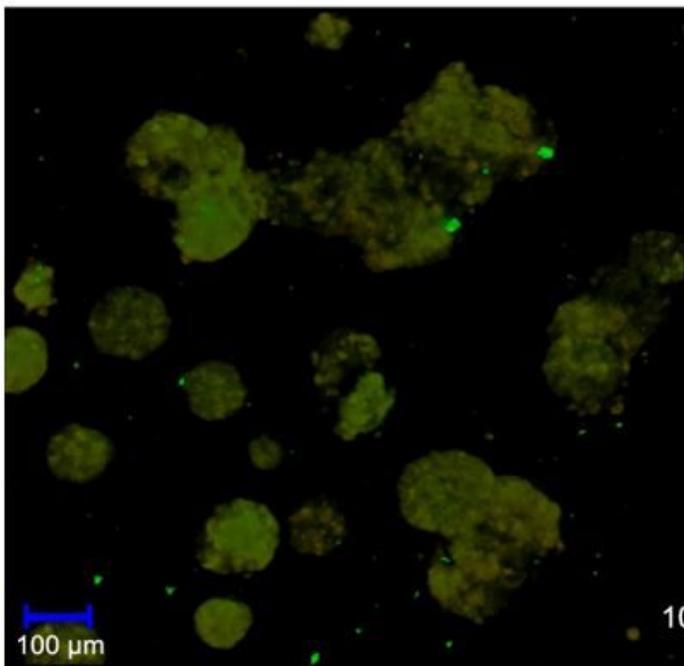
Formation of invasive structures in DCIS-TAF co-culture (day 14). Spheroids on day 14 with DCIS alone (A), DCIS with healthy fibroblasts (B) and with tumor-associated fibroblasts of type 1 (C) or type 2 (D). Spheroids in co-culture with tumor associated fibroblasts of type 1 (C) or 2 (D) have an uneven surface compared to the control (A) and to spheroids in co-culture with healthy fibroblasts (B). The left panels show the formation of the spheroids using the two channels (HeNe543, argon diode 405-30), while the right panels show the green signal with the argon diode 405-30 only caused by degradation of DQ-collagen IV.



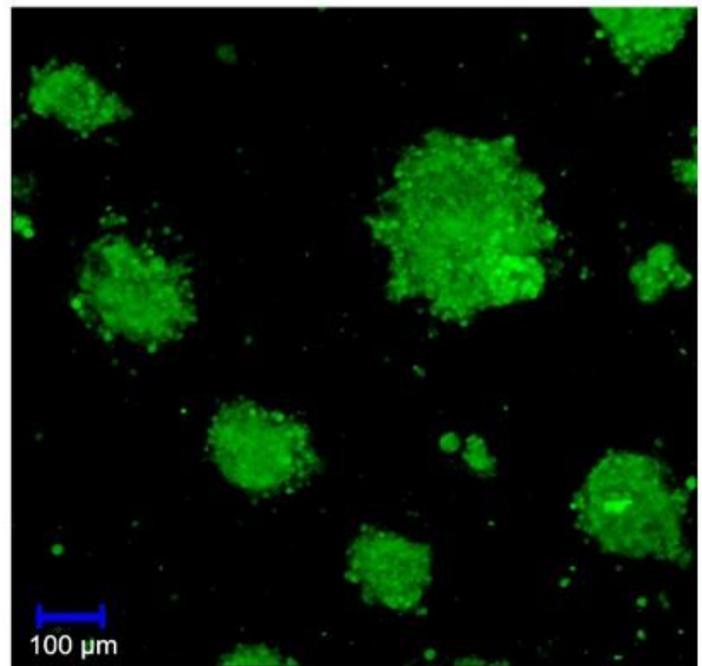
A



B



C



D

Figure 5

Assessment of the invasive phenotype by confocal microscopy. Spheroids of DCIS cells (A), MDA-MB-231 cells (B), and of DCIS cells in co-culture with tumor associated fibroblasts of type 1 (C) or type 2 (D) under the light microscope. The spheroids in C and D are morphologically similar to the spheroids in B. The morphological differences between the spheroids in C and D with the spheroids in A could correspond to the development of an invasive phenotype.

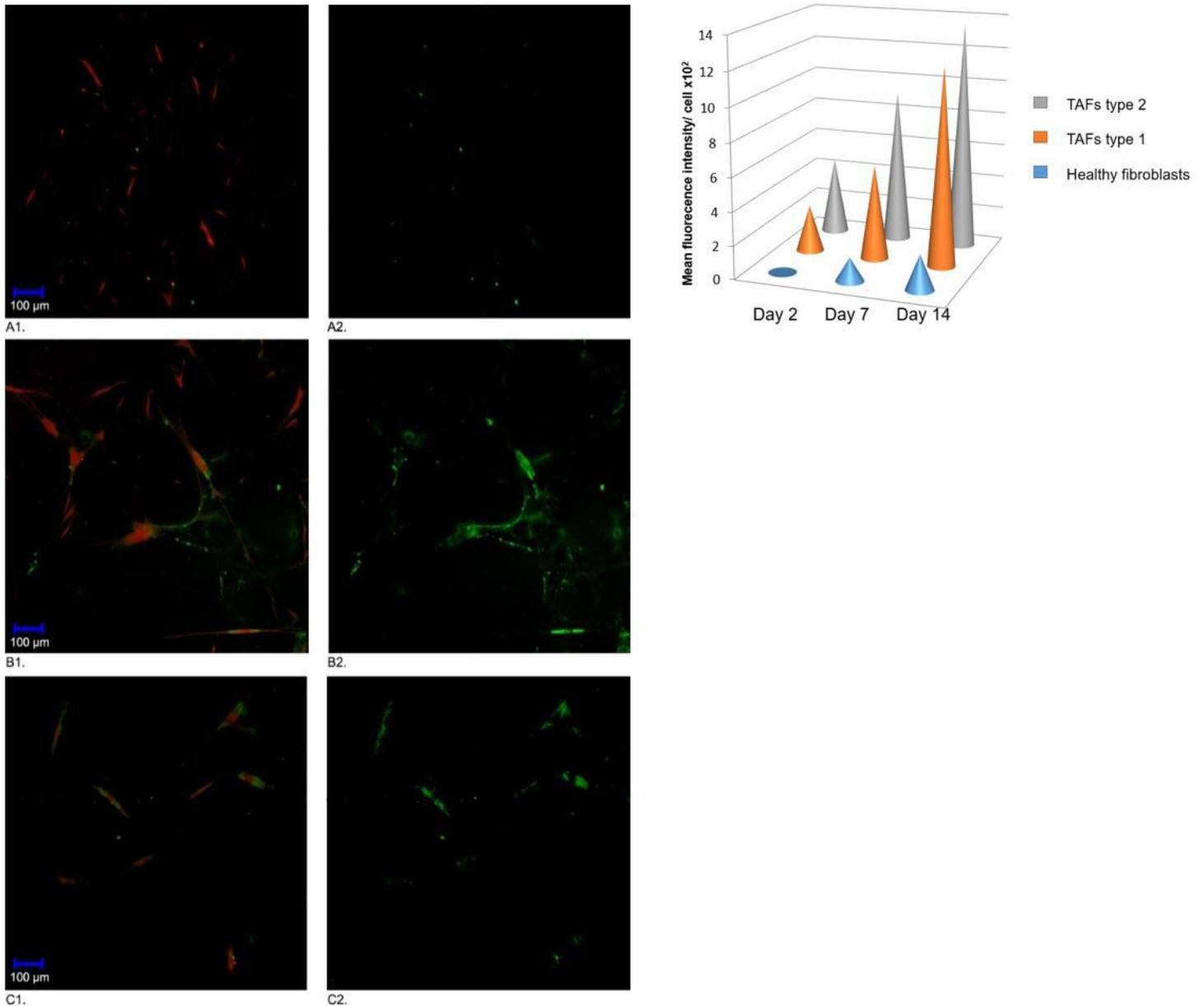


Figure 6

Analysis of the proteolytic activity of fibroblasts in the 3D co-culture system by confocal microscopy. Healthy fibroblasts (A) and tumor-associated fibroblasts of type 1 (B) and 2 (C) on day 2. Settings (magnification, laser intensity, gain for channel 1 and 2) are identical for the entire study. The TAFs have a stronger DQ-collagen signal (B2, C2) than the healthy fibroblasts (A2). The left panels show the cell

tracker-labeled cells, while the green fluorescent signal isolated in the right panels labels degraded DQ-collagen I. Graphic: The mean fluorescence intensity per cell for healthy fibroblasts (blue), TAFs of type 1 (orange) and 2 (grey) on day 2, 7 and 14. TAFs of type 2 show the greatest proteolytic activity. The laser intensity and gain for channel 2 was newly set on day 2, 7 and 14 to keep the signal-to-noise ratio as low as possible.

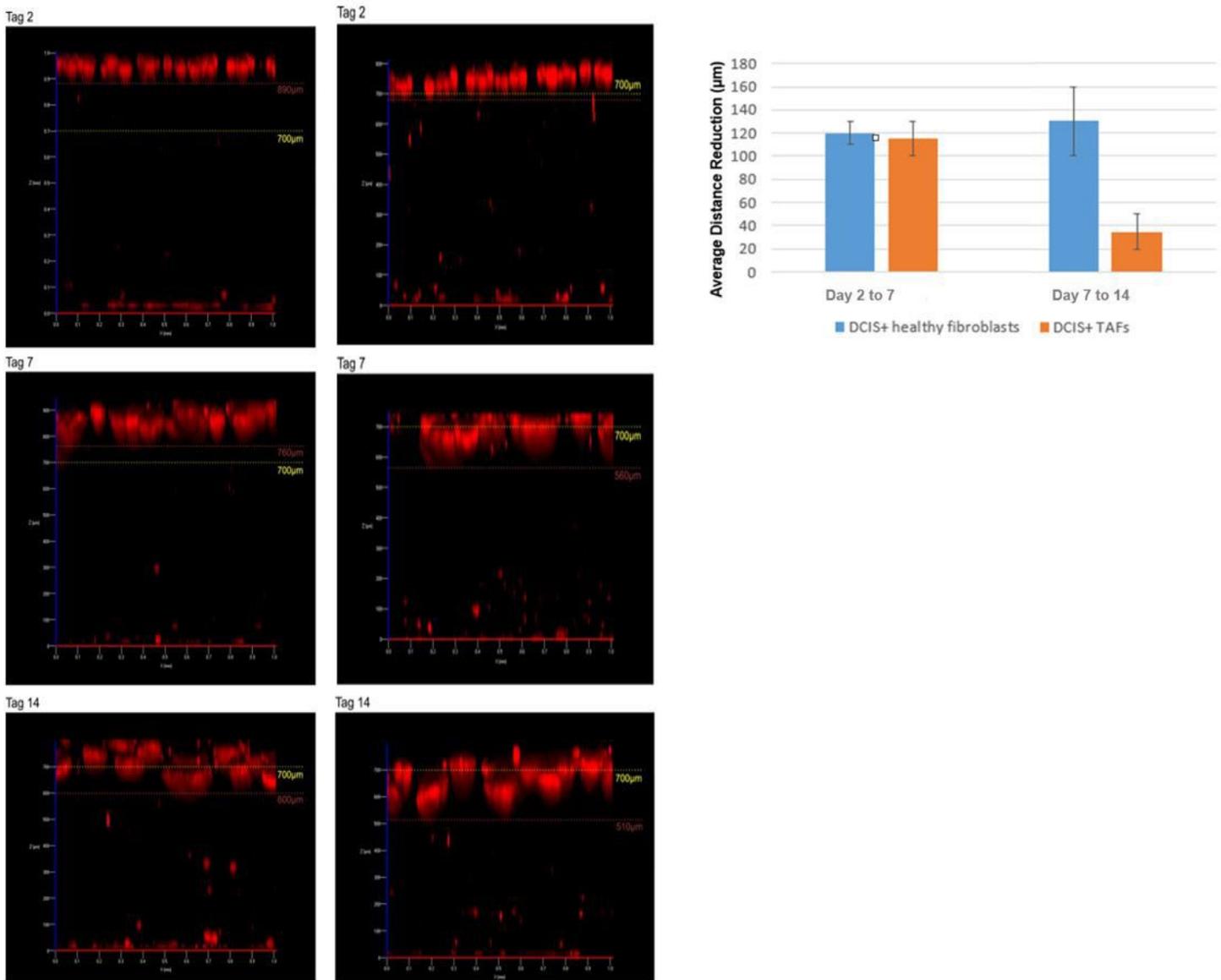


Figure 7

Assessment of cell movement in the 3D DCIS- fibroblast co-culture model. Left panels: Co-culture of MCF10DCIS.com-cells with healthy fibroblasts. The red line marks the bottom of the spheroids. The yellow line marks a distance of 700 μm to the fibroblasts. On day 2 the distance between the cells is 890 μm, on day 7 760 μm and on day 14 600 μm. The cells grow closer together over time, as the red line crosses the yellow one. Right panels: Co-culture of MCF10DCIS.com-cells with TAFs. The red line marks the bottom of the spheroids. The yellow line marks a distance of 700 μm to the fibroblasts. On day 2 the distance between the cells is 700 μm, on day 7 560 μm and on day 14 510 μm. The cells grow closer

together over time, as the red line crosses the yellow one. Graphic: Average Distance Reduction with standard deviation between DCIS/healthy fibroblasts (blue) and DCIS/TAFs (orange). There was no significant difference between the two groups ($p=n.s$).