Myoblasts inhibit prostate cancer growth by paracrine secretion of TNF alpha

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Abstract: PURPOSE: Myoblasts are capable of forming muscle fibers after transplantation and are therefore envisioned as a treatment for urinary incontinence after radical prostatectomy. However, the safety of this treatment and the interaction of myoblasts with remaining neighboring cancerare unknown. We investigated the interactions between myoblasts and prostate carcinoma cellsin vitro and in vivo. MATERIALS AND METHODS: Myoblasts isolated fromrectus abdominis were used in a series of co-culture experiments with prostate cancer cells and subcutaneously co-injected in vivo. Cell proliferation, cell cycle arrest and apoptosis of cancers in co-culture with myoblasts were assessed. Tumor volume and metastasis formation were evaluated in a mouse model. Tissue specific markers were assessed by immunohistochemistry, FACS analyses, Western blot and RT-qPCR. RESULTS: In this study we have demonstrated that myoblasts, in proximity to tumor, provide paracrine TNF to their microenvironment, decreasing tumor growth of all prostate cancer cell lines examined. Co-culture experiments showed induction of cell cycle arrest, tumor death by apoptosis and increased differentiation of myoblasts. This effect was largely blocked by TNF inhibition. The same outcome was demonstrated in a mouse model, where co-injected human myoblasts also inhibited tumor growth and metastasis formation of all prostate cancer cell lines evaluated. CONCLUSIONS: Myoblasts restrict prostate cancer growth and limit metastasis formation by paracrine TNF secretion in vitro and in vivo.

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Title: **Myoblasts inhibit prostate cancer growth by paracrine secretion of TNF alpha**

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ABSTRACT

Purpose: Myoblasts are capable of forming muscle fibers after transplantation and are therefore envisioned as a treatment for urinary incontinence after radical prostatectomy. However, the safety of this treatment and the interaction of myoblasts with remaining neighboring cancer **are** unknown. We investigated the interactions between myoblasts and prostate carcinoma cells *in vitro* and *in vivo*.

Materials and Methods Myoblasts isolated from *rectus abdominis* were used in a series of co-culture experiments with prostate cancer cells and subcutaneously co-injected *in vivo*. Cell proliferation, cell cycle arrest and apoptosis of cancers in co-culture with myoblasts were assessed. Tumor volume and metastasis formation were evaluated in a mouse model. Tissue specific markers were assessed by immunohistochemistry, FACS analyses, Western blot and RT-qPCR.

Results: In this study we have demonstrated that myoblasts, in proximity to tumor, provide paracrine TNFα to their microenvironment, decreasing tumor growth of all prostate cancer cell lines examined. Co-culture experiments showed induction of cell cycle arrest, tumor death by apoptosis and increased differentiation of myoblasts. This effect was largely blocked by TNFα inhibition. The same outcome was demonstrated in a mouse model, where co-injected human myoblasts also inhibited tumor growth and metastasis formation of all prostate cancer cell lines evaluated.

Conclusions: Myoblasts restrict prostate cancer growth and limit metastasis formation by paracrine TNFα secretion ***in vitro*** and ***in vivo***.

INTRODUCTION

Skeletal muscle comprises nearly 50% of the human body, is richly vascularized but rarely the site of cancer metastases. The cellular and molecular mechanisms underlying this phenomenon are not yet understood [1]. An intrinsic protective mechanism avoiding ingrowth of metastatic cells and formation of new tumors seems to exist. At the same time, skeletal muscles are the source of myoblasts [2], which are capable of regenerating muscle fibers, and therefore are investigated for the treatment of several muscular dysfunctions, including stress urinary incontinence (SUI) [3], a common complication after radical prostatectomy [4]. Preclinical studies have demonstrated that myoblasts, when implanted in the urinary sphincter, efficiently restore continence [5]**.** The pelvic floor is also a frequent site of residual prostate cancer cells [6], but until now no investigations targeted cell fate and possible interactions between myoblasts and vicinal preexisting cancer.

Parallels have long been drawn between stem cells and cancer cells. In fact, both cell types share common features such as capacity for self-renewal, differentiation potential, relative quiescence, resistance to **drugs** and toxins, resistance to apoptosis, secretion of growth factors and stimulation of angiogenesis by production of vascular endothelial growth factor (VEGF) [7]. These features could result in two possible outcomes: Cell proliferation or cell death. For instance, the presence of VEGF, which is secreted by many stem cells and progenitor cells including myoblasts [8], has the potential to promote prostate cancer angiogenesis leading to enhanced tumor growth and bone metastasis [9]. On the other hand, myoblasts are activated by inflammation and use inflammatory cytokines to perform and regulate their cross-talk for activation and differentiation [2]. These same inflammatory cues, paracrine secreted by myoblasts, could **trigger** cancer apoptosis.

A recent study successfully demonstrated inhibition of melanoma cell growth in the presence of myoblasts, but failed to describe a possible intercellular mechanism that explains this cell behavior [10]. Upon differentiation, myoblasts secrete tumor necrosis factor alpha (TNFα), which plays a key role in myoblast activation and differentiation, thereby linking inflammation to muscle regeneration [11]. In tumor cells, TNFα activates two parallel pathways, nuclear-factor-κB (NF-κB) or c-Jun N-terminal kinase (JNK). If NF-κB is activated, TNFα acts as a growth promoter, stimulating proliferation and metastasis. However, if JNK is turned on, a Caspase3-dependent-apoptosis-pathway leads to cell death [12]. In this study, we demonstrate that myoblast-secreted-TNFα is capable of influencing vicinal prostate carcinoma by inducing cell cycle arrest and apoptosis *in vitro* and *in vivo*. Additionally, despite the proximity to prostate cancer, myoblasts will rapidly differentiate into well-organized myotubes. Cell-therapy with myoblasts might provide an ideal treatment of post-prostatectomy urinary incontinence by improving sphincter function and inhibiting potential recurrent prostate cancer growth in the pelvic floor.

MATERIAL AND METHODS

Cell Isolation and Culture

Upon ethical-approval and informed-consent, myoblasts were isolated from *rectus abdominis* biopsies of four male patients (65 ± 6.4y) undergoing abdominal surgery. Biopsies were immediately processed according to established protocols [13] and cells were expanded until passage 2 (P2) with a medium change every third day. Cell characterization was performed by FACS, Immunocytochemistry, RT-qPCR and Western Blot. Muscle tissue formation was assessed by injecting 5 million myoblasts with a collagen carrier (Collagen-type-I Rat tail,BD) into the subcutaneous space of nude-mice. Tissues were retrieved after three and six weeks for histological analysis. The three prostate carcinoma cell lines (ATCC-LGC Standard) were chosen according to their increasing clinical aggressiveness. They are retrieved from lymph node (LNCaP), bone (PC3), and brain (DU145) **metastases**. An aggressive vulvar **leiomyosarcoma** cell line (SK-LMS1) served as an additional cancer control. An indirect co-culture model (BD Falcon™) was applied, where cells shared culture medium (DMEM enriched with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin) thereby exchanging their cellular products without direct cell-cell-contact. Cancer cells were co-cultured with myoblasts of each patient for 10 **days** and analyses were performed at days 1, 4, 7 and 10. Myoblasts alone and cancer cell **line** cultures were used as control. **To maintain the total number of seeded cells in the single cell controls (myoblast or cancer alone), we have placed cells in both compartments, the culture dish and the insert.** All experiments were performed in triplicates and medium change was done every third day. TNFα was neutralized with a mouse monoclonal anti-TNFα antibody (Sigma,T-6817).

Growth Rate and Fiber Formation Assay (FFA)

In all cases, triplicate samples of log phase cells were plated at a density of $5x10^3$cells/cm^2. Cell number and viability was confirmed after trypsinisation by toluidine blue staining **(n=36)**. The formation of myofibers was examined on slide chambers and, after 8 days in differentiating condition myofibers were fixed (methanol, 7min), stained (1:20 Giemsa, 1h) and air dried. Images were taken with a Leica-Imager-M1 Microscope. **Five high-power-fields (HPF) per sample were analyzed (n=30) and results were expressed as differentiation rate, which was calculated by the number of nuclei in differentiated myofibers/HPF divided by the total number of nuclei/HPF**.

Western Blot

Cells were washed with PBS/protease inhibitor (Sigma) and lysed with lysis buffer (50mM Tris-HCl, 150mM NaCl, 10% glycerol, 1% Triton X-100, 2mM EDTA, 40mM ß-glycerophosphate, 50mM sodium-fluoride, 10µg/ml leupeptin, 10µg/ml aprotinin, 1µM pepstatinA, 1mM PMSF). Samples were centrifuged (10min, 13000rpm), and proteins determined in the supernatant. Culture medium proteins were concentrated by filtering through a 10kDa filter (Amicon Ultra, Millipore). Total protein was measured using DC™ Protein-Assay (Bio-Rad), and 30 µg of protein lysate was loaded on 12% Biorad gels. Proteins were transferred onto PVDF-membranes (Millipore), blocked (1h, 5% non-fat-dry-milk), and incubated (4°C, overnight) with anti-Desmin (1:100,BD Biosciences), anti-MyH (1:6,DSHB), anti-TNFα (1:500,Sigma), anti-$p21^{WAF1}$ (1:1000,Calbiochem), Cleaved Caspase-3-Asp175 (1:1000,Cell- Signaling) and anti-GAPDH (1:2000,Sigma). Membranes were washed, incubated with HRP-conjugated

secondary antibody and developed by ECL-technique (ECL-Kit,Amersham).

RT-qPCR

RNA extraction, cDNA preparation and RT-qPCR reactions were done using Taqman® gene expression assay kits (Applied Biosystems), according to manufacturer's protocols. Data were normalized with 18S expression, quantitatively analyzed by quantification cycles (C_q) and fold changes and graphically represented in amplification plots. MIQE guidelines were followed.

In vivo Experiments and Tumor Size Determination

Myoblasts and tumor cells were cultured as described above, mixed with a collagen carrier (1mg/ml) and bilaterally injected into the dorsal subcutaneous space of 8 nude-mice (n=16 samples) per group. Cell-cell interactions *in vivo* were examined on day 21 and 42 after injection using nine groups: Myoblast ($5x10^6$) alone and co-injected with each of the four cancer cell lines (LNCaP, PC3, DU145 and SK-LMS, $2.5x10^6$) and the four tumors alone ($2.5x10^6$).

The experiments were performed in triplicates and repeated four times with myoblasts from 4 different patients. Tumor volume and growth was measured ($mm^3=a^2xb/2$). Animals were sacrificed by CO_2 asphyxiation. Tumor/sample size, myoblast differentiation, tumor aggressiveness and metastasis (lymph node, lung and liver) were assessed after 21 and 42 days by histological staining.

Histological Staining and Immunocytochemistry

Cells/tissues were fixed (4% PFA), permeabilized (0,5% Triton), blocked (5% BSA/0,1% Triton) and immunolabelled with anti-Pax7 (1:200,Sigma), anti-MyoD (1:100,BDPharmingen), anti-Desmin (1:50,BDBiosciences), anti-MyH (1:4,DSHB)

and anti-sarcomeric actinin (1:1000,Sigma), and incubated for 1 hour with anti-mouse-IgG-Cy3 (Sigma). Digital images were taken with a Leica Imager M1 Microscope. Tissues were also stained with hematoxylin and eosin (HE). Histomorphometric analysis was performed using “IMAGEJ for microscopy”.

Statistics

Presented data are expressed as averages with corresponding standard deviation. Analyses by independent samples t-tests, one way ANOVA **with *Bonferroni post hoc analysis*** or Pearson Correlation were done with SPSS v20 (SPSS Inc,Chicago,IL). A $p<0.05$ was considered significant.

RESULTS

Cancer cells undergo cell cycle arrest and apoptosis in co-culture with myoblasts *in vitro*

Human myoblasts were successfully isolated and characterized by FACS, Immunocytochemistry (ICC) and Fiber Formation Assay (FFA). The cell population expressed 76.5 ± 2.3% PAX-7, 60.6 ± 6.3% MyOD, and 81.6 ± 2.5% desmin.

After 10 days in co-culture cancer cell lines significantly decreased ($p<0.001$) their growth by 87.9±0.3% for DU145, 66.0±0.1% for LNCaP, 33.6±6.7% for PC3, and 42.0±0.6% for SK-LMS1 (Figure 1A, 1B, 1C). The decrease in cancer cell growth was due to apoptosis and cell cycle arrest, as demonstrated by RT-qPCR and Western Blot (Figure 1D). LNCaP and PC3 showed a >9 fold increase of mRNA for Caspase 3 and $p21^{WAF}$ when grown in co-culture, while DU145 and SK-LMS showed a lower but still significant increase. Western Blot confirmed these data.

Myoblasts in co-culture with cancer cells (Figure 1E, 1F) displayed a decreased growth rate as well (Figure 1G), they fused and developed muscle fibers (Figure 1H, $p<0.001$). While only 4.2 ±1.0% of the control myoblasts formed muscle fibers, the differentiation ratio of myoblasts increased in parallel to the respective prostate cancer line aggressiveness ($p=0.011$), with rates of 11.5 ±5.5% in LNCaP, 14.5 ±6.9% in PC3 and 25.3 ±2.3% in DU145.

TNFα-dependent induction of cell cycle arrest and apoptosis in cancer cell lines co-cultured with myoblasts.

Myoblasts demonstrated a striking, up to 25 fold, increase of TNFα mRNA when exposed to tumor (Figure 2A). This led to significantly higher amounts of TNFα protein ($p<0.001$) in the medium in the co-culture system (Figure 2B). **The relative protein expression levels measured in the culture medium were 0.92±0.13 for**

myoblast control and 3.01±0.17 for LNCaP, 4.71±0.26 for PC3, 5.88±0.26 for DU145 and 5.15±0.33 for SK-LMS co-cultures. These myoblast-TNFα-secretion values increased gradually according to the corresponding prostate carcinoma aggressiveness ($p<0.001$), significantly correlating (Pearson:0.754, $p<0.001$) with the myoblast differentiation ratio (Figure 2C). **The TNFα concentration in culture medium also negatively correlated to cancer cell growth ($p<0.001$, Pearson value -0.58) suggesting that myoblast-paracrine-TNFα is sufficient to induce significant cancer growth inhibition. A parallel assay demonstrated that cancer cell lines alone do not reach detectable levels of TNFα mRNA or protein expression.**

TNFα-antibody blocking in co-culture permitted cancer re-growth at day 10 to 84.0±0.3% for LNCaP, 99.1±11.4% for PC3, 23.3±0.3% for DU145 and 82.2±1.4% for SK-LMS1 (Figure 2D) of control **values (=100%)**. Using TNFα blocking in co-culture also decreased the myoblast differentiation ratio by reducing Caspase3 and $p21^{WAF}$ mRNA and protein expression to control-levels (Figure 2E).

Myoblasts restrain tumor growth inducing cancer apoptosis and cell cycle arrest *in vivo*

Interactions between myoblasts and prostate cancer were further investigated *in vivo* by co-injecting myoblasts and tumor cells subcutaneously in nude-mice (Figure 3A). All co-injected samples showed a significantly reduced tumor growth after 21 and 42 days (Figure 3A, 3B. $p<0.05$). Despite rigorous mixing before injection, muscle and cancer grew in distinct clusters of each cell type (Figure 4). **Immunohistochemistry** was able to confirm the higher level of Caspase 3 and $p21^{WAF}$ expression in all co-injected cancer samples.

Lymph node micrometastas**e**s (Figure 3C, **3D**) were significantly reduced in co-injected groups (10.9%), when compared to control (90.6%, $p<0.001$). No metastases to lung and liver were detected. The extent to which myoblasts influenced cancer growth was again proportional to cancer aggressiveness (Figure 4). Histomorphometric distance analyses demonstrated that the tumor areas closer to the newly formed muscle underwent apoptosis and cell cycle arrest more intensely (Pearson: -0.91 and -0.86 respectively) supporting the hypothesis that soluble factors are responsible for the antitumor effects (Figure 5). Despite the evident changes in tumor behavior, muscle tissue developed a well-organized and differentiated structure *in vivo*. We could not detect any changes in muscle phenotype in the presence of tumor, which also preserved a similar expression of Desmin and $p21^{WAF}$.

DISCUSSION

Cell-cell interactions play a crucial role in tissue formation, regeneration processes and inflammatory reactions. Cellular signaling between neighboring cells is based on two main mechanisms: Growth modulation by endogenous secretion of active compounds and cell competition. These two mechanisms have been well documented in fibroblasts, which are capable of secreting growth factors and other peptides, thus delivering cues to neighboring cells. Fibroblasts isolated from breast tumor areas are permissive allowing breast cancer metastasis, whereas fibroblasts from normal breast tissue restrict tumor growth [14]. Cell competition has also been proposed to regulate early cancer stages, when developing cancer cells overcome genomic constraints[15]. It triggers apoptosis within and around tumors by promoting rivalry between different anaplastic and normal cell lineages [15].

We have demonstrated that myoblasts in co-culture with cancer cells significantly increase TNFα-secretion *in vitro* and *in vivo*. TNFα has been isolated and described 30 years ago, and its clinical application in cancer therapy has been studied ever since [16]. Due to its systemic toxicity, TNFα is clinically only recommended in the treatment of advanced neoplasia, including sarcoma and melanoma, and in advanced cases, when limb amputation would represent the next step[17]. Myoblasts are known to secrete higher TNFα levels when differentiating [18] and this paracrine-secretion evokes microenvironmental changes, which control muscle regeneration by activating Pax7 in quiescent myoblasts and thereby induce differentiation and muscle formation [11].

In our study myoblast-secreted-TNFα levels increase according to tumor aggressiveness, in accordance with previous findings correlating prostate cancer Gleason-score and inflammatory response to endogenous cytokines [19]. The presence of inflammatory factors related to muscle regeneration plays a role in

myoblast-secreted-TNFα regulation [11]. This leads to the hypothesis that specific inflammatory cues delivered by the prostate tumor stimulate neighboring myoblasts to produce higher TNFα levels. A potential pathway is the increase of TACE production due to stress and nutrient shortage, leading to increased release of endogenous TNFα by muscle cells [20].

To investigate the paracrine influences we used a co-culture system where myoblast and cancer cells share the same environment without cell-cell-contact. All cancer cell lines in co-culture showed a significant decrease in growth and increase in apoptosis and cell cycle arrest. This inhibitory effect was almost completely blocked when TNFα was antagonized. In cancer cells TNFα binds to TNFR-1 receptor and triggers Caspase-3 activation leading to an apoptotic cascade and cell death [16]. The dual effect of TNFα inducing differentiation in myoblasts and apoptosis in tumors can be explained by two parallel pathways: activation of p38α and c-Jun N-terminal kinase (JNK). Once p38α is activated, Pax7 initiates myogenesis and myoblast differentiation [11] and, by activating the JNK pathway, triggers cancer apoptosis through a Caspase-3-dependent pathway [12]. A further line of action of TNFα in cancer inhibition affects tumor vascularity, probably due to higher response to TNFα in tumoral vessels by receptor up-regulation (TNFR-1) [16].

Our findings demonstrate that co-injected myoblasts are able to limit cancer growth ***in vivo***, again significantly triggering a cell cycle arrest and apoptosis in the cancer tissue. The histomorphometric distance analysis confirmed the role of a soluble factor in hindering tumor growth, supporting that TNFα might be a key player. Further, a significant reduction of lymph node metastasis formation was shown after co-injection, indicating an effect on confining the cancer to the primary site. We anticipate that investigations targeting cancer-mediated-stress factors on muscle

cells **as well as the additional effects of physical cell-cell and cell-ECM (extracellular matrix) interactions** will be the focus of future efforts towards a better understanding of interactions between cancer and Adult Stem Cells, such as myoblasts.

This research was based on established models for cancer research, however the complex interactions between stromal and tumor cells were not addressed. We used human myoblasts and cancer cell lines in an animal model. In order to avoid xenograft rejection nude mice with limited immune response were used.

CONCLUSIONS

Myoblasts can be isolated from patients' muscle biopsies, rapidly grown in culture, implanted and thereafter form functional muscle within weeks. Our results indicate that differentiating myoblasts secrete TNFα, which induces apoptosis and cell cycle arrest in prostate cancer. These characteristics make myoblasts a promising cell source for muscle reconstruction, even in the proximity of cancer.

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FIGURE LEGENDS

Figure 1 – **Co-culture effects on myoblasts and cancer cells.** Cell growth rate, differentiation ratio, morphology and gene expression were influenced by co-culture. Prostate carcinoma and sarcoma cells significantly decreased in growth (A, B, E) and underwent apoptosis and/or cell cycle arrest (B, G). **Activation of the apoptotic pathway was determined by staining for cleaved Caspase-3 (Asp175). A positive staining was found in DU145 cells in co-culture with myoblasts (A) but not in control of tumor alone (B).** Desmin staining in co-culture (C) and control (D) demonstrated an evident differentiation of myoblasts into well-organized myotubes. Cytoskeleton labelled in green (Phalloidin 488) and secondary antibody in red (Cy3). Caspase 3 and p21 mRNA fold increase and protein expression (G) significantly increased when compared to tumor control (dashed line=1.0). Myoblasts differentiated rapidly in the presence of tumor, significantly increasing differentiation ratio and, consequently, decreasing cell growth (F, H). Samples in co-culture with myoblast were represented as (+ Mb) and control without myoblasts as (- Mb). mRNA fold increase was normalized with 18S reference gene (*$p<0.05$, **$p<0.001$).

Figure 2 – **Myoblast secreted TNFα induce myoblast differentiation and inhibit cancer cell line growth rate by inducing apoptosis and cell cycle arrest.** (A) RT-qPCR assay demonstrates that myoblast TNFα mRNA expression is increased after 4 days of co-culture with different cancer cell lines. A significant difference could be found between different prostate cancer cell lines, increasing according to tumor aggressiveness. (B) Myoblasts TNFα secretion increases according to the cancer aggressiveness in co-culture. CM: Culture Medium. (C) Myoblast differentiation ratio

correlate (Pearson correlation: 0.754) to the amount of produced TNFα . (D) Cell growth rate of cancer (LNCaP, PC3, DU145 and SK-LMS-1) was assessed by cell counting at day 1, 4, 7 and 10 of co-culture. All cancer cell lines showed a significant decrease in growth in the presence of myoblasts (bold lines), when compared to control (fine line). Cancer growth rate was in great part recovered (dashed lines) after TNFα neutralization. (E) Apoptosis and cell cycle arrest are triggered in all cancer cells co-cultured with myoblasts. Again TNFα blocking reversed in great part these effects. Caspase 3 and p21 tumor control mRNA fold increase is represented with a dashed line (=1.0). *p<0.05, **p<0.001

Figure 3 – **Tumor growth and lymph node metastasis was reduced *in vivo* in samples co-injected with myoblasts.** (A) 21 days after subcutaneous cell injection, tumor size was measured and a significant tumor size difference was found between co-injected and control samples. On day 42, myoblast co-injected tumor mass shrank, whereas control samples kept growing. (B) Final tumor size at day 42 was significantly smaller in myoblast co-injected samples. (C) Axillar lymph node metastasis assessment was performed by analysis of metastasis with H&E, Desmin and cytokeratin staining, positive lymph nodes. Ln ratio of axillar metastasis was also significantly reduced (p<0.001) in all tested cancers, when co-injected with myoblasts. **(D) Pan-cytokeratin staining of mice axillar lymph node 3 weeks after subcutaneous injection of LNCaP with and without myoblasts.** Samples co-injected with myoblasts were represented as (+ Mb) and control without myoblasts as (- Mb). *p<0.05, **p<0.001

Figure 4 – **Histological analysis of tumor formation *in vivo*.** At day 42, HE staining demonstrates a tendency of newly formed muscle and cancer tissue (first row) to

growth in clusters, with differentiated muscle areas impairing growth of neighbor tumor masses. In opposition, control cancers (second row) grow freely forming bigger and complex tumor masses. Increasing Caspase3 and $p21^{WAF}$ expression was detected in all tumors in samples co-injected with myoblasts (third and fifth row). Co-injected myoblasts differentiated into $p21^{WAF}$ positive muscle fibers. Samples co-injected with myoblasts were represented as (+ Mb) and controls are cancer cell lines injected without myoblasts (-Mb). In the co-injected samples muscle is represented with a “M” and cancer tissue areas with a “C”. DAPI (blue), anti-mouse IgG Cy3 (red), and the injected myoblasts were labeled *in vitro* with PKH 67 (green).

Figure 5 – **Histomorphometric analyses of co-injected tumor with myoblasts.** Histomorphometric analysis demonstrates a significant increase on apoptosis (A) and cell cycle arrest (B) in all co-injected tumors. (C) Three tumor areas were analyzed according to distance of newly formed muscle. The total positive area, calculated by fluorescence intensity, demonstrated a gradient of Caspase 3 and p21 expression in all cancers tested. These directly correlated with the proximity to differentiating muscle tissue. Samples co-injected with myoblast were represented as (+ Mb) and control without myoblasts as (- Mb). *$p<0.05$, **$p<0.001$

Key of Definitions for Abbreviations

TNFα – Tumor Necrosis Factor alpha
FACS – Fluorescence-activated cell sorting
WB – Western Blot
RT-qPCR – real time quantitative polymerase chain reaction
SUI – stress urinary incontinence
VEGF – vascular endothelial growth factor
NF-κB – nuclear-factor-κB
JNK – c-Jun N-terminal kinase
FBS – fetal bovine serum
FFA - Fiber Formation Assay
HPF – high-power-fields
PFA – Paraformaldehyde
HE – Hematoxylin and eosin
ICC – Immunocytochemistry
$p21^{WAF}$ – Cyclin-dependent kinase inhibitor 1A
TNFR-1 – Tumor Necrosis Factor receptor 1
Pax7 – Paired box protein 7
MyoD – Myogenic Differentiation

Figure1

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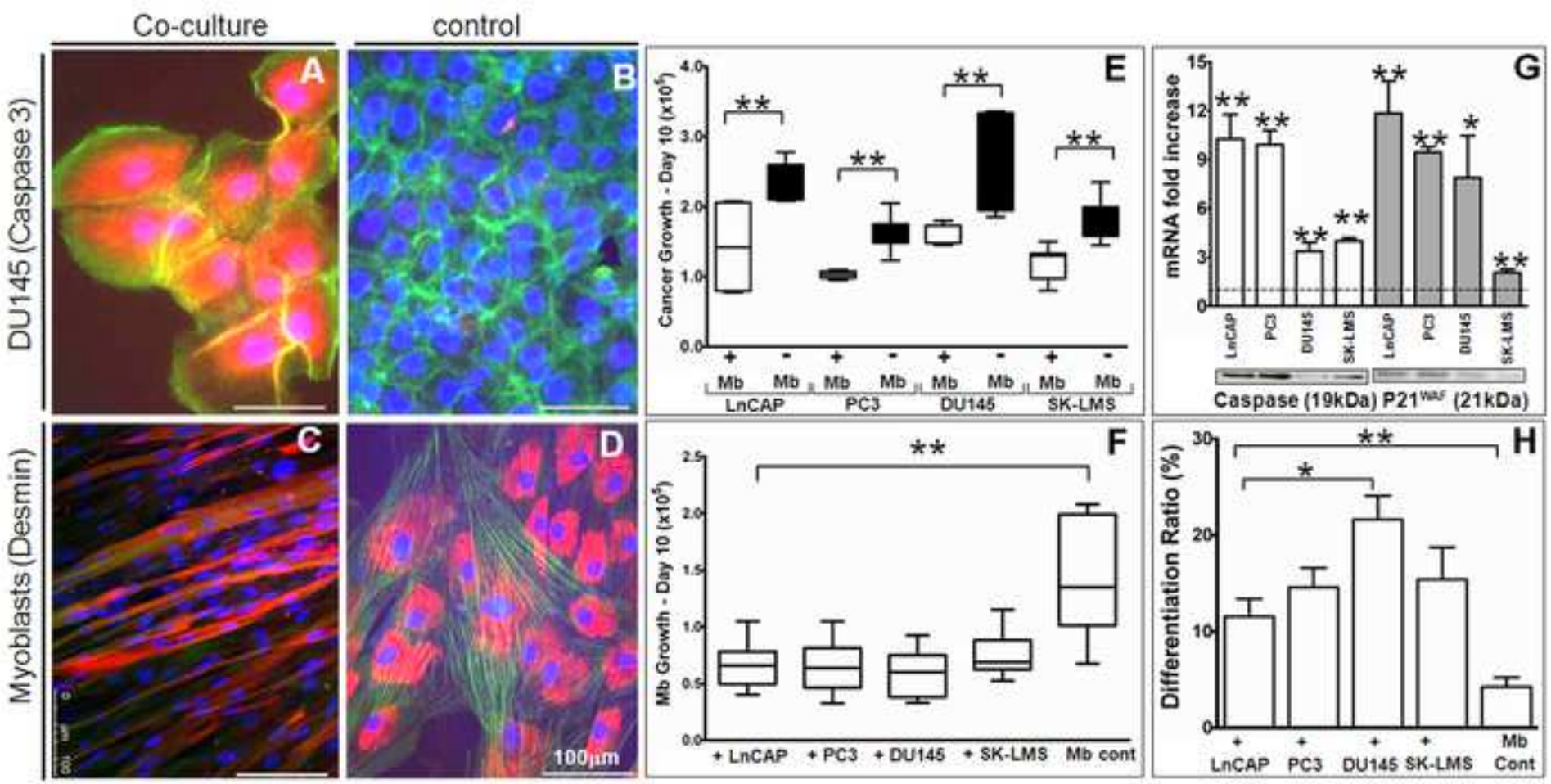

Figure2

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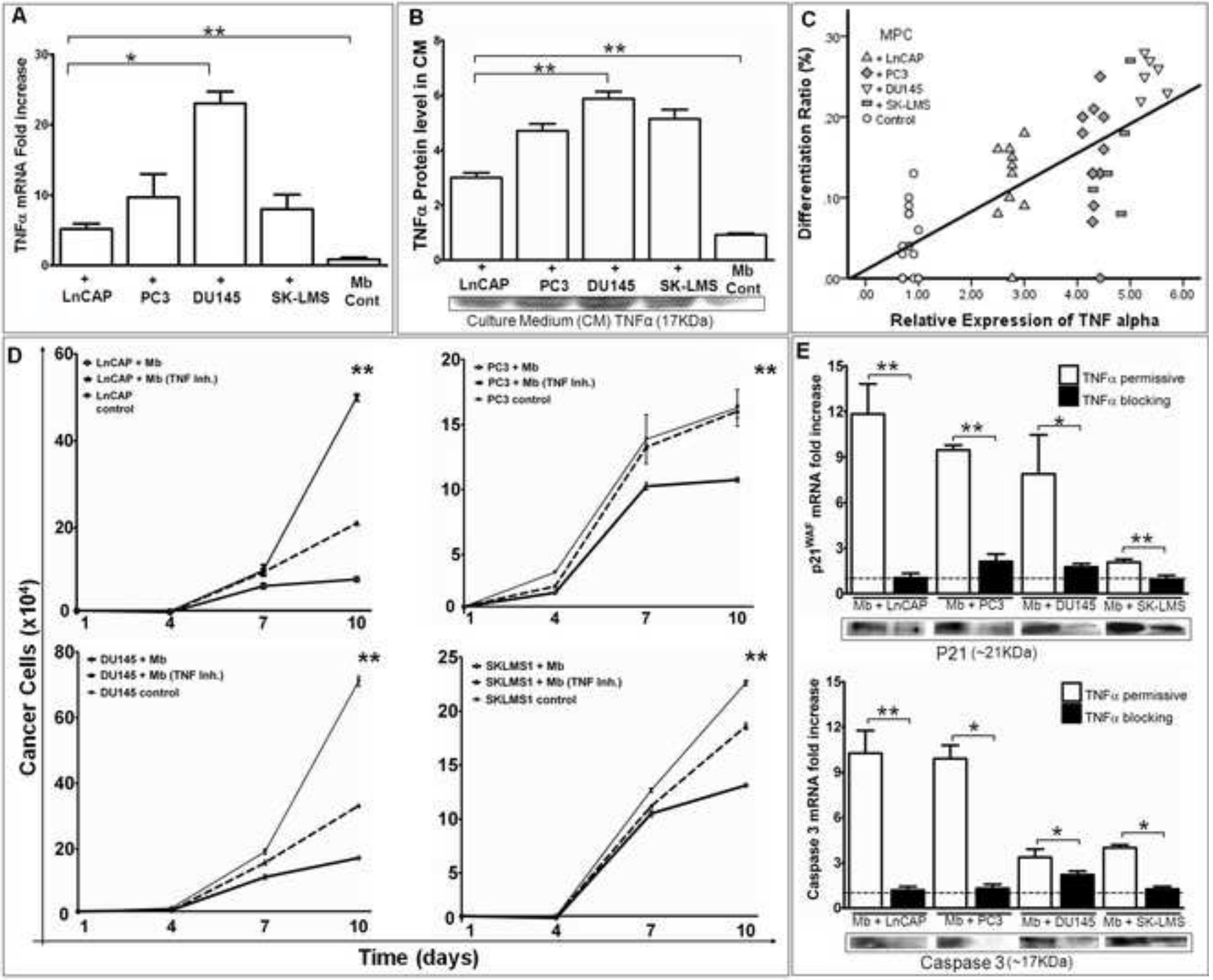

Figure3

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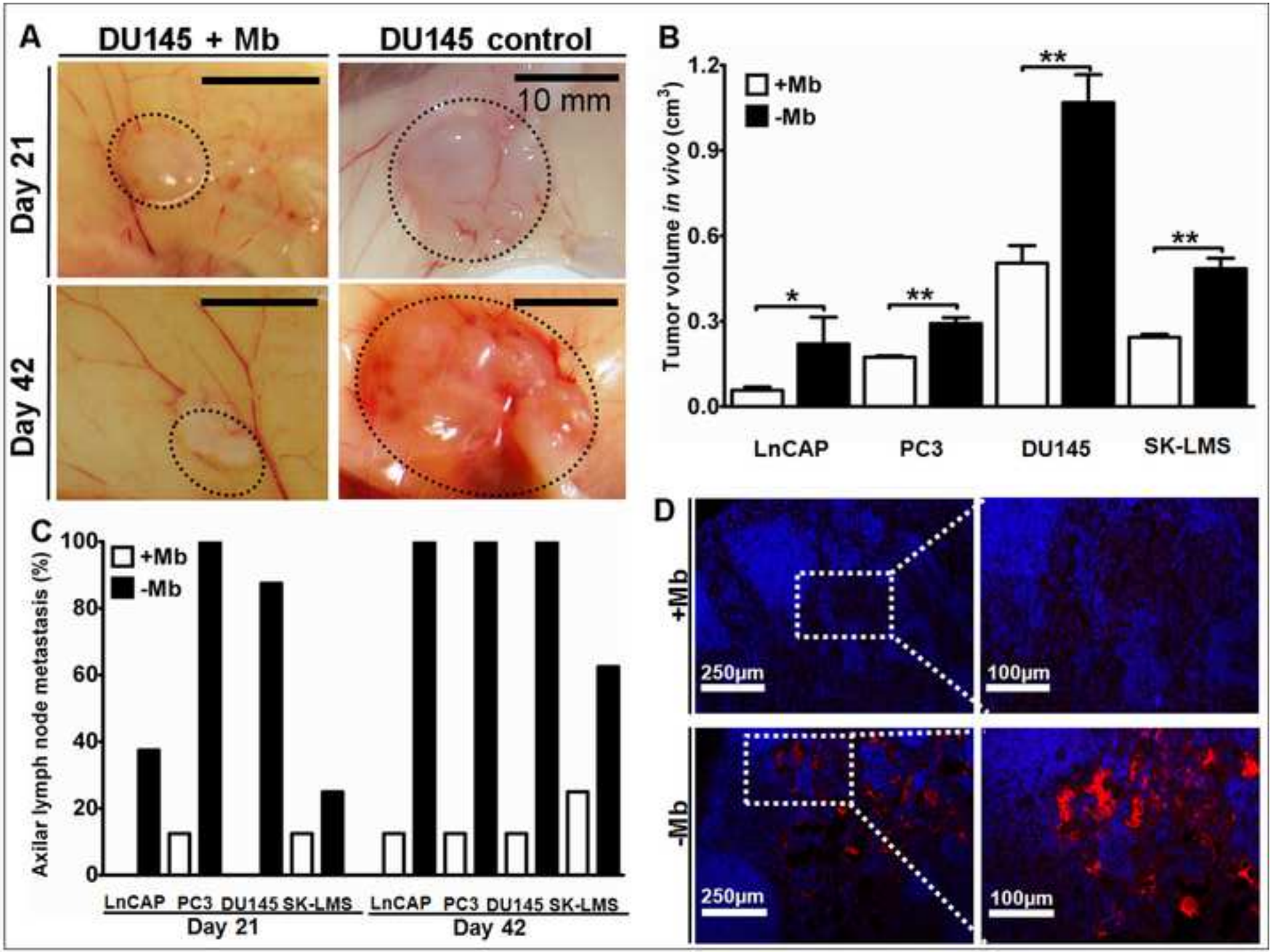

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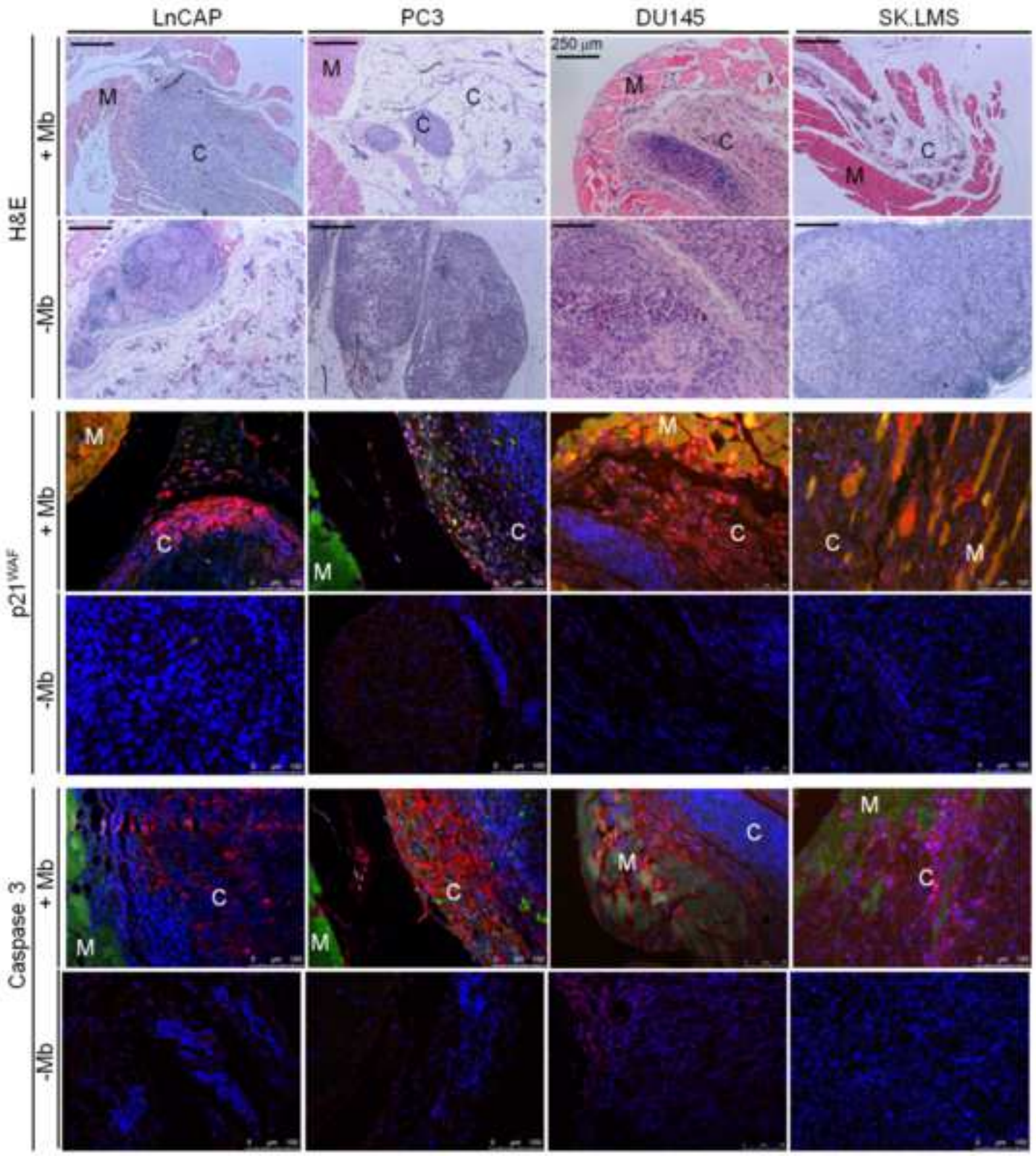

Figure5

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