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Quantitative and Functional Requirements for Bioluminescent Cancer Models

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Abstract. *Background: Bioluminescent cancer models are widely used but detailed quantification of the luciferase signal and functional comparison with a non-transfected control cell line are generally lacking. In the present study, we provide quantitative and functional tests for luciferase-transfected cells. Materials and Methods: We quantified the luciferase expression in BLM and HCT8/E11 transfected cancer cells, and examined the effect of long-term luciferin exposure. The present study also investigated functional differences between parental and transfected cancer cells. Results: Our results showed that quantification of different single-cell-derived populations are superior with droplet digital polymerase chain reaction. Quantification of luciferase protein level and luciferase bioluminescent activity is only useful when there is a significant difference in copy number. Continuous exposure of cell cultures to luciferin leads to inhibitory effects on mitochondrial activity, cell growth and bioluminescence. These inhibitory effects correlate with luciferase copy number. Cell culture and mouse xenograft assays showed no significant functional differences between luciferase-transfected and parental cells. Conclusion: Luciferase-transfected cells should be validated by quantitative and functional assays before starting large-scale experiments.*

Luciferase-transfected cancer cells are widely used in biomedical research applications. Bioluminescence has the advantage of allowing longitudinal monitoring of tumor growth, metastasis formation, and therapeutic responses (1, 2). The use of bioluminescence can significantly reduce the number of animals that need to be sacrificed in animal experiments (3, 4). Luciferase bioluminescence is based on the oxidation of luciferin in the presence of oxygen, adenosine triphosphate (ATP) and magnesium, resulting in the production of CO_2, inorganic pyrophosphate adenosine monophosphate and oxyluciferin. Oxyluciferin, in an excited state, falls back to its steady-state by emitting light (5, 6). The emitted light can be detected by a sensitive charged-coupled device (CCD) camera (5, 6).

Despite the global biomedical use of luciferase-transfected cell lines, there exist conflicting data on the effect of the luciferin–luciferase reaction on functional characteristics of the transfected cells. One group of researchers found that high expression of luciferase alters the transfected cells, causing inhibition of tumor growth (7). Others claim that there are no effects on cell growth, metabolism or immunological properties (8-10).

In the present study, we used different methods to quantify the amount of luciferase in transfected single-cell-derived populations. Furthermore, a minimal required number of functional tests were used to examine the impact of luciferase transfection and luciferin addition to cancer cells.

Materials and Methods

Cell lines. Human colon cancer cell line HCT-8/E11 and BLM melanoma cell lines [American Type Culture Collection (ATCC), Manassas, VA, USA] were maintained as described elsewhere (11, 12). The authenticity of ATCC cell lines was confirmed by short tandem repeat profiling in the last 6 months before use.

Transfection and selection of cells. Cancer cells were transfected with pGL4.50 vector (Promega, Madison, WI, USA) containing the

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firefly luciferase gene (*luc2*) and the antibiotic resistance gene (*Hygro*) by using FuGene (Promega). Luciferase-transfected cells were cultured in the presence of hygromycin B (400 μg/ml; Life Technologies, Waltham MA, USA). The surviving colonies were screened for bioluminescence positivity by adding culture medium supplemented with 150 μg/ml D-luciferin and using the *in vivo* imaging system (IVIS) as detector (Xenogene, Alamede, CA, USA). In a second selection round, bioluminescent and antibiotic-resistant colonies were seeded in a dilution of one cell/well in a 96-cell culture well/plate under continuous hygromycin selection. Different single-cell-derived populations were selected and named BLM_luc SCP 1, 15 and 16, and as HCT8/E11_luc SCP 3, 8 and 16.

Antibodies and reagents. Antibodies used were goat polyclonal anti-luciferase (G745A, 1:1000; Promega) and mouse monoclonal anti-α-tubulin (T5168, 1:5000; Sigma-Aldrich, St. Louis, MO, USA). D-Luciferin, potassium salt (PerkinElmer, Waltham, MA, USA) was used in bioluminescent imaging measurements and OneGlo (Promega) was used for relative luminescence unit (RLU) measurements. Hygromycin B (Life Technologies) antibiotics were used to select luciferase-positive single cell-derived populations.

Short tandem repeat (STR) profiling. DNA was extracted by using the DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA). The PowerPlexR 16 System (Promega) was used for human identification applications, according to the manufacturer's protocol.

DNA extraction and restriction digest. DNA concentration was measured by spectrophotometric measurement on an *Ultrospec Plus* Spectrophotometer (Pharmacie LKB Biotechnology, Uppsala, Sweden). Each DNA sample (200 ng) was digested with 2 units of *Hae*III in a total volume of 40 μl for 1 h at 37˚C. The digest was diluted 2-fold to a final concentration of 2.5 ng/μl. Two microliters (5 ng) was assayed per 20 μl droplet digital polymerase chain reaction (ddPCR).

Primer and probe design. The GenBank sequence EU921840.1, encompassing the luciferase reporter vector pGL4.50[*luc2*/*CMV*/*Hygro*], was used for the design of the *luc2* primers. A primer-BLAST was run from the start codon at bp 859 until the end of the coding region of luc2, namely bp 2511 (www.ncbi.nlm.nih.gov/tools/primer-blast). The following parameters were adapted: PCR product size: 70-120; Max T_m difference: 2˚C; primer length: min 16 – opt 20 – max 30; GC clamp: 2; Max GC in primer 3' end: 2; Primer GC content (%): min 30 – opt 50 – max 80. All primers were analyzed *in silico* using DINAMelt for homo- or hetero-dimer or formation (unafold.rna.albany.edu). The amplicon was then analyzed using mfold for investigating secondary structures. Primer pairs were selected with the least homo- or hetero-dimer and secondary structures. The resulting *luc2* assay sequences were (forward primer) 5'-CCCCGACACCGCTATCC-3', (reverse primer) 5'-TGAGCA CGACCCGAAAGC-3'.

ddPCR workflow. The PCR reaction mixture resulted from a 2× ddPCR Mastermix (Bio-Rad, Hercules, CA, USA), 20× primer and probes solution (final concentrations of 250 and 100 nM, respectively), 2 μl template (2.5 ng/μl) and water (variable volume) in a final volume of 20 μl. Each ddPCR reaction mixture was then loaded into the sample well of an eight-channel disposable droplet generator cartridge (Bio-Rad). A volume of 70 μl of oil containing emulsion-stabilizing, biocompatible surfactant was loaded into adjacent oil wells, and the microfluidic chip was loaded into the droplet generator. The droplet generator simultaneously partitions the sample into ~20,000 monodispersed droplets of known volume. After removing the cartridge from the droplet generator, the droplets in the droplet well were then transferred with a multichannel pipette to a 96-well PCR plate, heat-sealed with foil and then DNA was amplified to endpoint using a T100 Thermal Cycler (Bio-Rad) and the cycling protocol: 95˚C for 10 min then 40 cycles of 95˚C for 15 s and 59˚C for 1 min (2.5˚C/s ramp rate) with a final step at 98˚C for 10 min and 12˚C hold. Plates containing amplified droplets were loaded into a QX100 droplet reader (Bio-Rad), which streams droplets single-file (~1,500 droplets/s) past a two-color FAM/HEX detector. Discrimination between droplets that did not contain target (negatives) and those that did (positives) was achieved by applying a global fluorescence amplitude threshold. The fluorescence threshold was set dependent on the assay: BLM_luc copy number analysis had a threshold of 809 relative fluorescence units (RFUs) and HCT8/E11_luc copy number analysis had a threshold of 2395 RFUs. Concentration estimates were based on the fraction of droplets where amplification is modeled as a Poisson distribution. Analysis of the ddPCR data was performed with QuantaSoft analysis software version 1.3.2.0 (Bio-Rad).

The experiment was carried out in triplicate and analyzed data were also merged.

Luciferase reporter assay luminometry. To quantify the relative luminescence per cell, 4000 luciferase-transfected cancer cells were seeded in a black/clear bottom 96 cell culture well plate. Six hours after seeding, firefly luciferase activity was monitored using OneGlo luciferase assay kits (Promega) according to the manufacturer's instructions. Values are reported as relative luminescence units (RLU).

Continuous/intermittent exposure assay. To test the effect of continuous exposure of luciferin on cell lines with different luciferase expression, 10^3 BLM_luc SCP 1 and 16 were seeded in a black/clear bottom 96-well cell culture plate. Cells were exposed to medium supplemented with luciferin with daily refreshment (intermittent exposure) *versus* not (continuous exposure), and luminescent signal, mitochondrial activity and total protein concentration were compared at different time points. Luminescence signal was measured using OneGlo luciferase assay kit (Promega). Values are reported as RLU.

Mouse strain and animal care. Animals were treated according to the European guidelines on animal experiments (2010/63/EU). Animal studies were approved by the Animal Ethics Committee of Ghent University, Belgium (ECD 10/36). Mice used in these studies were 4-week-old female NOD/SCID mice (Harlan, Indianapolis, IN, USA). One million BLM as a control or BLM_luc cells, suspended in 100 μl serum-free culture medium, were subcutaneously injected into the ventral side of mice near the mammary fat pad. Each group consisted of three mice. *In vivo* images were made every week after inoculation.The primary tumor volume was quantified weekly by caliper measurements of the longest and the shortest tumor diameter ($V=0.4 \times (\text{longest axis}) \times (\text{shortest axis})^2$) (13). After 50 days, mice were sacrificed and tumor and lungs were resected.

Bioluminescent imaging and quantification. In total, 4000 luciferase-transfected cancer cells were seeded in a black/clear bottom 96-well cell culture well plate. Six hours after seeding firefly

luciferase activity was monitored. Bioluminescent imaging of cancer cells, primary and metastatic tumor growth and *ex vivo* imaging was described previously (14).

Polyacrylamide gel electrophoresis and western blotting. Samples of parental and *luc2*-ransfected cancer cells for western blot analysis of luciferase expression were prepared, run, and immunostained as described by Hendrix *et al*. (15). Bands were quantified by ImageJ software (Wayne Rasband, Bethesda, MD, USA). Parental cells were used as negative control, a commercial luciferase-positive cell line was used a positive reference control.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. MTT assay was used to investigate mitochondrial activity changes in BLM *luc2*-transfected cells after continuous/intermittent luciferin exposure. The assay was performed as described elsewhere (16). Data are reported as optical density (OD).

Sulforhodamine B (SRB) assay. SRB assay was used to investigate changes in total protein in BLM *luc2*-transfected cells after continuous/intermittent luciferin exposure as described in (16). Data are reported as OD.

Collagen invasion assay. To test invasion through extracellular matrix, the collagen type I invasion assay was used. The assay was performed according to De Wever *et al*. (17). Briefly, 5×10^4 BLM, BLM_luc, HCT8/E11 and HCT8/E11_luc cells were seeded as a single-cell suspension on 0.1% type I collagen gel (Santa Cruz Biotechnology, Dallas, TX, USA). After 24-h incubation at 37˚C and 10% CO_2, invasiveness was scored.

Statistical analysis. Statistical analyses was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) and confirmed by IBM SPSS Statistics 21.0 software (IBM, Armonk, NY, USA). All data were analyzed using Mann-Whitney *U*-test. All values are expressed as the mean±SD. A *p*-value of less than 0.05 was considered statistically significant.

Results

Luciferase transfection has no effect on cell-specific STR. To verify that luciferase transfection did not change the stability of the fingerprinting profiles of parental cancer cells, STR analysis was performed. STR profiles showed that there was no difference between transfected cancer cells and parental cancer cells (Figure 1).

ddPCR surpasses all other quantification techniques. Luciferase copy number, amount of protein and activity were assessed between different SCPs by different quantification techniques. ddPCR provided absolute quantification of the *luc2* DNA molecules. Analysis of the transfected BLM_luc SCPs revealed a significant difference in copy number of *luc2* between SCP 1 and SCP 16 and SCP 1 and SCP 15 ($p<0.05$). Analysis of transfected HCT8/E11_luc SCPs revealed no significant difference in copy numbers of *luc2* (Figure 2A and 3A). Luciferase protein quantification was achieved by western blot. Quantification showed no clear differences in relative protein expression between different SCPs (Figure 2B and 3B). Results showed that SCP 16, with the lowest copy number, also had the lowest protein expression. For BLM_luc SCP 1 and 15, copy numbers were slightly different; the protein expression had the same trend (Figure 2B). We used two techniques to measure the bioluminescence intensity (BLI). The first was based on a cellular lysate followed by the addition of luciferase substrate. Quantification of BLI showed only significant difference in RLU/cell between BLM_luc SCP 1 and 16 ($p<0.001$) and between BLM_luc SCP 15 and 16 ($p<0.001$) (Figure 2C and 3C). The second technique to quantify bioluminescent signals was by addition of luciferin to the living cancer cells and detection of BLI by a CCD camera. Quantification of these results also revealed a significant difference in BLI between BLM_luc SCP 1 and 16 and between BLM_luc SCP 15 and 16, but IVIS measurements revealed that SCP 15 had more BLI then BLM_luc SCP 1 despite having a lower copy number. No signal was seen in parental BLM cells (Figure 2D). Serial dilution of different BLM_luc SCPs revealed that a higher *luc2* copy number not only resulted in higher BLI, but also in detection of lower cell numbers. As expected, no signal was observed in parental BLM cells (Figure 2E). In transfected HCT8/E11 cells, no correlation was seen between increased copy number and increased bioluminescent signal.

Continuous luciferin exposure causes cell fatigue. In vitro and *in vivo* experiments with luciferase-positive cells require the addition of luciferin. We tested if there was a difference in mitochondrial activity, total protein and RLU between transfected cells continuously exposed to luciferin and transfected cells where the luciferin was washed-off after every measurement. This was performed for cells with a high (BLM_luc SCP 1) and a low (BLM_luc SCP 16) *luc2* copy number. MTT results showed that after 4 days of continuous exposure to luciferin, the mitochondrial activity decreased compared to cells under intermittent exposure (Figure 4A). The total amount of protein also decreased under continuous exposure, resembling to slower cell growth (Figure 4B). After 1 day's continuous exposure, a decrease in RLU was seen compared to intermittent exposure (Figure 4C). MTT and SRB results showed that after 3 days (BLM_luc SCP 1) or 4 days (BLM_luc SCP) only, there was an inhibitory effect on cell growth. These effects were more pronounced in cells with high *luc2* copy number.

Functional comparison between luciferase-transfected cancer cells and parental cells. SCPs should have identical functional characteristics compared not only to each other but also to the parental cells. Morphologically, we did not detect any differences between the transfected SCPs and the parental cell line (Figure 5A). Collagen invasion revealed no

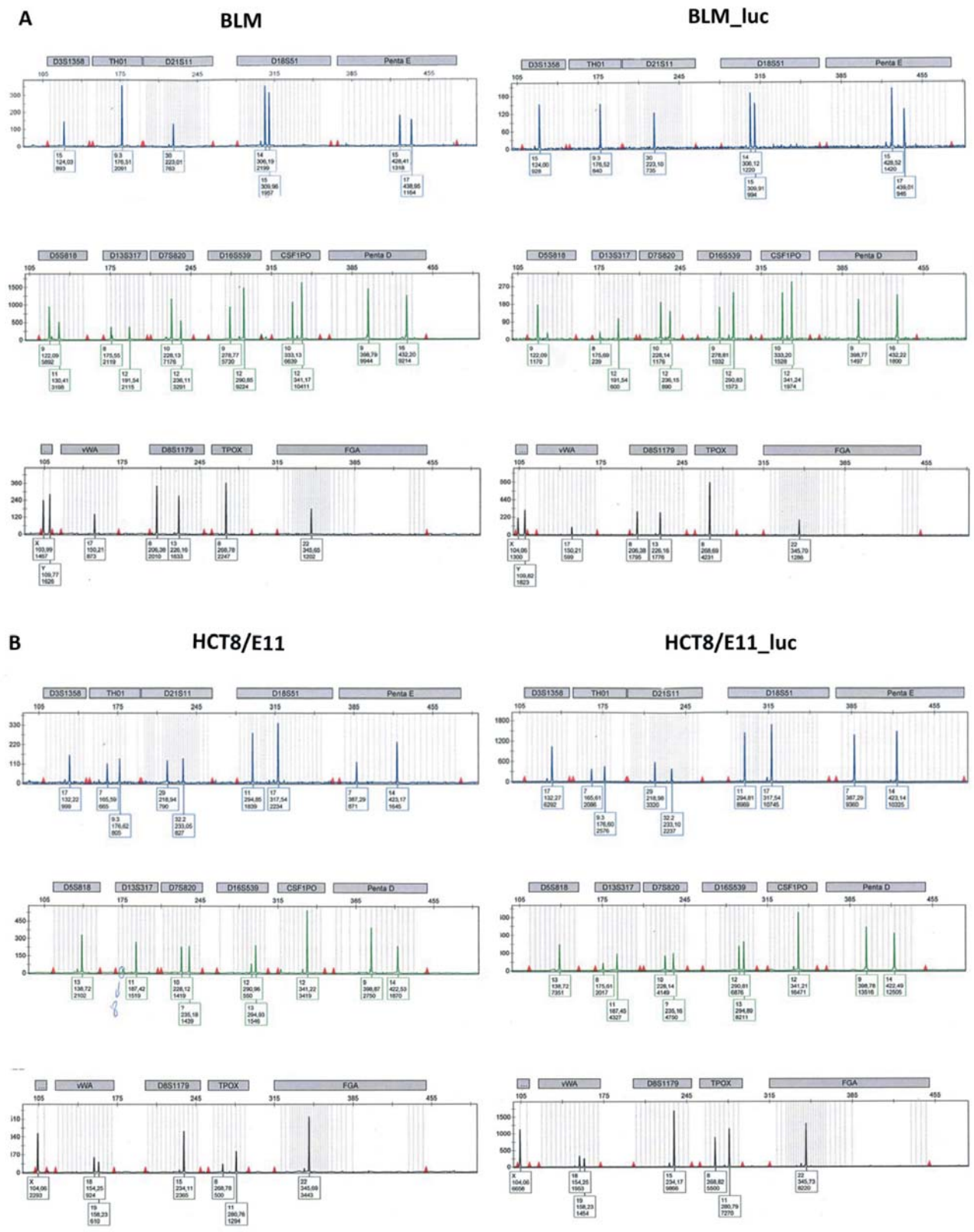

Figure 1. *Short tandem repeat (STR) sequence comparison between parental and luc2-transfected BLM and HCT8/E11. A: Comparison between BLM and BLM_luc cell line shows no difference in SRT sequence. B: Comparison between HCT8/E11 and HCT8/E11_luc cell line shows no difference in SRT sequence.*

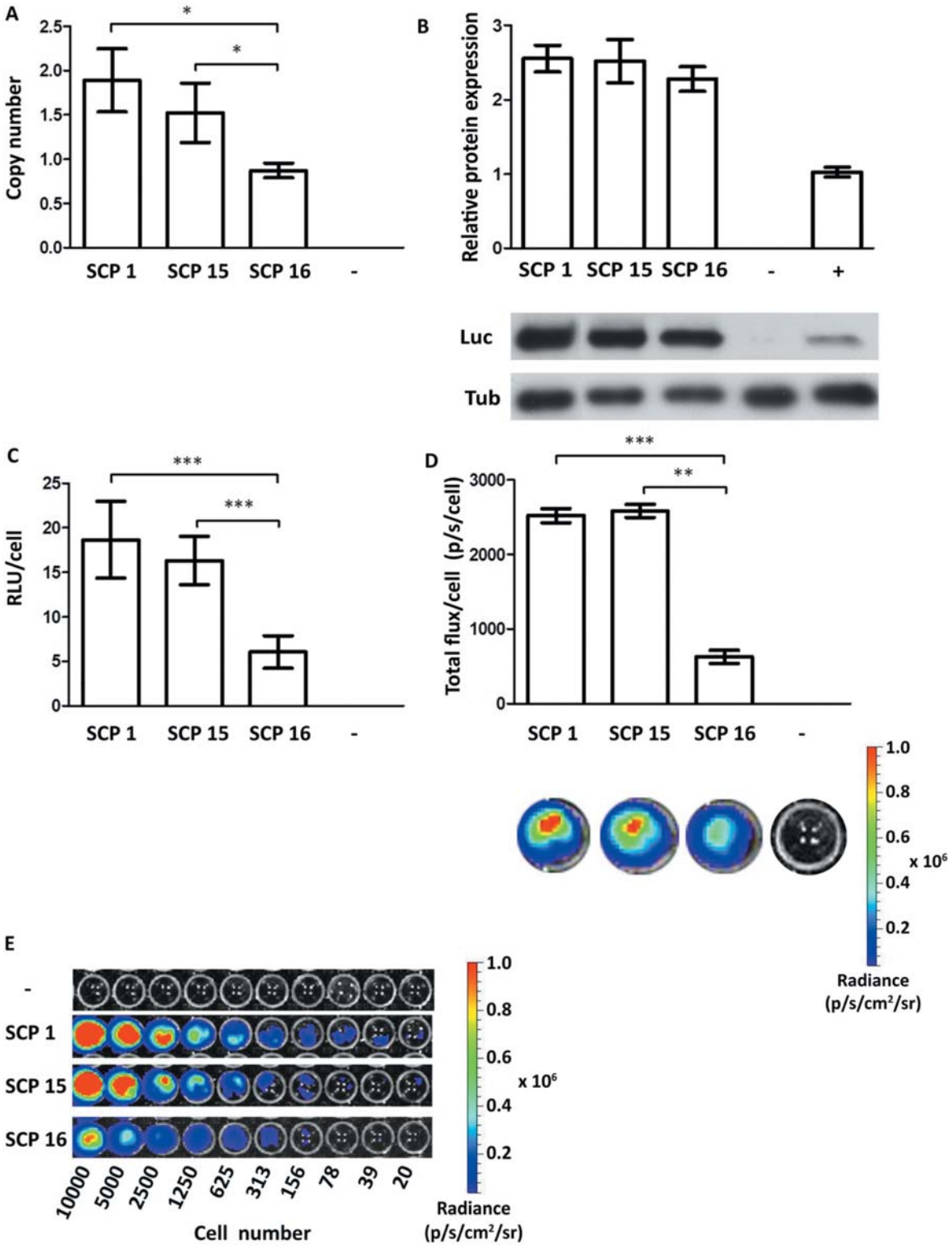

Figure 2. *Droplet digital polymerase chain reaction (ddPCR) surpasses all other quantification techniques in luciferase (luc2) quantification of luc2-transfected BLM cells (SCP 1, 15 and 16). Bar charts illustrating copy number quantification by ddPCR (A), luciferase protein expression quantification by western blot (B), bioluminescence quantification by luminometry (C) and bioluminescence quantification by CCD camera (D). E: In vitro bioluminescence of serially diluted SCPs and parental cells. Values are means±SD. Results are presented from three wells per assay from three independent experiments.*

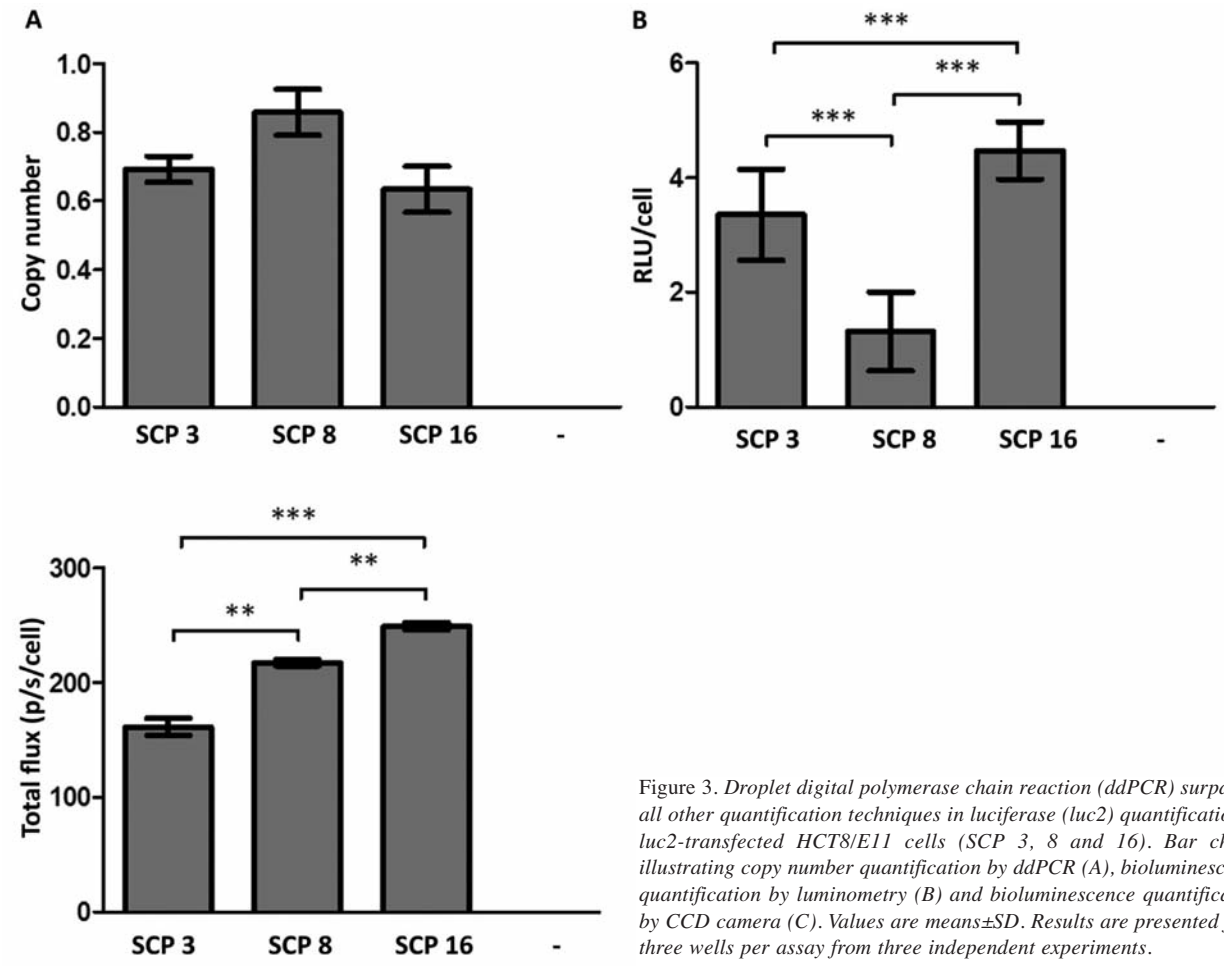

Figure 3. *Droplet digital polymerase chain reaction (ddPCR) surpasses all other quantification techniques in luciferase (luc2) quantification of luc2-transfected HCT8/E11 cells (SCP 3, 8 and 16). Bar charts illustrating copy number quantification by ddPCR (A), bioluminescence quantification by luminometry (B) and bioluminescence quantification by CCD camera (C). Values are means±SD. Results are presented from three wells per assay from three independent experiments.*

difference in invasive phenotype or invasion index (Figure 5B). To confirm that *luc2*-transfected BLM_luc SCPs grew *in vivo* in a manner comparable to the parental cells, both were injected subcutaneously (n=3 per cell line) and tumor volume was monitored using caliper measurements. Tumor growth pattern in mice injected with BLM_luc was similar to that of mice injected with parental BLM (Figure 6A). In general, the tumor BLI resembled the caliper-measured tumor volume (Figure 6B and C). At day 50, mice were sacrificed and *ex vivo* imaging was carried-out to confirm lung metastasis (Figure 6D). Immunohistological comparison of primary tumor and lung metastases showed no difference between parental and luciferase-transfected BLM cells. There was a necrotic center, and high vascular density was observed at the periphery of the primary tumor. Both tumor types from BLM parental and BLM_luc cells lacked the presence of inflammatory cells and fibroblasts minimally infiltrated. The topographical localization, size and number of metastases in the lung were similar for both cell lines (Figure 6E and F).

Discussion

Many researchers have already studied factors that may influence BLI signaling including: type of luciferase (18, 19), level of luciferase expression (7), concentration of luciferin injected (20), method of luciferin injection (21, 22), time of imaging (23), metabolism of cell/tissue (24), anesthetic used (25, 26) and plasma proteins (27).

Different complementary luciferase quantification methods were performed herein on multiple SCPs of a melanoma and a colorectal cancer cell line transfected with a luciferase-expressing plasmid. Superior sensitivity of ddPCR was observed compared to the bioluminescence assays and western blot in discriminating quantitative luciferase differences between the SCPs. To our knowledge, we are the first to report the comparison of luciferase activity with luciferase copy numbers and conclude that high protein expression and high BLI or RLU does not always mean that these cells have a higher *luc2* copy number. HCT8/E11_luc SCPs had only a

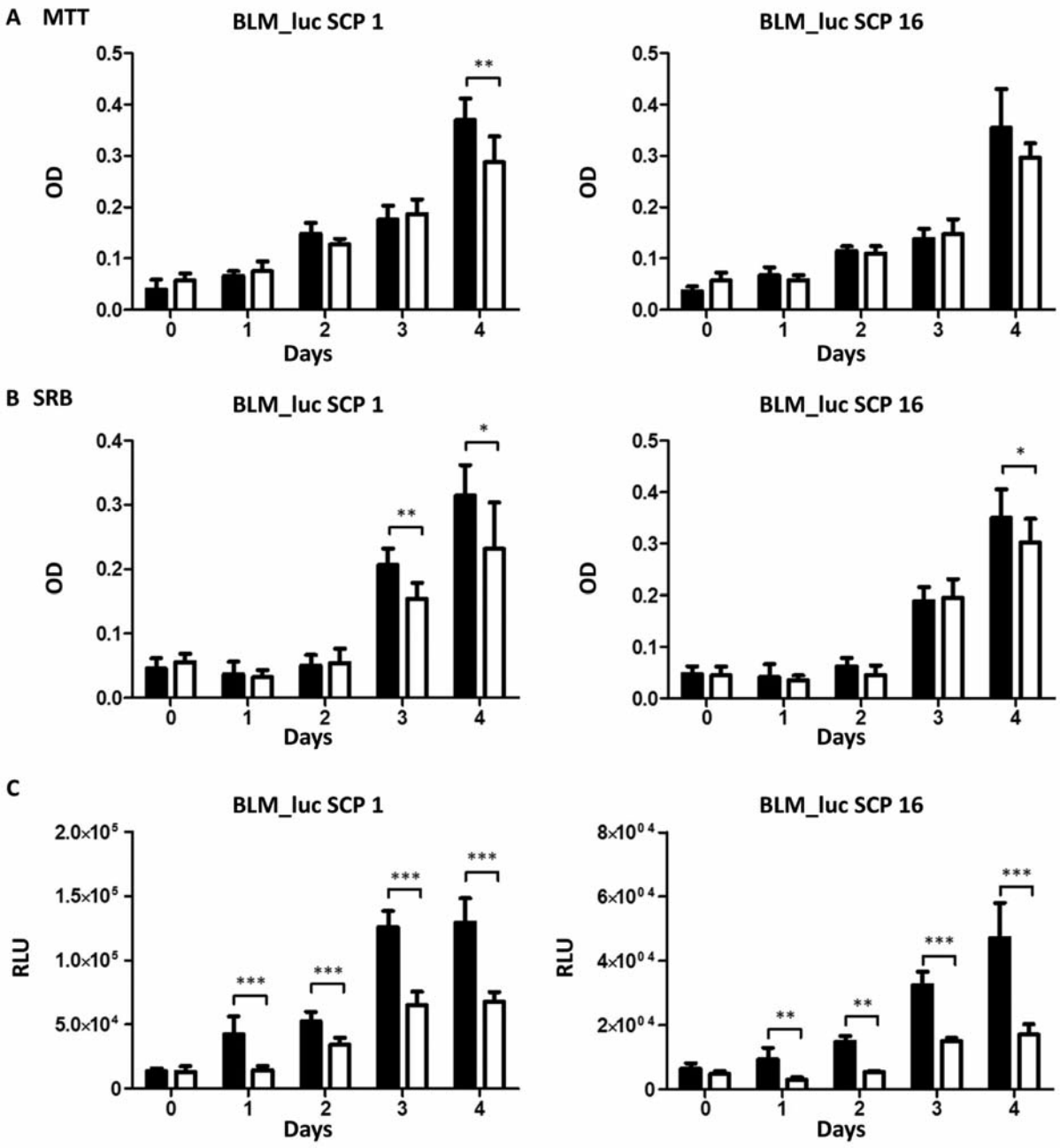

Figure 4. *Continuous luciferin exposure causes cell fatigue. Effect of luciferin exposure on mitochondrial activity as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (A), total protein concentration as measured by sulforhodamine B (SRB) assay (B) and luciferase activity measured by luminometry (C) in cancer cells with a high luc2 copy number (SCP 1) and low luc2 copy number (SCP 16). Closed bars represent intermittent exposure to luciferin; open bars represent continuous exposure to luciferin. Results are presented from three wells per assay from three independent experiments. Values are means±SD. *p<0.05, **p<0.01 and ***p<0.001.*

small difference in copy number but significant differences in BLI. The difference in RLU and BLI can be explained by the difference in sensitivity of the quantification technique. RLU quantification is based on single-point measurements, while BLI quantification is based on the signal of the entire well.

Removal of luciferin-containing medium after measuring BLI is a necessary precaution because continuous exposure to luciferin reduces mitochondrial activity and total protein after prolonged incubation. Both these findings suggest a decrease in growth because of continuous luciferin exposure.

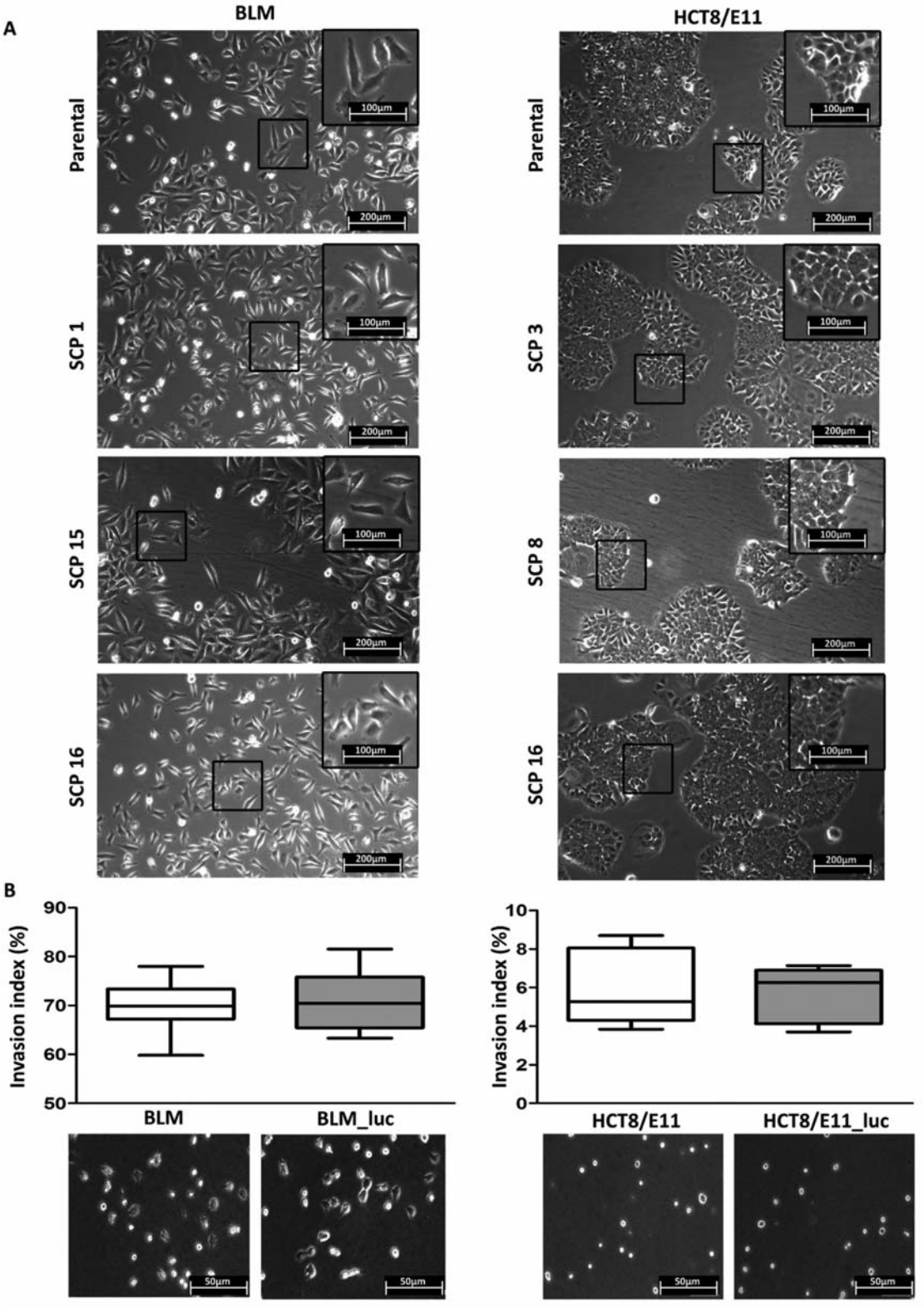

Figure 5. *Luciferase-transfected cancer cells and parental cells have similar characteristics in vitro. A: Representative phase-contrast micrographs of luciferase-transfected and parental cells. B: Comparison of type I collagen invasion assay. Luciferase-transfected cells and parental cells were seeded as single cells. After 24 h, invasive and non-invasive cells were counted and the percentage invasion was quantified. Results are presented from three wells per assay from three independent experiments. Values are mean percentages±SD.*

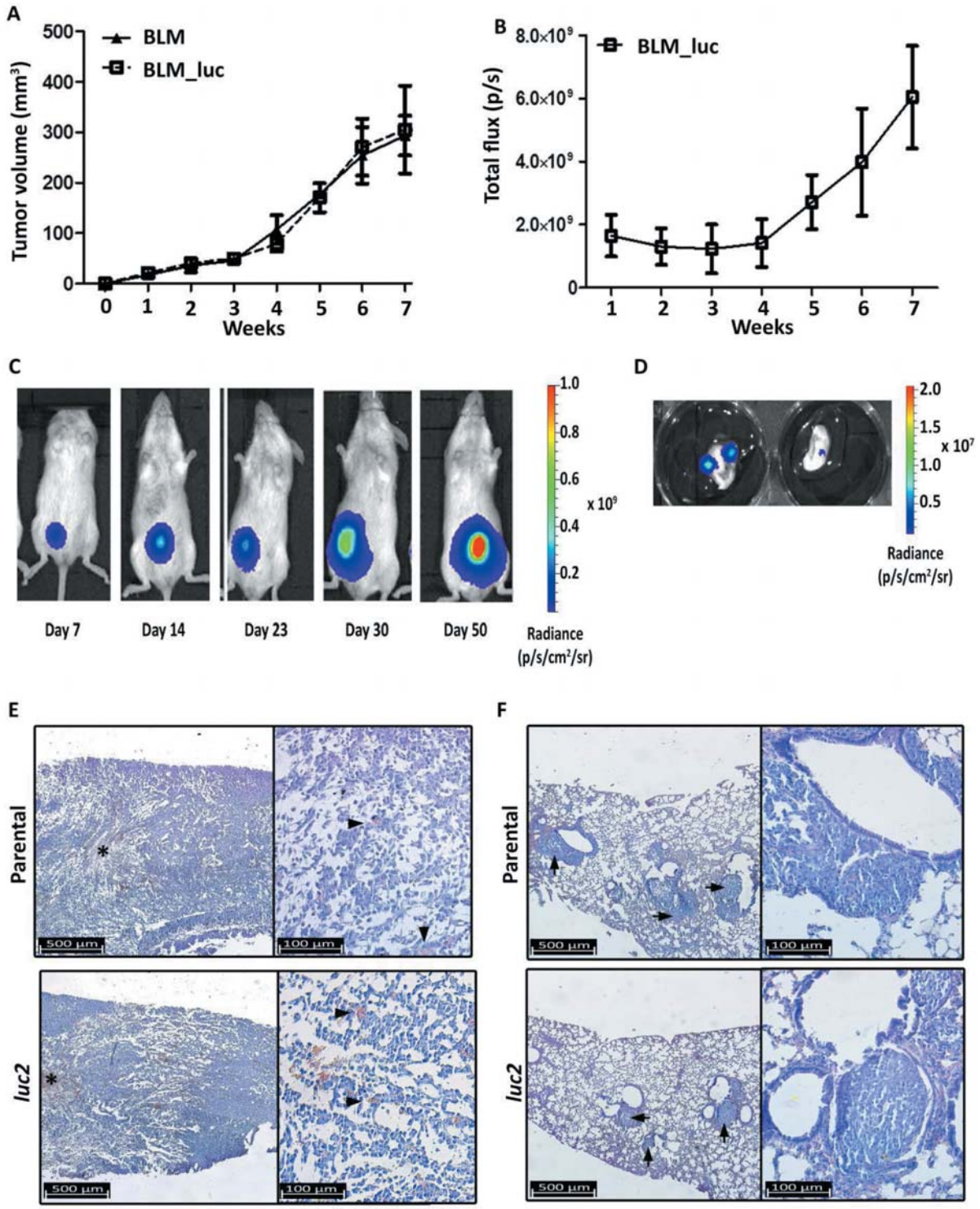

Figure 6. *Luciferase-transfected cancer cells and parental cells have the same characteristics in vivo. A: Four-week-old NOD/SCID female mice were injected subcutaneously with 1×10⁶ BLM_luc or parental BLM cells in 0.1 ml of serum-free culture medium. Tumor volume in three mice was monitored for 50 days by caliper measurements. B: Quantification of bioluminescent signal of BLM_luc cancer cell growth in vivo. C: Imaging of one representative animal at different time points after injection of BLM_luc. D: Image of a representative pair of lungs of a mouse 50 days after subcutaneous implantation of BLM_luc cells; mice were sacrificed and total lung metastasis was quantified by bioluminescent imaging. Immunohistochemical staining was used for conventional morphological analysis of primary tumor and lung metastases. *Necrotic tissue; arrowhead, blood vessels (E); arrows, metastatic lesion (F).*

A high level of luciferase continuously fuelled by luciferin and cellular ATP consumes a significant proportion of the ATP pool necessary for maintaining cellular metabolism and growth (7). Moreover, luciferase-expressing cells consume oxygen during the luciferase–luciferin reaction, which leads to a hypoxic state (17). Hypoxia is known to reduce cell proliferation in a hypoxia-inducible factor-dependent manner and continuous exposure to luciferin leads to excessive oxygen consumption, resulting in growth stasis (17, 28-30). In addition, a build-up of oxyluciferin or oxidative damage occurring during the luciferase–luciferin reaction my also play a role in growth stasis (17). But the latter hypothesis was rejected by Tiffen *et al*. who claimed that a limiting co-factor (*i.e*. oxygen and ATP) cannot cause excessive production of oxyluciferin (9). The fact that these effects are more pronounced in SCPs with a high *luc2* copy number is probably due to higher consumption of ATP and oxygen than in those with a low copy number. Therefore, it can be concluded that replacement of luciferin-containing culture medium by regular culture medium needs to become the golden standard after luciferase quantification in *in vitro* experiments.

The important question of whether manipulation by luciferase transfection initiates functional differences between the transfected and the parental cell line needs to be answered. Our research provides basic knowledge essential for working with luciferase-transfected cell lines. Bolin and co-workers studied the difference in orthotopic breast tumor growth and metastasis formation between SCPs with high and low BLI *in vivo* (31). Similar tumor growth and metastasis profiles were observed between the transfected SCP cells and parental cells. In our research, we only tested the SCP with highest BLI and compared it with the parental cell line. With this high-intensity SCP, lower numbers of cancer cells can be detected, suggesting a more sensitive detection of early stages of metastasis or tumor responses to therapy. According to Brutkiewicz *et al*. a high luciferase expression may affect tumor growth *in vivo* if animals are exposed to luciferin continuously and serial re-imaging (7). We showed that our high expressing luc2 SCP had a similar primary tumor growth profile and lung metastasis rate compared to the parental line. Jenkins and co-workers are one of the few who described a comparison in tumor growth between parental and transfected cell lines, similar tumor progression was seen in both cell lines, but no further functional tests were performed (32). Other examples are described by Thalheimer *et al*. (4) and Clark *et al*. (8). Talheimer *et al*. first investigated *in vitro* luciferase activity in serial dilution before use *in vivo* but never compared with the parental cells *in vivo*. Clark *et al*. investigated the effect of luciferase transcription on cell characteristics. No significant difference was observed in cell growth/migration and invasion but a significant difference in gene expression of seven cytokine genes was observed (8). From this research, we can conclude that investigators should test their transfected cells on a small number of mice to guarantee that luciferase transfection did not change tumor behavior *in vivo*.

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