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Establishment of a luciferase-based method for measuring cancer cell adhesion and proliferation

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ABSTRACT

Background: Methods measuring cell proliferation and adhesion are widely used but each hold limitations. We, therefore, introduce novel methods for measuring cell proliferation and adhesion based on CRISPR-modified cancer cell lines secreting luciferase to the growth media.

Materials and methods: Using CRISPR genome editing, we generated stable luciferase-secreting LS174T, HCT 116, Caco-2, and PANC-1 cell lines. The modified cells were seeded, and luciferase activity was measured in the media and compared to Coulter counter cell counts and iCELLigence impedance assay to evaluate the value of the secreted luciferase activities as a measurement for adhesion and proliferation.

Results: Our results demonstrate that luciferase secreted into the media can be used quantifying cell proliferation and adhesion. The adhesion luciferase assay and the iCELLigence impedance assay showed similar results with increased significant difference observed in the luciferase assays. The luciferase proliferation assay showed increased growth following increased serum concentrations in all cell lines vs. only two cell lines in the iCELLigence impedance assay.

Conclusions: Our results show that the luciferase adhesion and proliferation assays are reliable methods for measuring adhesion and proliferation. The luciferase assays have advantages over existing assays as they are highly sensitive, easy to perform, non-invasive and suitable as high-throughput measurements.

1. Introduction

The development of improved methods for cell quantification is indispensable for the advancement of research in the field of cancer biology [1]. The ability to evaluate cellular responses under a variety of testing conditions is crucial for this development and consequently, for the discovery of new therapeutic agents. Important techniques currently used for assessing alterations in cellular processes includes cell-based assays for measuring the proliferation and adhesion. During a variety of testing conditions, the cancer cell proliferation assays provide an important readout on the actively dividing cells whereas cancer cell adhesion assays quantify the ability of the cells to adhere to the extracellular matrix or to each other. To analyze cell proliferation and adhesion, sensitive techniques are needed. Several cell quantifying methods holding both advantages and inadequacies are extensively used [1]. Existing assays like MTT [3-(4,5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], Hoechst, [H^3]-thymidine/5--Bromo-2′-deoxyuridine (BrdU) incorporation, Real-Time Cell Analysis (RTCA) iCELLigence impedance assay, and those involving direct cell counting using hemocytometer or Coulter counter are some of the most frequently used techniques. However, each of them have some limitations. The MTT, Hoechst, and thymidine/BrdU incorporation assays can be tedious, require several steps and do not allow for continuous monitoring. Further, counting cells using hemocytometer or Coulter counter can be too time consuming to allow high-throughput. The RTCA iCELLigence assay may be used for real time monitoring but is not suitable as a high-throughput method. Therefore, selection of the most suitable assay, that is both sensitive and has high throughput, is essential

Abbreviations: CI, Cell Index; CRC, Colorectal Cancer; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; DMEM, Dulbecco's Modified Eagle's Medium; FBS, Fetal Bovine Serum; PBS, Phosphate Buffered Saline; PS, Penicillin Streptomycin; RLU, Relative Light Unit; RTCA, Real-Time Cell Analysis.

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when designing a study.

Regardless of the continous improvement in the diagnosis and treatment techniques, the prognosis of cancer remain inadequate [2]. The progress within therapeutic agents and cancer treatment regimens have shown minimal effect in patients, making further investigation of the molecular mechanisms responsible necessary for the identification of new targets and to develop novel drugs with improved efficacy [3].

It is of great importance to investigate cancer cell proliferation and adhesion given that aberrations in the cell proliferation and adhesion capacities lead to the development of metastasis, which is the cause of most cancer-associated deaths [2]. Metastases development and selection of organ site may be attributed to the changes in the expression of cell adhesion molecules [4]. During the development of metastases, the tumor cells evade the primary tumor and undergo intravasation as well as extravasation in order to manifest in distant organs and establish metastatic lesions. Every step of this process involves the interaction of tumor cells with its surroundings and these interactions are mediated by adhesion molecules [5], which highlights the importance for the development of new and improved methods for measuring cancer cell adhesion and proliferation.

Cancer is a severe disease with high mortality rates [3]. Moreover, colorectal cancer (CRC), one of the most frequently diagnosed cancers, is one of the leading causes of cancer-related deaths [2,6]. Currently, a surgical resection of the tumor is mainstay treatment, but despite successful resection, approximately 30% of CRC patients experience recurrence [7]. In addition, pancreatic cancer is one of the most lethal types of cancer. Despite it causes only about 3% of cancer incidents in the United States, it accounted for approximately 8% of cancer-related deaths in 2020 [8]. Pancreatic cancer tends to produce late symptoms and therefore presents at a late stage when metastasis has already occurred, limiting the treatment options available. We, therefore, chose three CRC cell lines and one pancreatic cancer cell line for validating our methods.

In the present study, we have developed luciferase-based assays for analyzing proliferation and adhesion. Luciferase reporter assays are widely used for monitoring transcription factor activities and gene promotor activities as well as other cellular activities [9]. In this study, we used a synthetically designed Lucia gene derived from marine copepods (InvivoGen, San Diego, CA, USA) [10]. The Lucia gene produces a secreted form of luciferase, an oxidative and bioluminescent enzyme, that is specially designed for stable and sustained expression in mammalian cells. The Lucia gene is controlled by the common and robust promotor EF-1α/HTLV ensuring this constant and continued expression. The Lucia luciferase provides a 1000-fold higher bioluminescent activity compared to the frequently used luciferases from Firefly and *Renilla*, making it easy to detect in the cell culture growth media. Furthermore, it uses coelenterazine as substrate emitting visible blue light between 465 and 493 nm, and does not require ATP or Mg^{2+} for light production. Additionally, the luciferase enzyme is highly sensitive and non-toxic.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the associated endonuclease Cas9 have made it possible to manipulate DNA and perform precise targeted genomic modifications [11]. By utilizing the advantages provided by the CRISPR technology, we have designed a method ensuring stable and predictable expression of Lucia luciferase by using the gRNA and Cas9 expression from the AAVS1-T2-CRISPR in pX330 plasmid [12] targeting the Adeno-Associated Virus Site 1 (AAVS1) locus. Transfection of the AAVS1-T2-CRISPR in pX330 plasmid together with our AAVS1 targeted luciferase plasmid (pSelect-Zeo-Lucia-AAVS1) will result in specific insertion of the Lucia luciferase reporter construct in the AAVS1 locus by homology directed repair. The benefits of using genomic safe harbor sites such as the AAVS1 locus is to ensure long term stable transgene expression and at the same time decreasing the risk of interactions interfering with the function of the host genome [13].

We aimed at establishing and validating two innovative functional assays by generating stable cell lines secreting luciferase into the media they are cultured in and thereby creating easy to perform assays that are inexpensive, extremely sensitive, reliable and non-invasive and could provide new high-throughput *in vitro* methods for measuring cancer cell proliferation and adhesion.

2. Materials and methods

2.1. Cell culture

The human colon cancer cell lines LS174T, HCT 116, and Caco-2 and the pancreatic cancer cell line PANC-1 were cultured in T75 culture flasks grown in Dulbecco's Modified Eagle's Medium (DMEM) with ultraglutamine 1 with 4.5 g/L Glucose (Lonza, Basel, Switzerland) supplemented with 10% Fetal Bovine Serum (FBS) (HyClone, Fisher Scientific, Waltham, MA, USA), and Penicillin (100 units/mL) and Streptomycin (100 μg/mL) (PS) (Gibco, Life Technologies, Carlsbad, CA, USA). The cell cultures were passaged twice a week within 3–4 days and were incubated at 37 °C in 5% CO_2.

2.2. Plasmid construction

The AAVS1 T2 CRISPR in pX330 plasmid were obtained from Addgene (Plasmid #72833 [12]). The reporter plasmid were constructed by cloning the AAVS1 sites from pMK232 (CMV-OsTIR1-PURO) plasmid obtained from Addgene (Plasmid #72834 [12]) into the pSelect-Zeo-Lucia (InvivoGen, San Diego, CA, USA) containing the Lucia luciferase and Zeocin resistance genes generating the plasmid pSelect-Zeo-Lucia-AAVS1 as seen in Fig. 1.

The two AASV1 fragments used in the cloning were generated by PCR with primers shown in Table 1 and two pSelect-Zeo-Lucia plasmid fragments were generated by restriction enzyme digestion (*Not*I and *Pst*I). The fragments were assembled and cloned using the In-Fusion HD Cloning Kit (Clontech, Fremont, CA, USA) and the transformation was performed using DH5-α Competent *E. coli* cells (Thermo Fisher Scientific, Waltham, MA, USA). The sequence of the plasmid was verified by sequencing.

2.3. Generation of stable cell lines

LS174T, HCT 116, Caco-2 and PANC-1 cells were seeded on a 6-well plate one day prior to transfection. For each well, 1.05 μg pSelect-Zeo-Lucia-AAVS1 and 0.35 μg AAVS1-T2-CRISPR in pX330 were mixed and adjusted up to 125 μL with 150 mM NaCl. All plasmid concentrations were measured using the Nanodrop spectrophotometer (Nanodrop ONE, Thermo Fisher Scientific Inc., Massachusetts, USA). Next, 125 μL of 2 μM polyethylenimine was added and incubated 1 h at room-temperature. The cells were transfected with 250 μL of the mix in each well and the media were changed with DMEM after 4 h of incubation. After 24-h incubation, the cells were detached using 0,05% trypsin/0,02% EDTA (Gibco, Life Technologies, Carlsbad, CA, USA) and were seeded into T75 cell culture flasks. The selection of transfected cells with stable integration of the Lucia gene was achieved with zeocin selection (LS174T (600 μg/mL); HCT 116 (300 μg/mL); Caco-2 (50 μg/mL); PANC-1(600 μg/mL)) (Invitrogen, Carlsbad, CA, USA) and the cells were grown by changing the medium at regular intervals until zeocin resistance cells was obtained and stable growth was observed. The Caco-2 and HCT 116 cell lines were maintained with a polyclonal approach and the production of Lucia luciferase activity was furthermore monitored in the media until stable and robust expression was achived. In addition the Caco-2 and HCT 116 cell lines were grown in media containing low concentration of zeocin between experiments to ensure selection for the luciferase expression system. The LS174T and PANC-1 cell lines were made using a monoclonal approach and cell colonies were harvested and cultured until confluence levels were obtained. Single colonies were chosen based on luciferase measurements to extract the

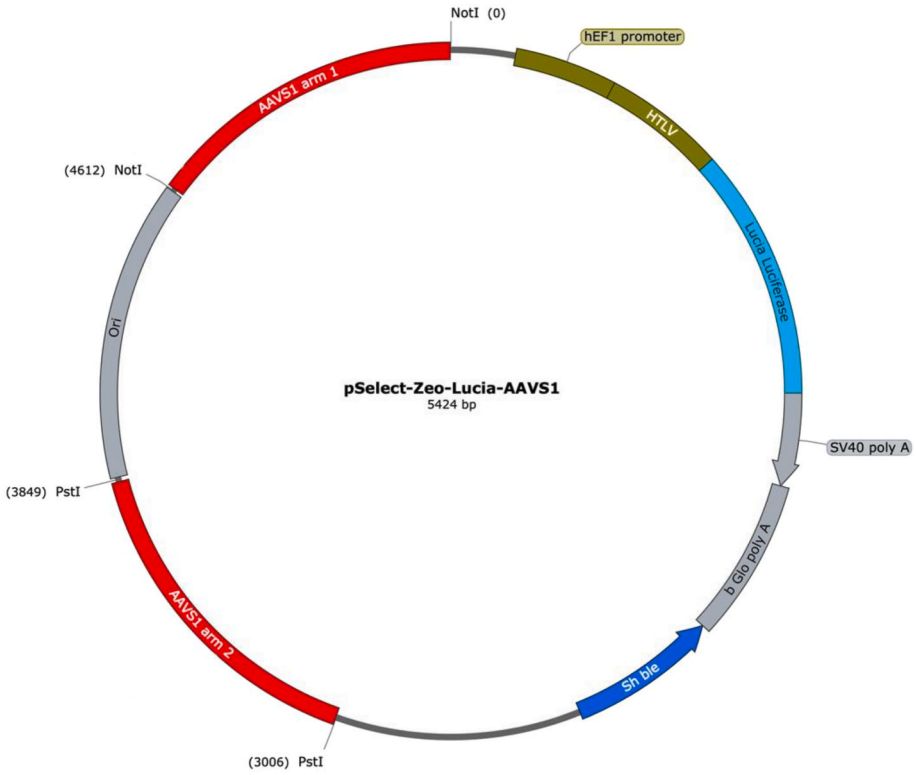

Fig. 1. Map of the constructed pSelect-Zeo-Lucia-AAVS1 plasmid with the AAVS1 sites from pMK232 plasmid cloned into the pSelect-Zeo-Lucia.

Table 1
AAVS1 primers used for In-fusion Cloning.

Primer name	Sequence
*Not*I Forward	5'_TTTAAATCA**GCGGCCGC**TGCTTTCTCTGACCAGCATTCTCTC_3′
*Not*I Reverse	5'_TATTTTATT**GCGGCCGC**GCCCCACTGTGGGGTGGAG_3′
*Pst*I Forward	5'_AAGTTATGTAACGC**CTGCAG**ACTAGGGACAGGATTGGTGACAG_3′
*Pst*I Reverse	5'_TGTTCTTAATTAAC**CTGCAG**AGAGCAGAGCCAGGAACCC_3′

DNA and the integration of Lucia gene in the AAVS1 locus was controlled by PCR with AAVS1 and construct specific primers. We used the primer sequences targeting the AAVS1 locus outside integration forward: 5′- CTG CCG TCT CTC TCC TGA GT-3′ and reverse: 5′-GGG AAC GGG GCT CAG TCT-3′ which amplifies a 1753 bp sequence from unmodified human genomic DNA. If the plasmid is inserted correctly, the PCR fragment will be 4768 bp. For the LS174T and PANC-1 cell lines only clones with bi-allelic insertion was selected and used for further experiments.

2.4. Luciferase measurements

125, 250, 500, 1000, 2000 and 4000 Caco-2 cells were seeded in triplicates in a 24 well plate. At intervals of 24, 48, 72 and 96 h, 20 μL media were transferred from three wells to a 96-well microplate and luciferase activity was measured. Luciferase activity in the media of the 96-well microplates was quantified using the QUANTI-Luc assay reagent (InvivoGen, San Diego, CA, USA) made according to the manufacturer's protocol. SpectraMax® i3x plate reader (Molecular Devices, San Jose, CA, USA) was set to 50 μL injections and end-point measurements with 4 s start time, and 0.5 s reading time concurring to the manufacturer's instructions.

2.5. Coulter counter cell count

All experiments were performed in triplicates. For adhesion 50.000 Caco-2 cells were seeded in a 24 well plate in either 2,5% or 10% FBS and incubated for 60 min at 37 °C in 5% CO2. Media were removed and 1 mL of DMEM with FBS and PS were added to each well and incubated for 24 h 20 μL media were transferred from each well to a 96-well microplate and luciferase activity was measured. Afterward the cells was harvested and counted in the Coulter counter. For proliferation 1500 Caco-2 cells were seeded in a 24-well plate in either 2,5% FBS or 10% FBS. At 24, 48, 72 and 96 h, 20 μL media were transferred from three wells with 2,5% FBS and 10% FBS to a 96-well microplate and luciferase activity was measured before harvesting the cells for a cell count.

Cell counts were achieved by removing the media and the cells was washed twice in 1 mL phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, Missouri, USA). Subsequently 250 μL trypsin/EDTA was added and incubated for 5 min at 37 °C in 5% CO2. Subsequently media were added up to 1 mL and the cells were resuspended. 200 μL of cell suspension were transferred to a Coulter counter cuvette with 10 mL 0.9% NaCl. The dilution factor was set at 51 and cell size range at 8–29 μm and the cells were counted using the Beckman Coulter Counter Z2 (Beckman Coulter Life Sciences, IN, USA).

2.6. RTCA iCELLigence impedance assay

The adhesion and proliferation assays were performed using the RTCA iCELLigence (ACEA Biosciences, San Diego, CA, USA). The iCELLigence device uses electrical impedance as readout monitoring changes in the cellular phenotype quantifying real-time cell adhesion and/or proliferation. It measures the electrical impedance through electronic microtiter plates termed as E-Plate L8 PET (ACEA Biosciences,

San Diego, CA, USA), with bottoms covered in interdigitated gold microelectrodes. Cell adhesion as well as increases in cell number will impede the electrical flow and enhance the degree of impedance and the relative change in the electrical impedance is shown as a dimensionless value called the Cell Index (CI).

2.7. Adhesion assay

The LS174T, HCT 116, Caco-2 and PANC-1 cells were split following the procedure of sub-culturing and re-suspended in DMEM containing either 2.5% or 10% FBS. The cells were counted and transferred to a 15 mL tube. Totally, 500 μL of cell suspension where added to each well in the E-Plate L8 PET. The cells were seeded in concentrations specific to each cell line: LS174T, 2×10^4/well; HCT 116 and PANC-1, 1×10^4/well; and Caco-2, 5×10^3/well. The experiment was performed in quadruplicates. Subsequently, the E-Plate L8 PET were placed in the RTCA iCELLigence. The impedance was measured every 10 min and the differences in CI at 60 min between the cells seeded in 2.5% FBS and those seeded in 10% FBS were calculated.

The E-Plate L8 PET plates were removed from the iCELLigence after 60 min and the media from the plates were removed by reversing the plate on a container with paper towels. Subsequently, 500 μL of DMEM with FBS and PS were added to each well and incubated for 24 h. Twenty microlitres of media from each well were transferred to a 96-well microplate and luciferase activity was measured. The differences in luciferase activity measured as relative light units (RLU) between cells seeded in 2.5% FBS and 10% FBS were calculated.

2.8. Proliferation assay

The proliferation assays were performed as follows: CRISPR-modified LS174T, HCT 116, Caco-2 and PANC-1 cells were resuspended in DMEM containing either 2.5%, 5%, or 10% FBS. To each well of the E-Plate L8 PET, 500 μL of cell suspension were added. The number of cells seeded were: LS174T, 1×10^4/well; HCT 116 and PANC-1, 5×10^3/well; and Caco-2, 2.5×10^3/well. The experiment was performed in duplicates. Afterward, the E-Plate L8 PET were placed in the RTCA iCELLigence and the impedance was measured every 10 min. The differences in CI at 0, 24, 48, 72, and 90 h between cells seeded in 2.5%, 5%, and 10% FBS were calculated.

At 0, 24, 48, 72 and 90 h, 20 μL media from each well in the E-Plate L8 PET plates were transferred to a 96-well microplate and luciferase activity was measured. The differences in luciferase activity/RLU between cells seeded in 2.5%, 5%, and 10% FBS were calculated for each time-point.

2.9. Statistical analysis

Unpaired *t*-test was used for both the luciferase assays and the RTCA iCelligence assays to verify the statistical differences in the adhesion of cells grown in either 2.5% or 10% FBS. Repeated measures ANOVA with Tukey test was used for both the luciferase assays and the RTCA iCelligence assays verifying statistical differences in the proliferation of cells with either 2.5%, 5%, or 10% FBS. The level of statistical significance was set at p-value < 0.05. (p-value<0.05*, p-value<0.01**, p-value<0.001***). Microsoft Excel and Graphpad Prism 9 software were used for statistical analysis.

3. Results and discussion

This study aimed to establish two new assays for measuring cancer cell adhesion and proliferation based on luciferase activity secreted to the growth media by the CRISPR-modified cancer cell lines with insertion of Lucia luciferase in the AAVS1, a safe harbour locus that ensures stable expression. Furthermore, we used a monoclonal (LS174T and PANC-1) as well as a polyclonal approach (HCT 116 and Caco-2) in generation of the stable cell lines. The two approaches gave the same results, but the polyclonal approach is a faster procedure to obtain the luciferase secreting cells as the selection period is shorter, and the diversity of original cell line is better preserved after selection as the luciferase producing cell line consists of many clones.

Both the proliferation and adhesion assays have been constructed on principles displayed in Fig. 2. Starting with the transfection for generating the stable cell lines containing Lucia luciferase gene, were we used the gRNA and Cas9 expression from the AAVS1-T2-CRISPR in pX330 plasmid to target the AAVS1 Lucia luciferase reporter construct (pSelect-Zeo-Lucia-AAVS1) into the AAVS1 locus by homology directed repair. After establishment of the modified cell lines, the next step is seeding the cells for the assays.

As the experiments proceed, the cells will secrete luciferase to the media, making it possible to transfer growth media containing luciferase to a 96 well assay plate and measure luciferase activity without harvesting the cells.

3.1. Number of cells seeded correlates with measured luciferase activity

To demonstrate that secreted luciferase can be used to quantify the cell number, we first investigated whether there was a correlation between the number of cells seeded and the luciferase activity in the responding growth media. We examined 125, 250, 500, 1000, 2000, and 4000 Caco-2 cells seeded per well, continuously measuring luciferase activity in the growth media for 96 h. As shown in Fig. 3, our results showed a correlation between the number of seeded cells and the secreted luciferase activity measured in the responding media of the cells, and the luciferase activities increase exponentially with time, no matter the number of cells seeded. This finding demonstrates that secreted luciferase is very sensitive and can detect down to 125 cells and can detect exponential growth. This confirms that the luciferase assays are highly sensitive.

3.2. Coulter counter cell count correlates with luciferase measurements

Two experiments were performed to confirm that Coulter counter cell counts correlate with measured luciferase activities. As seen in Fig. 4, the adhesion significantly increases when seeded in 10% FBS compared to 2,5% FBS in both the luciferase measurements and when the cells are counted in the Coulter counter. Our findings verify that the luciferase measurements correlate with the cell count performed using the Coulter counter. In addition, the results show that changes in concentration of serum can be used to change adhesion.

As displayed in Fig. 5, proliferation increased with the Caco-2 cells grown in media containing 10% FBS compared to 2,5% in both the luciferase measurements and the cells counted in the Coulter counter. Our results show a direct correlation between cells counted in the Coulter counter and the luciferase measured in the corresponding media. Our findings also confirm that proliferation changes as we alter the percentage of FBS in the media.

In order to validate our methods for measuring adhesion and proliferation, we used an already established assay based on impedance measurements conducted by the RTCA iCELLigence device [14]. With the purpose of directly comparing the results of the impedance assay with the luciferase assay, all adhesion and proliferation experiments were performed by transferring media during the impedance assays at specific timepoints and measuring the luciferase activity in the corresponding media. To confirm the reliability of our assays, we chose to change the adhesion and proliferation conditions by modifying the final concentration of FBS, the most frequently used serum in cell culture media which contributes to essential functions such as cell attachment as well as proliferation. The final concentration of FBS in the growth medium has a direct influence on these processes [15]. Therefore, we expected improved adhesion and proliferation following increased concentrations of FBS as confirmed by our previous experiments

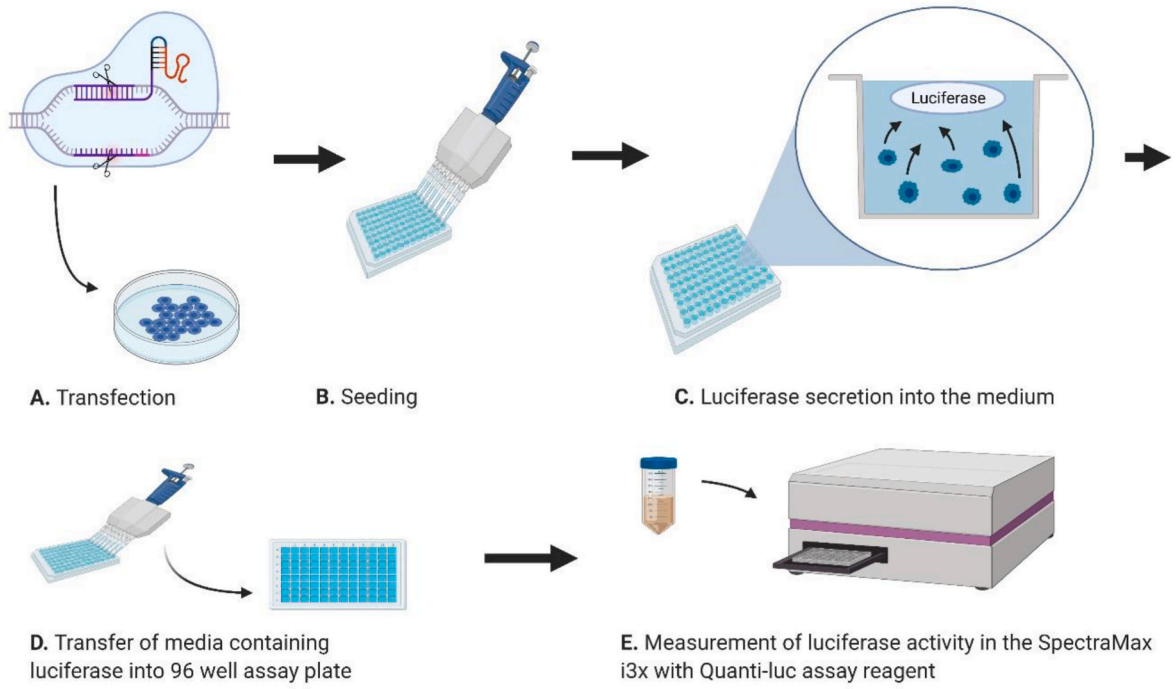

Fig. 2. Overview of the principles in the luciferase assays. **A.** Transfection of cell line for generating a stable luciferase-secreting cell line; **B.** Seeding of the CRISPR-modified cancer cells for experiments; **C.** Luciferase is secreted into to the media; **D.** Media containing luciferase is transferred to a 96-well assay plate; **E.** Luciferase activity in the media is measured using the SpectraMaxi3X with Quanti-luc assay reagent.

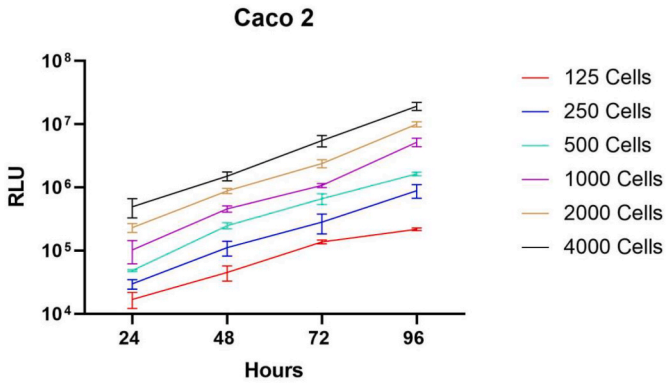

Fig. 3. Comparison of cell number seeded with luciferase activity as relative light units (RLU) for the CRISPR-modified Caco-2 cell line. Measured luciferase activity as relative light units (RLU) for 125,250, 500, 1000, 2000 and 4000 Caco-2 cells at 24, 48, 72 and 96 h after seeding(n = 3).

displayed in Figs. 4 and 5.

3.3. Adhesion assay

In order to validate the luciferase-based adhesion assay, we compared it to the RTCA iCELLigence impedance assay for measuring cancer cell adhesion: [16]. The difference in impedance recorded as CI by the RCTA iCelligence device between cells seeded in 2.5% FBS and those seeded in 10% FBS was compared to the difference in RLU measurements in the corresponding media of the cells.

Our results in the luciferase adhesion assay were found to be similar to those of the impedance assay. As expected, a clear difference in adhesion was observed between the cells seeded in 2.5% and those in 10% FBS, with an expected increase in adhesion corresponding to the increased serum concentration. However, differences were observed across the different cell lines. No statistically significance differences in the adhesion were found between LS174T cells seeded in either 2.5% or 10% FBS from the luciferase assay (p-value 0.0575) or the impedance assay (p-value 0.7850; Fig. 6A). The luciferase adhesion assay did however show a p-value which could be considered borderline significant and a trend concurring the expected outcome, contrary to the impedance assay. The HCT 116 cell line showed dissimilar findings for the two assays (Fig. 6B) with the difference in adhesion being statistically significant in the luciferase assay (p-value 0.0057) but not in the impedance assay (p-value 0.7523). A significant difference in adhesion was observed in both the Caco-2 and the PANC-1 cell lines (Fig. 6C and D) with increased adhesion in cells seeded in 10% FBS. There was however a difference in the level of significance between the RTCA iCELLigence assay and the luciferase assay. In the Caco-2 cell line, we found significant difference in adhesion with a p-value of 0.0082 in the impedance assay and 0.0287 in the luciferase assay whereas the PANC-1 cell line showed the greatest difference in adhesion in both the assays with a p-value 0.0001 in the impedance assay and <0.0001 in the luciferase assay.

The difference in adhesion across the RCTA iCELLigence and luciferase assays could have resulted from the difference in how the assay was performed. For RCTA, the impedance was read without removing any cells from the plates whereas the non-adherent cells in the luciferase assay were removed at 60 min when the media were removed to conduct the luciferase measurements of adherent cells only. Given that the impedance is affected by the electrodes at the bottom of each well it is unlikely that non-adherent cells in the media could have had a great impact on the impedance readouts. The differences in between cell lines and in the level of significance indicates that the luciferase assay is more sensitive compared to the impedance measurements.

3.4. Proliferation assays

To validate the luciferase proliferation assay, we used the RCTA iCELLigence impedance assay to assess proliferation and compared it with the findings of the luciferase assay (Fig. 7). The impedance measurements (CI) and the secreted luciferase activities in the responding

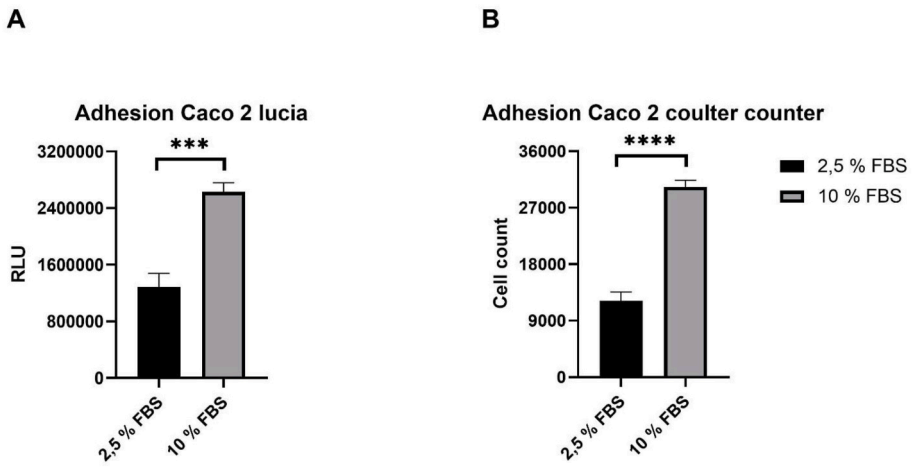

Fig. 4. Comparison in adhesion of CRISPR-modified Caco-2 cell line in media containing either 2,5% or 10% FBS measured using luciferase activity (RLU) and the Coulter counter. A. Measured luciferase activity in the media before harvesting the cells for a cell count performed in either 2,5 or 10% FBS. B. Cell count of adhesion in 2,5% or 10% FBS measured using the Coulter counter (n = 3). *** p-value<0.001, **** p-value<0.0001.

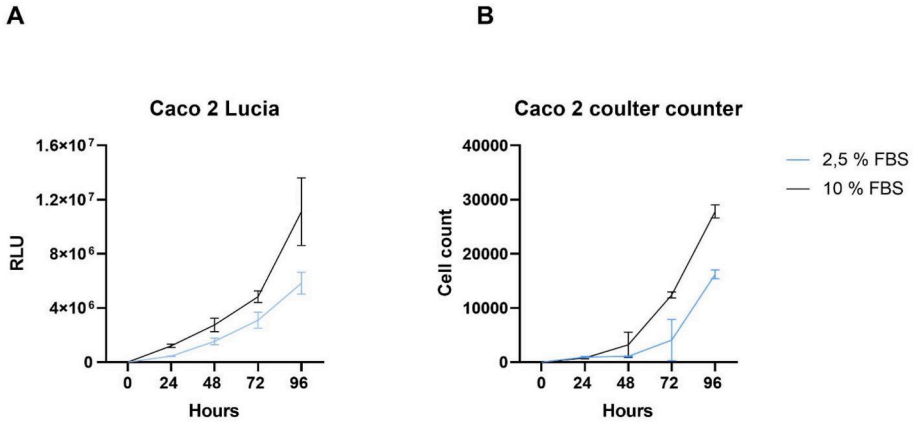

Fig. 5. Difference in proliferation of CRISPR-modified Caco-2 cell line in media containing either 2,5% or 10% FBS measured using luciferase activity (RLU) and the Coulter counter at 24, 48, 72 and 96 h. A. Measured luciferase activity in the media before harvesting the cells for a cell count performed in either 2,5 or 10% FBS. B. Cell count of proliferation in 2,5% or 10% FBS measured using the Coulter counter (n = 3).

media are displayed according to cell lines: Caco-2 (Fig. 7A), LS174T (Fig. 7B), HCT 116 (Fig. 7C), and PANC-1 (Fig. 7D).

When performing the proliferation assay, we expected to find a difference across the cells seeded in 2.5%, 5%, and 10% FBS with increased growth following increased serum concentrations which was the case in all of the luciferase assays (Fig. 7A–D). That however did not apply to all of the impedance assays where only LS174T and PANC-1 cell lines showed the expected results. When comparing the results of the impedance proliferation assays across the 4 cell lines, we found no overall significant differences between the growth curves of Caco-2 (p-value 0.1965), LS174T (p-value 0.0565), and HCT 116 (p-value 0.7928) cell lines. The only significance difference in the impedance assay between growth curves for cells grown at different FBS concentration was observed for the PANC-1 cell line (p-value 0.0407). In comparison, the only cell line that did not show a significant difference between the growth curves in the luciferase assay was Caco-2 (p-value 0.1430); while LS174T (p-value 0.0032), HCT 116 (p-value 0.0002) and PANC-1 (p-value 0.0029) cell lines revealed statistically significant differences. These results indicate that the luciferase proliferation assay is a more sensitive and precise method for measuring cancer cell proliferation compared to the impedance assay.

Our results also revealed timepoint specific differences between the cells seeded in 2.5% FBS and 10% FBS in the two assays. In the impedance assay only the LS174T cell line showed a significant difference (p-value 0.0294) at the 90-h CI measurement. On the contrary the luciferase assay showed timepoint specific significant difference in several cell lines seeded in 2.5% FBS and 10% FBS. Our luciferase assay found a statistically significant difference in the HCT 116, LS174T and PANC-1 cell lines at the 72-h and 90-h RLU measurement. At the 72-h RLU measurement a significant difference were found in the HCT 116 (p-value 0.0001), LS174T (p-value 0.0446) and PANC-1(p-value 0.0333) cell lines. Moreover, the HCT116 cell line exhibited significant difference at the 90-h RLU measurement (p-value 0.0185). The timepoint specific difference found in both of the assays supports the results found by comparing the difference in the growth curves. However, the luciferase-based assay demonstrated a much more robust exponential growth signal for all cell lines and serum concentrations compared to the impedance based assay.

We have previously used the RTCA iCELLigence evaluating the difference in cancer cell adhesion when comparing the effect of pre- and postoperative patient serum [16]. The RTCA iCELLigence impedance assays hold many advantages when compared to existing available assays. The iCELLigence is very easy to use and does not require several steps. In addition, the method is not time consuming and allows for real-time measurements over a long period of time. Furthermore, the impedance assay allows for the further use of the cells used in the assay if needed. Given the fact that iCELLigence measurements are based on impedance as a readout, it is important to note that morphological

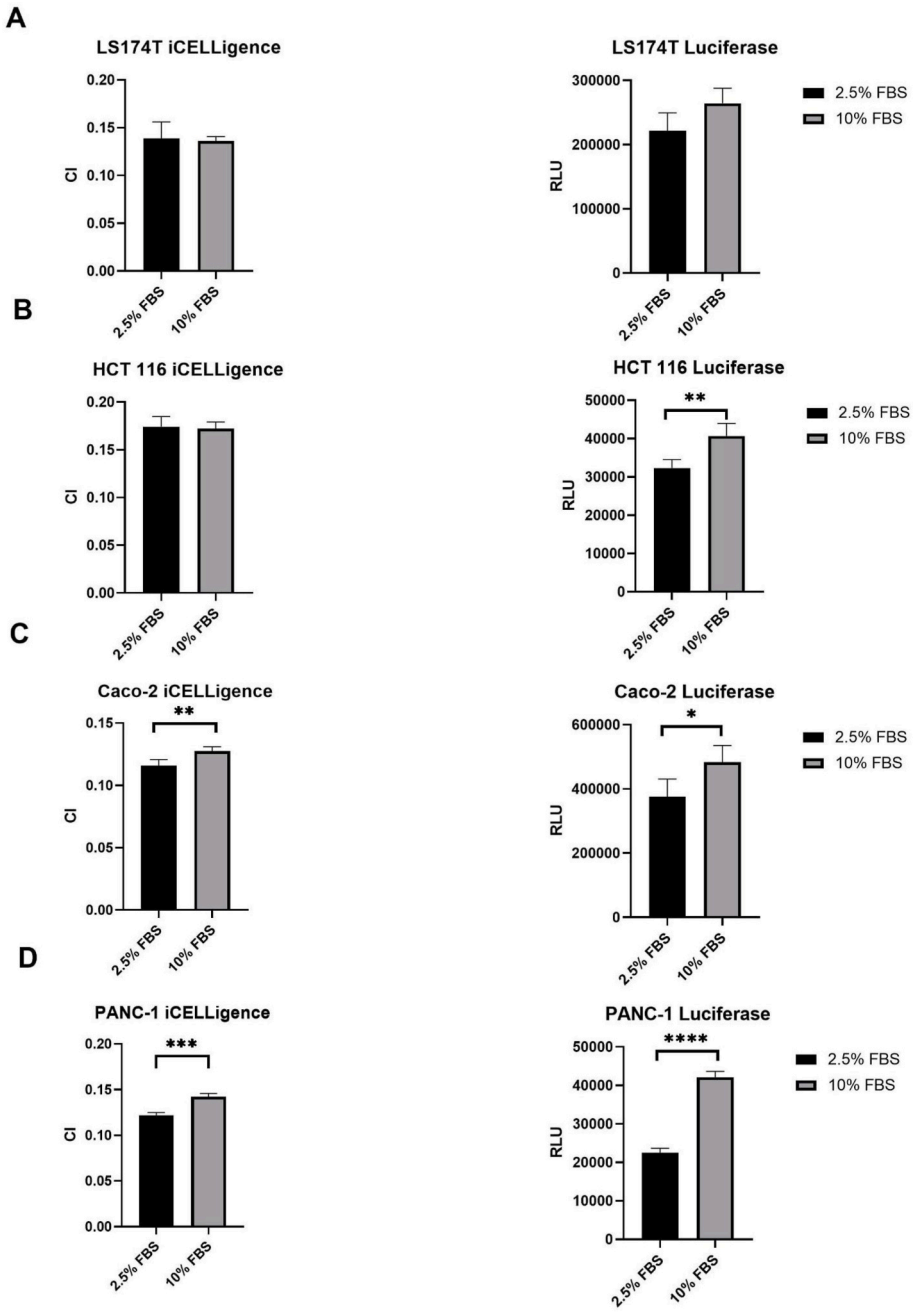

Fig. 6. Comparison of adhesion in four different cancer cell lines grown in 2.5% and 10% fetal bovine serum (FBS). Adhesion capacity was recorded as cell index (CI) and relative light units (RLU) at 60 min using the RTCA iCELLigence assay and the luciferase adhesion assay, respectively (n = 4). Differences in cell adhesion for cell lines are as follows **A.** LS174T: impedance assay, p-value 0.7850 and luciferase assay, p-value 0.0575. **B.** HCT 116: impedance assay, p-value 0.7523 and luciferase assay, p-value 0.0057. **C.** Caco-2: impedance assay, p-value 0.0082 and luciferase assay, p-value 0.0287. **D.** PANC-1: impedance assay, p-value 0.0001 and luciferase assay, p-value <0,0001.* p-value<0.05, ** p-value<0.01, *** p-value<0.001, **** p-value<0.0001.

differences in the cells could impact the CI. Notably, the iCELLigence impedance assay is not a high throughput method like the MTT and Hoechst assays which would require purchasing several instruments for the screening of biobanks. However, those assays do not allow for continuously monitoring and requires several steps.

The luciferase adhesion and proliferation assays are very sensitive, do not require many steps compared to the MTT and Hoechst assay and allow for continuously monitoring as well as have a high throughput. In addition, the luciferase assays are inexpensive making them suitable for screening of biobanks.

4. Conclusion

Based on the overall results of our experiments, we found that the luciferase adhesion and proliferation assays are very reliable methods that should be taken into consideration when performing cancer cell adhesion and proliferation assays. These luciferase assays overcome the limitations of the currently-used techniques, are highly sensitive and easy to perform as they require fewer steps. In addition, they allow for non-invasive measurements, which make them particularly suitable as high-throughput measurements. Finally, our luciferase assays provide a new inexpensive method for measuring cancer cell adhesion and proliferation.

CRediT authorship contribution statement

Stine Bull Jessen: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization. **Derya Coskun Özkul:** Investigation, Writing – review & editing. **Yasemin Özen:** Investigation, Writing – review & editing. **Ismail Gögenur:** Resources, Writing – review & editing, Supervision, Funding acquisition. **Jesper T. Troelsen:**

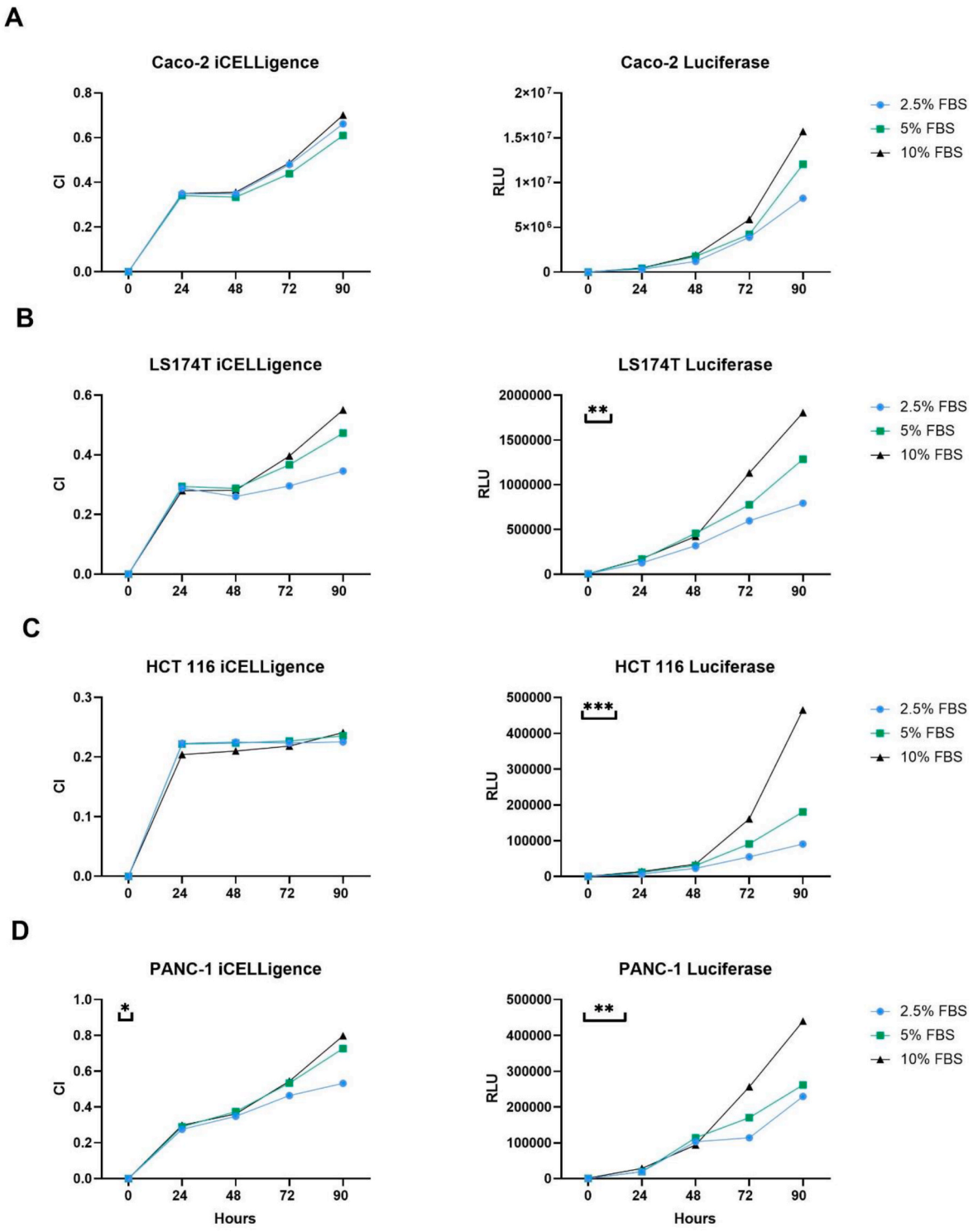

Fig. 7. Proliferation measurements of the four different cancer cell lines grown in 2.5%, 5%, and 10% fetal bovine serum (FBS) measured in a real-time cell analysis iCELLigence impedance assay and a luciferase assay. Mean cell index (CI) and relative light unit (RLU) at 0, 24, 48, 72, and 90 h is shown (n = 2). Difference between the growth curves for the different cell lines is as follows **A.** Caco-2: impedance assay (p-value 0.1965) and luciferase assay (p-value 0.1430); **B.** LS174T: impedance assay NS (p-value 0.0565) and luciferase assay (p-value 0.0032); **C.** HCT 116: impedance assay NS (p-value 0.7928) and luciferase assay (p-value 0.0002); **D.** PANC-1: impedance assay (p-value 0.0407) and luciferase assay (p-value 0.0029). * p-value<0.05, ** p-value<0.01, *** p-value<0.001, **** p-value<0.0001.

Conceptualization, Methodology, Resources, Writing – original draft, Supervision, Funding acquisition.

Declaration of competing interest

None.

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