ACEA Biosciences, Inc.

INVESTIGATING THE HALLMARKS OF CANCER

USING xCELLigence REAL-TIME CELL ANALYSIS

Investigating the Hallmarks of Cancer

Early hopes of finding a therapeutic "magic bullet" capable of eradicating all types of cancer were progressively replaced with the sobering realization that cancer consists of >100 distinct diseases that manifest in ~200 cell types with diverse genetic/mutational etiologies. For the clinician this complexity is compounded by the fact that a single disease phenotype can result from multiple genotypes, each of which requires a unique approach to treatment (estrogen dependent/independent breast cancer being a primary example). Citing that the cancer scientific literature had already become "complex almost beyond measure," in 2000 Robert Weinberg and Douglas Hanahan stated a belief that "the complexities of the disease, described in the laboratory and the clinic, will become understandable in terms of a small number of underlying principles."[1] In this now famous paper they suggested that distillation of decades of research reveals "a small number of molecular, biochemical, and cellular traits – acquired capabilities – shared by most and perhaps all types of human cancer." The six original hallmarks of cancer that they proposed, as well as one new one that has become evident in the ensuing years[2], are listed in Table 1 at the bottom of this page.

Much of what has been learned about the hallmarks of cancer has come through *in vitro* cell biology research. Positioned between reductionistic biochemical assays and whole organism *in vivo* experimentation, cell-based assays are an indispensable tool in both basic and applied cancer research. However, the utility of many traditional and current cell-based assays is diminished by: (1) the need to use labels, (2) incompatibility with continuous monitoring (i.e. only end point data is produced), (3) incompatibility with orthogonal assays, and (4) the inability to provide an objective/quantitative readout. Each of these shortcomings is, however, overcome by the non-invasive, label-free, and real-time cellular impedance assay. This brochure demonstrates how ACEA's cellular impedance-monitoring xCELLigence® RTCA DP instrument can be used for cell-based assays investigating each and every hallmark of cancer. The xCELLigence real-time cell analysis (RTCA) assay principle is first described, followed by examples from the primary literature.

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4. Inducing angiogenesis	11
5. Evading apoptosis	12
6. Replicative immortality	13
7. Evading immune destruction	14

Table 1.

[1] Hallmarks of Cancer. Hanahan, D.; Weinberg, R. Cell 2000, 100, 57-70.
[2] Hallmarks of Cancer: The Next Generation. Hanahan, D.; Weinberg, R. Cell 2011, 144, 646-674.

The Instrument

The seven different xCELLigence real-time cell analysis (RTCA) instruments made by ACEA Biosciences all use noninvasive electrical impedance monitoring to quantify cell proliferation, morphology change, and cell-substrate attachment quality in a label-free and real-time manner. What distinguishes the DP (dual purpose) model from the other xCELLigence instruments is its ability to additionally make kinetic measurements of cell invasion and migration using an electronically integrated Boyden chamber (described in detail on page 8).

The xCELLigence RTCA DP instrument is placed inside a standard CO_2 cell culture incubator and is controlled by a laptop computer housed outside the incubator (Figure 1). The three cradles of the DP instrument enable three separate electronic 16-well plates to be controlled and monitored in parallel or independently of one another, allowing maximal productivity for multiple users. Intuitive software enables real-time interfacing with all three cradles, and includes real-time data display and analysis functions.

By *continuously* providing data on cell number/size/attachment in the short (minutes to hours) and long (days to weeks) time regimes, RTCA provides a phenotypic view of cell health and behavior at an unprecedented level of detail. Moreover, by skipping the guesswork associated with end-point assays and eliminating the time- and labor-intensive steps of traditional methods, real-time cell analysis with the xCELLigence® RTCA DP instrument vastly improves efficiency and overall productivity.

Figure 1. The xCELLigence RTCA DP instrument. The instrument, which contains three independent cradles that interface with electronic microtiter plates, is placed inside a standard tissue culture incubator and is compatible with the full range of biologically relevant temperatures, atmospheric compositions, and humidities. The instrument is controlled by a laptop computer that is housed outside the incubator.

Cellular Impedance

The functional unit of the cellular impedance assay that is run by the xCELLigence RTCA DP instrument is a set of gold microelectrodes embedded in the bottom surface of a microtiter plate well (Figure 2). When submersed in an electrically conductive solution (such as buffer or standard tissue culture medium), the application of a weak electric potential across these electrodes causes current to flow between them. Because this phenomenon is dependent upon the electrodes interacting with bulk solution, the presence of adherent cells at the electrode-solution interface impedes current flow. The magnitude of this impedance is dependent upon the number of cells, the size and shape of the cells, and the cell-substrate attachment quality. Importantly, neither the gold microelectrode surfaces nor the applied electric potential have an effect on cell health or behavior.

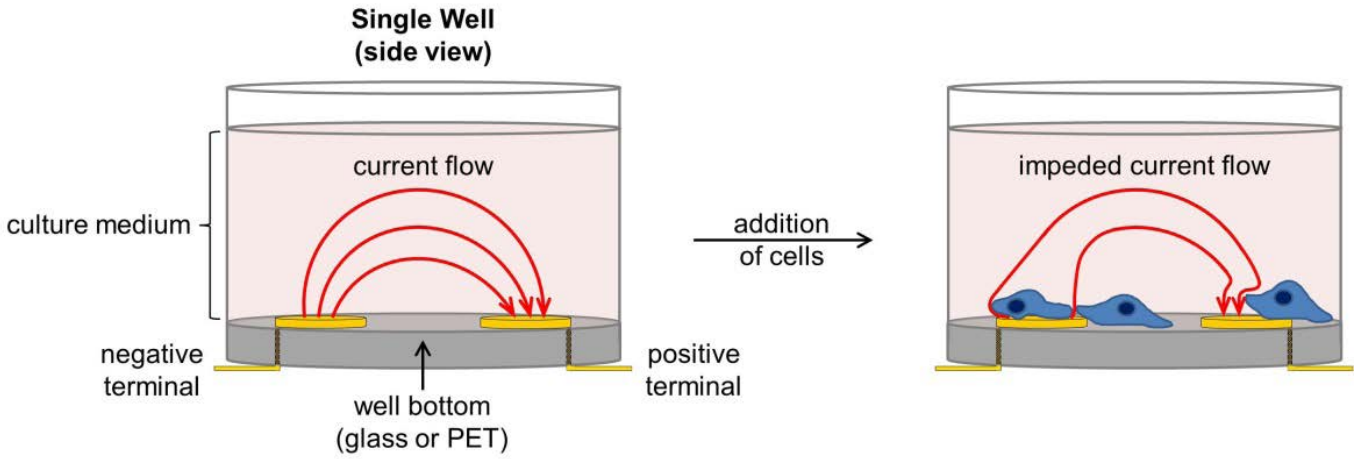

Figure 2. Overview of cellular impedance apparatus. A side view of a single well is shown before and after cells have been added. Neither the electrodes nor the cells are drawn to scale (they have been enlarged for clarity). In the absence of cells electric current flows freely through culture medium, completing the circuit between the electrodes. As cells adhere to and proliferate on the electrodes current flow is impeded, providing an extremely sensitive readout of cell number, cell size/morphology, and cell-substrate attachment quality.

E-Plates

The gold microelectrode biosensors in each well of ACEA's electronic microtiter plates (E-Plates®) cover ~75% of the bottom's surface area. Rather than the simplified electrode pair depicted in Figure 2, the circular electrodes in each well of an E-Plate are linked into "strands" that form an interdigitating array (Figure 3). This proprietary design enables large populations of cells to be monitored simultaneously and thereby provides exquisite sensitivity to the number of cells attached to the plate, the size/morphology of the cells, and the cell-substrate attachment quality.

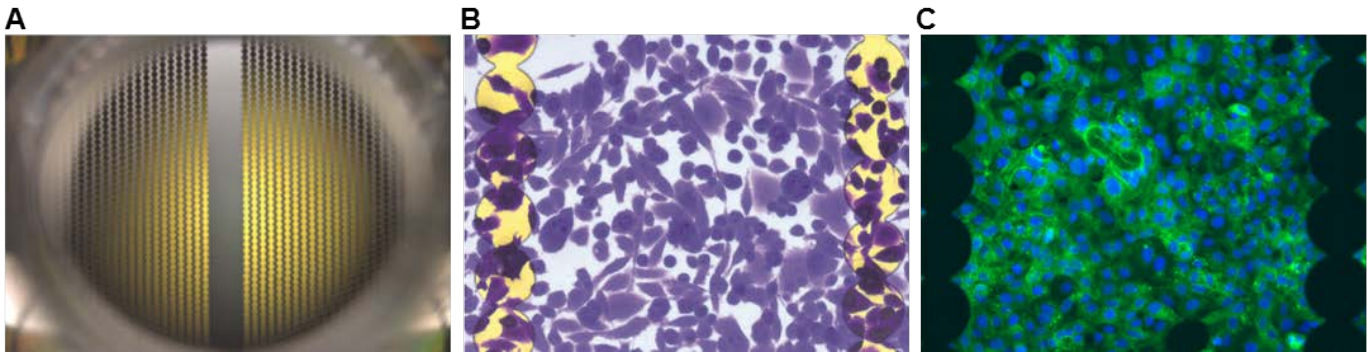

Figure 3. Impedance electrodes on ACEA's E-Plates. (A) Photograph of a single well in an E-Plate. Though cells can also be visualized on the gold electrode surfaces, the electrode-free region in the middle of the well facilitates microscopic imaging. (B) Gold electrodes and crystal violet stained human cells, as viewed in a compound microscope. (C) Immunofluorescence microscopy with gold electrodes silhouetted.

Real-Time Impedance Traces Explained

The impedance of electric current that is caused by adherent cells is reported using a unitless parameter called Cell Index (CI), where CI = (impedance at time point *n* - impedance in the absence of cells)/(nominal impedance constant). Figure 4 provides a generic example of a real-time impedance trace throughout the course of setting up and running an apoptosis experiment. For the first few hours after cells have been added to a well there is a rapid increase in impedance, which is caused by cell attachment and spreading. If cells are sub-confluent after the initial attachment stage, they will start to proliferate, causing a gradual yet steady increase in CI. When cells reach confluence the CI value plateaus, reflecting the fact that the electrode surface area that is accessible to bulk media is no longer changing. The addition of an apoptosis inducer at this point causes a decrease in CI back down to zero. This is the result of cells rounding and then detaching from the well bottom. While this generic example involves addition of the apoptosis inducer at the point of cellular confluence, impedance-based assays are extremely flexible and can interrogate a wide variety of phenomena across the full spectrum of cell densities.

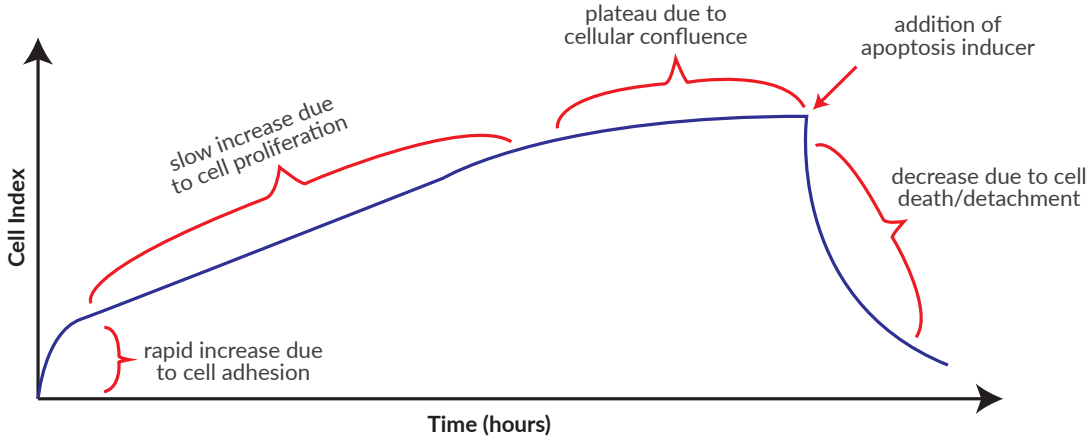

Figure 4 Generic real-time impedance trace for setting up and running an apoptosis assay. Each phase of the impedance trace, and the cellular behavior it arises from, is explained in the text.

Applications

To date, more than 1,700 xCELLigence instruments have been placed globally in labs that span everything from academia and biotech startups to contract research organizations and big pharma. This has resulted in >800 xCELLigence publications in peer-reviewed journals.

In addition to the 7 hallmarks of cancer research areas that are profiled in detail within this brochure, the xCELLigence RTCA DP instrument is being used extensively for cancer research in other applications that include, but are not limited to, the following:

- Compound-mediated cytotoxicity
- Cell-mediated cytotoxicity
  - T cell
  - NK cell
  - CAR T cell
  - macrophage
- Antibody-dependent cell-mediated cytotoxicity (ADCC)
  - bispecific antibodies
  - bispecific T cell engagers (BiTEs)
- Drug mechanism of action
- Blood brain barrier
- Tumor microenvironment (cell-cell interactions)
- Cell adhesion/spreading
- Receptor activation
- Oncolytic viruses
- Autophagy
- Solid tumor killing assays
- Liquid tumor killing assays
- Immune cell activation
- Apoptosis
- Inflammation

Table 2.

Hallmark #1: Self-Sufficiency in Growth Signals

For normal tissues entry into, and progression through, new rounds of cell proliferation are tightly controlled via growth promoting signals that are produced by neighboring cells (paracrine signals) or distal cells/tissues (endocrine signals). These mitogenic signals are transmitted to a target cell's interior by transmembrane proteins that activate cytoplasmic signaling cascades.

In contrast to healthy cells, a fundamental trait of cancer cells is their ability to proliferate in the absence of exogenous mitogenic signals. This growth signal autonomy is achieved through a number of mechanisms. In some instances cancer cells acquire the ability to produce growth factors to which they are directly responsive, creating a positive feedback loop (autocrine stimulation). In other cases, overexpression of key transmembrane growth factor receptors, or expression of mutant/truncated versions of these receptors, have been found to impart ligand hypersensitivity or even ligand-independent activation. Finally, mutation of genes encoding the cytoplasmic signal transduction circuitry can lead to mitogenic signaling even when upstream receptors have not been stimulated.

In the example below, the role of the transmembrane receptor anaplastic lymphoma kinase (ALK) in promoting the proliferation of neuroblastoma cells was interrogated. After demonstrating a linkage between neuroblastoma predisposition and ALK point mutations/gene amplification, neuroblastoma cell lines were examined and found to express elevated levels of both ALK protein and transcripts. Importantly, these cancer cell lines show constitutive phosphorylation of an activating tyrosine residue in ALK. To examine the functional consequence of these genetic modifications, the cell lines were subjected to xCELLigence real-time cell analysis in the presence or absence of ALK-specific siRNA. As seen in Figure 5, knockdown of WT ALK has no impact on cell proliferation. In contrast, when cells harbor either an ALK point mutant or amplified ALK copy number, ALK expression drives proliferation and its knockdown results in substantial growth reduction. This profound "addiction" to ALK activity/signaling for neuroblastoma proliferation strongly implicates this protein as a potential target for therapeutic intervention.

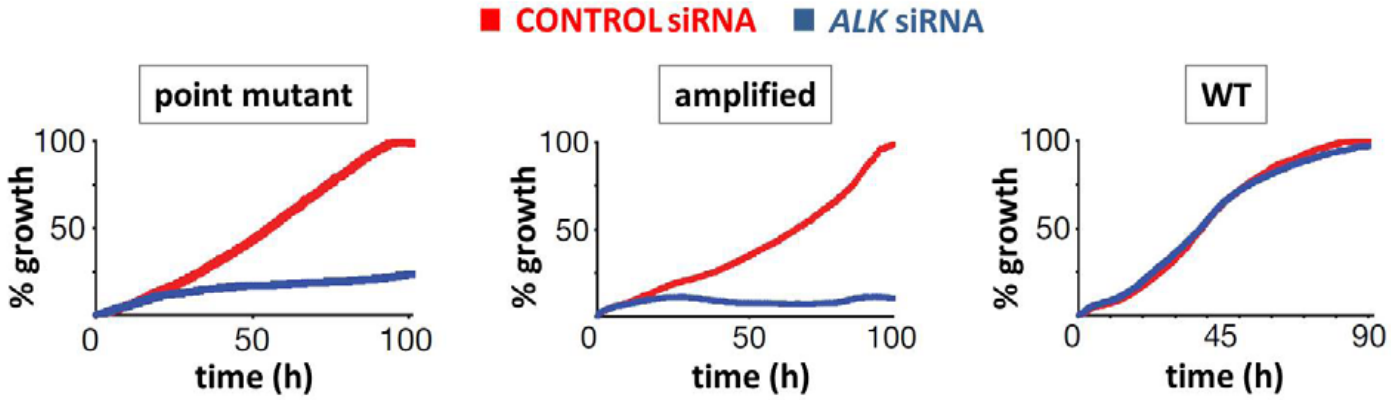

Figure 5. Proliferation of neuroblastoma cell lines is driven by point mutations or copy number amplifications of the ALK gene. Whereas cell lines harboring WT ALK are unperturbed by its knockdown, cell lines expressing mutant or amplified versions of the gene are dependent upon its expression in order to proliferate. See text for details. Figure adapted from Nature 2008 Oct 16;455(7215):930-935.

Select Publications:

1. **Identification of ALK as a major familial neuroblastoma predisposition gene.** Mossé YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, Laquaglia MJ, Sennett R, Lynch JE, Perri P, Laureys G, Speleman F, Kim C, Hou C, Hakonarson H, Torkamani A, Schork NJ, Brodeur GM, Tonini GP, Rappaport E, Devoto M, Maris JM. Nature. 2008 Oct 16;455(7215):930-5
2. **The AKT inhibitor MK-2206 is cytotoxic in hepatocarcinoma cells displaying hyperphosphorylated AKT-1 and synergizes with conventional chemotherapy.** Simioni C, Martelli AM, Cani A, Cetin-Atalay R, McCubrey JA, Capitani S, Neri LM. Oncotarget. 2013 Sep;4(9):1496-1506.

Hallmark #2: Insensitivity to Anti-Growth Signals

Cell proliferation is regulated not only by growth stimulating factors but also by a milieu of growth suppressing signals derived from multiple sources. Suppression of cell cycle progression can be triggered by intracellular damage/stress signals, as well as by extracellular signals from soluble/diffusible molecules, proteins displayed on neighboring cells, and components of the extracellular matrix. Genetic modification of anti-growth sensor and effector genes allows for unchecked proliferation and is a common etiology in many cancers.

Genomic aberrations associated with neuroblastoma are well characterized and include both deletions and duplications of numerous loci – some of which encode microRNAs. To define their role in development and progression of the disease, these microRNAs were individually transfected into a neuroblastoma cell line that expressed them at low levels due to a hemizygous deletion. As seen in Figure 6A, when compared with the non-targeting control (NTC) microRNA, overexpression of miR-34a causes substantial reduction in cell proliferation. This effect was consistently observed in neuroblastoma cell lines lacking endogenous miR-34a, but was absent in cell lines that expressed endogenous miR-34a (Figures 6 B and C). Subsequent studies suggested that at least one of the functions of miR-34a is to suppress cell growth by promoting cell cycle arrest. The downregulated expression of this tumor suppressor in neuroblastoma serves as a classic example of the desensitization to anti-growth signaling that is so common in cancer.

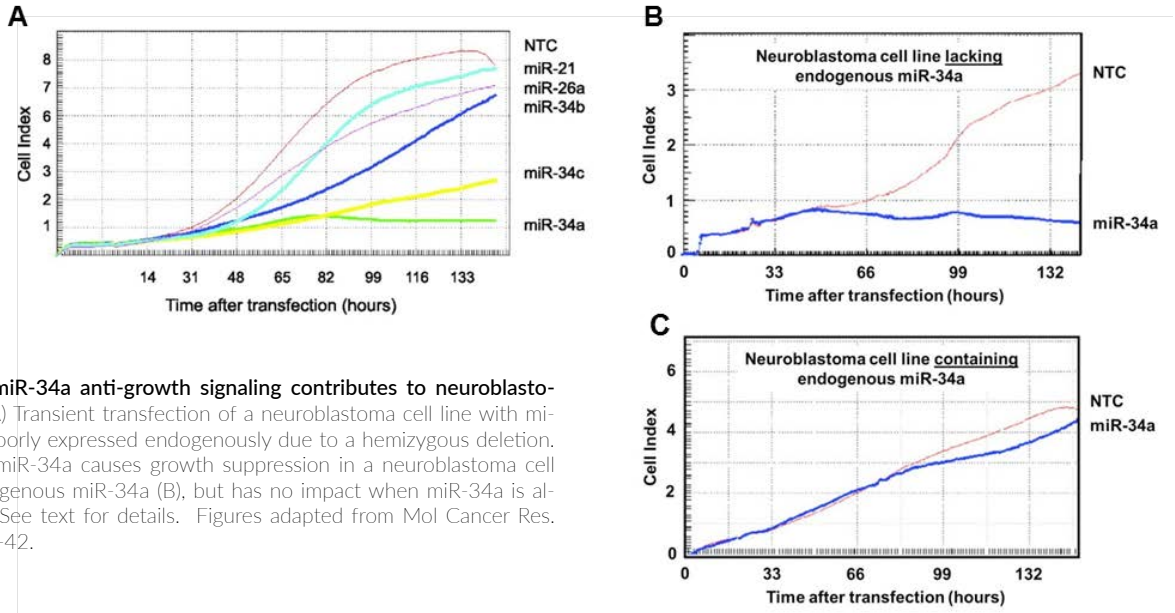

Figure 6. Loss of miR-34a anti-growth signaling contributes to neuroblastoma proliferation. (A) Transient transfection of a neuroblastoma cell line with microRNAs that are poorly expressed endogenously due to a hemizygous deletion. Overexpression of miR-34a causes growth suppression in a neuroblastoma cell line that lacks endogenous miR-34a (B), but has no impact when miR-34a is already present (C). See text for details. Figures adapted from Mol Cancer Res. 2008 May;6(5):735-42.

Select Publications:

1. **A functional screen identifies miR-34a as a candidate neuroblastoma tumor suppressor gene.** Cole KA, Attiyeh EF, Mosse YP, Laquaglia MJ, Diskin SJ, Brodeur GM, Maris JM. Mol Cancer Res. 2008 May;6(5):735-42.
2. **Dual CDK4/CDK6 inhibition induces cell-cycle arrest and senescence in neuroblastoma.** Rader J, Russell MR, Hart LS, Nakazawa MS, Belcastro LT, Martinez D, Li Y, Carpenter EL, Attiyeh EF, Diskin SJ, Kim S, Parasuraman S, Caponigro G, Schnepp RW, Wood AC, Pawel B, Cole KA, Maris JM. Clin Cancer Res. 2013 Nov 15;19(22):6173-82.

Overview

The ability of cancer cells to migrate and invade through different tissue types enables them to intravasate/extravasate blood and lymphatic vessels and produce the metastases that are responsible for more than 90% of human cancer deaths. A major hurdle to studying cancer cell migration and invasion has been the lack of techniques that are kinetic, quantitative, reproducible, and efficient. Scratch assays require repetitive visual evaluation of cells and provide subjective data that is poorly reproducible. Boyden chambers provide more objective data but are labor intensive (cells that have passed through membrane pores must be fixed, stained, and counted) and yield information for only a single time point. In contrast, ACEA's cell invasion and migration plate (CIM-Plate®; used exclusively with the xCELLigence RTCA DP instrument) contains electronically integrated Boyden chambers that provide, in real-time and without the use of labels, quantitative kinetic data for migration/invasion with minimal hands-on time by the researcher. As cells move from the upper chamber towards chemoattractant in the lower chamber they pass through a membrane containing 8 µm pores and then adhere to gold impedance microelectrodes (Figure 7). The resultant change in impedance signal perfectly correlates with the number of cells attached to these electrodes, enabling collection of highly reproducible data over time ranges spanning from minutes to days. An overview of the CIM-Plate is shown below.

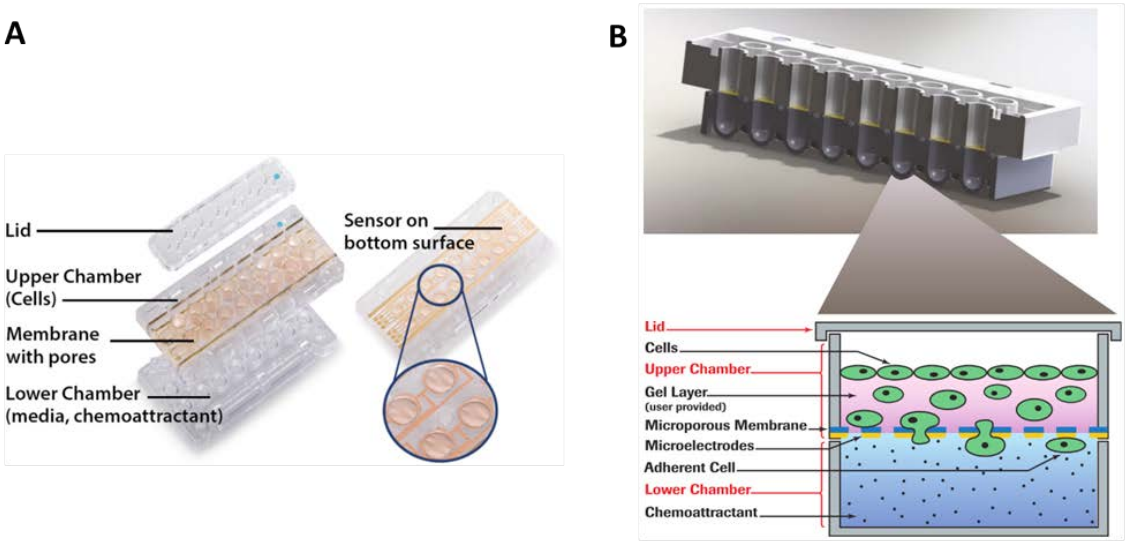

Figure 7. CIM-Plate Overview. (A) CIM-Plate components. (B) CIM-Plate detail. The expanded view illustrates the upper and lower chambers for a single well. The bottom surface of the upper chamber is composed of a microporous membrane that cells can migrate through. Gold electrodes on the underside of this membrane detect the presence of adherent cells. For a simple migration assay (not illustrated here) the cells being monitored would be plated directly onto the membrane. For an invasion assay (shown here), cells are plated on top of a basement membrane matrix, a cellular monolayer, or some combination thereof.

Key Benefits of Using the xCELLigence RTCA DP Instrument for Cell Migration/Invasion Studies:

1. Quantitative monitoring of cell migration or invasion in real-time.
2. Label-free assay requires no fixation, staining, or any other sample processing, dramatically reducing hands-on time.
3. Easy quantification of the kinetics of migration or invasion.
4. Rapid optimization of cell density and extracellular matrix density conditions.
5. Non-invasive nature of the assay allows for cells on either side of the microporous membrane to be analyzed by complementary techniques (imaging, RT-PCR, etc.).

Cell Migration Assay

With metastasis posing the primary challenge in the clinical management of breast cancer, there is high demand for effective diagnostic and therapeutic strategies focused on this facet of the disease. Through efforts to understand the molecular mechanisms driving breast cancer metastasis, a tight correlation between acetylated α-tubulin levels and aggressive metastatic behavior was discovered. Using the xCELLigence RTCA DP instrument and CIM-Plate, BT-A549 cells were found to migrate towards serum much less efficiently when expressing an α-tubulin mutant (K40R) that cannot be acetylated (Figure 8A). The results from these impedance traces were corroborated by an orthogonal assay in which migrated cells were imaged on the surface of the gold microelectrodes (Figure 8B).

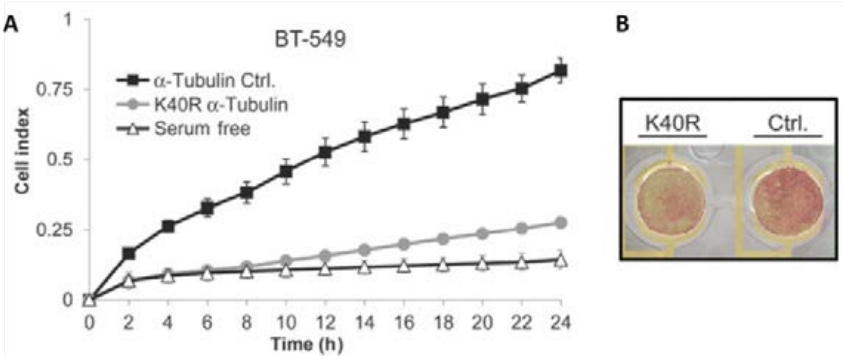

Figure 8. Chemotactic migration of breast tumor cells is stimulated by α-tubulin acetylation. (A) Analysis of BT-549 cell migration in real-time using ACEA's CIM-Plates and the xCELLigence RTCA DP instrument. Cells were plated in serum-free media (upper chamber) and allowed to migrate toward the lower chamber (containing 5% FBS) for 24 hours. α-tubulin Ctrl: BT-549 cell line stably expressing α-tubulin; K40R α-tubulin: BT-549 cell line stably expressing the K40R α-tubulin mutant that cannot be acetylated; serum-free control: the lower chamber contained serum-free media (and therefore lacked chemoattractant). Error bars represent standard deviation. (B) Imaging of migrated cells. At the 24 hour time point cells on the underside of the upper chamber were fixed and stained. The differences in migrated cell numbers visualized here correlate with the quantitative real-time impedance data from panel "A". Figures adapted from Cancer Res. 2015; 75(1):203-15.

Select Publications:

1. **Differential roles of Smad2 and Smad3 in the regulation of TGF-β1-mediated growth inhibition and cell migration in pancreatic ductal adenocarcinoma cells: control by Rac1.** Ungefroren H, Groth S, Sebens S, Lehnert H, Gieseler F, Fändrich F. Mol Cancer. 2011 May 30;10:67.
2. **MicroRNA-200c Represses Migration and Invasion of Breast Cancer Cells by Targeting Actin- Regulatory Proteins FHOD1 and PPM1Ferences.** Jurmeister S, Baumann M, Balwierz A, Keklikoglou I, Ward A, Uhlmann S, Zhang JD, Wiemann S, Sahin Ö. Mol Cell Biol. 2012 Feb;32(3):633-51.
3. **Comparative Analysis of Dynamic Cell Viability, Migration and Invasion Assessments by Novel Real-Time Technology and Classic Endpoint Assays.** Limame R, Wouters A, Pauwels B, Fransen E, Peeters M, Lardon F, De Wever O, Pauwels P. PLoS One. 2012, 7(10), e46536.
4. **A real time chemotaxis assay unveils unique migratory profiles amongst different primary murine macrophages.** Iqbal AJ, Regan-Komito D, Christou I, White GE, McNeill E, Kenyon A, Taylor L, Kapellos TS, Fisher EA, Channon KM, Greaves DR. PLoS One. 2013, 8(3), e58744.
5. **α-Tubulin acetylation elevated in metastatic and basal-like breast cancer cells promotes microtentacle formation, adhesion, and invasive migration.** Boggs AE, Vitolo MI, Whipple RA, Charpentier MS, Goloubeva OG, Ioffe OB, Tuttle KC, Slovic J, Lu Y, Mills GB, Martin SS. Cancer Res. 2015 Jan 1;75(1):203-15.

Cell Invasion Assay

Once ovarian cancer cells shed into the peritoneal fluid, they aggregate and then bind to and invade through the peritoneal lining to establish secondary tumors. The complexity of this metastatic process makes it difficult to study, particularly in a quantitative manner. In the example highlighted in Figure 9, a model of the peritoneal lining, including a basement membrane matrix covered by a mesothelial cell monolayer, was established within the upper chambers of a CIM-Plate. The chemotaxis-fueled invasion of cancer spheroids through these layers was then quantitatively monitored in real-time using the xCELLigence RTCA DP instrument.

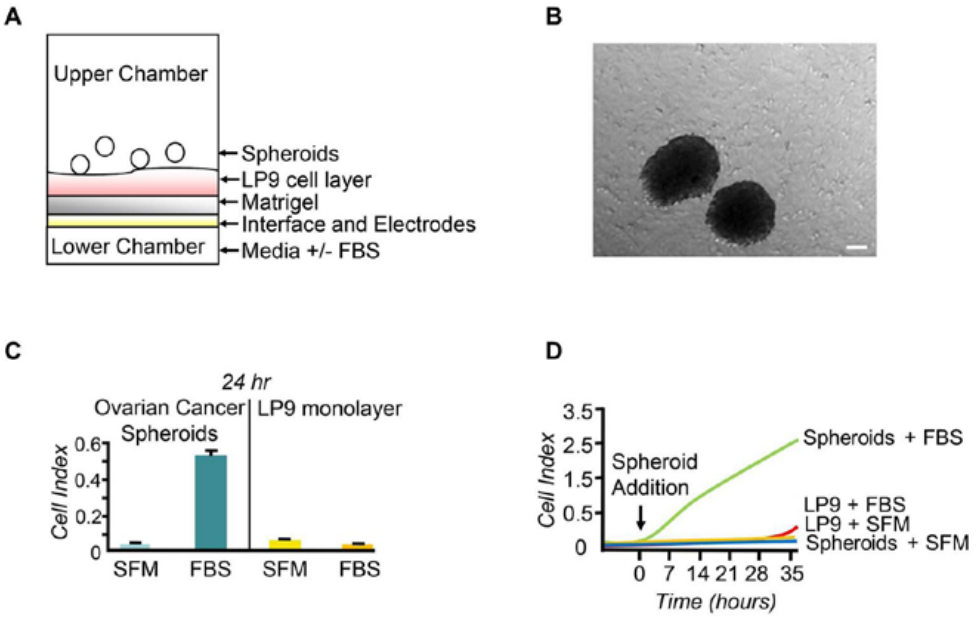

Figure 9. Transmigration of ovarian cancer spheroids. (A) Assay schematic. To mimic the microenvironment that metastasizing ovarian cancer cells encounter when invading through the peritoneal lining, the upper chambers of a CIM-Plate were first coated with a layer of Matrigel® followed by a monolayer of LP9 mesothelial cells. Finally, ovarian cancer spheroids were added on top. (B) Phase contrast microscopy image of ovarian cancer spheroids on top of an LP9 cell monolayer. (C) Quantitative analysis of cancer spheroid invasion capacity assessed 24 hours after plating. Media in the lower chamber of the CIM-Plate was either serum free (SFM) or was supplemented with FBS to drive chemotaxis. The capacity of LP9 cells to invade through Matrigel® (i.e. in the absence of cancer spheroids) is shown as a negative control. (D) Same as (C), except the real-time impedance readings are plotted continuously for 35 hours after spheroid addition. Figures adapted from J Vis Exp. 2014 May 20;(87).

Select Publications:

1. **Mechanistic modeling of the effects of myoferlin on tumor cell invasion.** Eisenberg MC1, Kim Y, Li R, Ackerman WE, Kniss DA, Friedman A. Proc Natl Acad Sci U S A. 2011 Dec 13;108(50):20078-83.
2. **MicroRNA-200c represses migration and invasion of breast cancer cells by targeting actin-regulatory proteins FHOD1 and PPM1F.** Jurmeister S, Baumann M, Balwierz A, Keklikoglou I, Ward A, Uhlmann S, Zhang JD, Wiemann S, Sahin Ö. Mol Cell Biol. 2012 Feb;32(3):633-51.
3. **c-Myb regulates matrix metalloproteinases 1/9, and cathepsin D: implications for matrix-dependent breast cancer cell invasion and metastasis.** Knopfová L, Beneš P, Pekarčíková L, Hermanová M, Masařík M, Pernicová Z, Souček K, Smarda J. Mol Cancer. 2012 Mar 23;11:15.
4. **Assessment of ovarian cancer spheroid attachment and invasion of mesothelial cells in real time.** Bilandzic M, Stenvers KL. J Vis. Exp. 2014 May 20;(87).
5. **The ubiquitin ligase CHIP inactivates NF-κB signaling and impairs the ability of migration and invasion in gastric cancer cells.** Liu F, Zhou J, Zhou P, Chen W, Guo F. Int J Oncol. 2015 May;46(5):2096-106.

Hallmark #4 : Inducing Angiogenesis

Neoplastic cell growth causes a localized need for oxygen and nutrients that exceeds the capabilities of the existing vasculature. Subjected to chronic hypoxia and nutritional stress, tumors are under strong selective pressure to promote angiogenesis – the process of forming new blood vessels from pre-existing ones. The efficacy of angiogenesis inhibitors in slowing tumor growth highlights the importance of new blood vessel formation for progression of the disease.

Endothelial cell proliferation and chemotactic migration towards cancer cells are key processes for angiogenesis within a tumor's microenvironment. In the below example the protein angiopoietin 4 (Ang-4) was evaluated for its ability to influence endothelial cell proliferation and migration. Small cell lung cancer cells were embedded in Matrigel® chambers that either contained or did not contain Ang-4. After being incubated for 16 days within subcutaneous pockets in mice, chambers were recovered and photographed. As seen in Figure 10A, the presence of Ang-4 within the chamber inhibited mouse endothelial cells (red dots) from building an invasive vascular network to supply the embedded tumor cells. To elucidate the mechanism by which Ang-4 inhibits angiogenesis, the proliferation of human umbilical vein endothelial cells (HUVECs) was analyzed ± Ang-4 using xCELLigence real-time cell analysis. Ang-4 was found to have an insignificant effect on the proliferation of HUVECs grown in the presence (Figure 10B) or absence (not shown here) of the growth factors bFGF and VEGF. The ability of Ang-4 to inhibit angiogenesis *in vivo* combined with its inability to suppress endothelial cell proliferation *in vitro* suggested that it acts by inhibiting endothelial cell migration. This was confirmed to be the case using a transwell assay (not shown here).

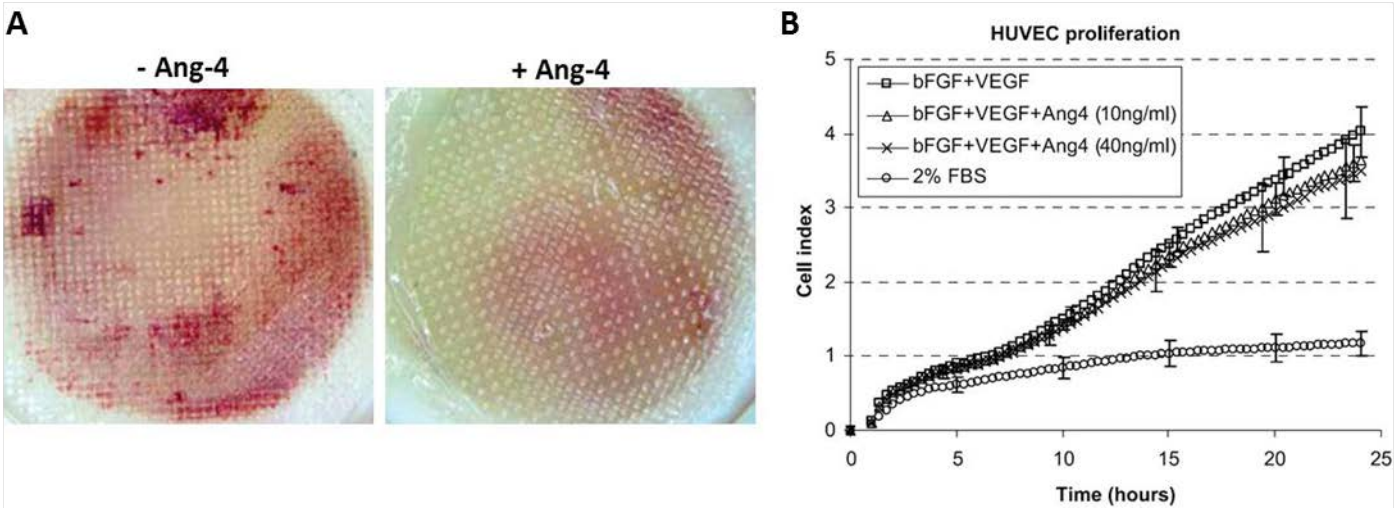

Figure 10. Angiopoietin 4 (Ang-4) suppresses angiogenesis by inhibiting endothelial cell migration but not proliferation. See text for details. Figure adapted from Neoplasia 2006 May;8(5):364-72.

Select Publications:

1. **Angiopoietin-4 inhibits angiogenesis and reduces interstitial fluid pressure.** Olsen MW, Ley CD, Junker N, Hansen AJ, Lund EL, Kristjansen PE. Neoplasia. 2006 May;8(5):364-72.
2. **Inhibition of APE1/Ref-1 redox activity with APX3330 blocks retinal angiogenesis *in vitro* and *in vivo*.** Jiang A, Gao H, Kelley MR, Qiao X. Vision Res. 2011 Jan;51(1):93-100.
3. **Slow binding kinetics of secreted protein, acidic, rich in cysteine-VEGF interaction limit VEGF activation of VEGF receptor 2 and attenuate angiogenesis.** Cydzik M, Abdul-Wahid A, Park S, Bourdeau A, Bowden K, Prodeus A, Kollara A, Brown TJ, Ringuette MJ, Gariépy J. FASEB J. 2015 Aug;29(8):3493-505.

Though it serves multiple purposes, one of the main roles of apoptosis is the elimination of cells that are damaged/stressed. This capacity for programmed cell death is present in most human cell types and is tightly regulated, with its initiation being dependent upon the net input of survival-promoting and death-inducing signals – which can be intracellular or extracellular in origin.

It is now clear that the expansion of tumor cell populations is a function of both cell proliferation and the rate at which cells are eliminated by apoptosis. Genetic modifications causing increased pro-survival (i.e. anti-apoptosis) signaling and/or defective pro-death signaling are common in many types of cancer. This capacity for evading apoptosis can lead to cancer cells being refractory to chemo- and radiotherapies. Further elucidating the mechanisms of apoptosis avoidance, and finding ways to re-institute the capacity for apoptosis in cancer cells is, accordingly, of significant interest.

TNF-related apoptosis-inducing ligand (TRAIL) is a transmembrane protein whose extracellular domain binds to cognate receptors on target cells and induces target cell death via the extrinsic apoptosis pathway. The fact that TRAIL's two primary receptors are frequently overexpressed on cancer cells has led to substantial efforts towards using the soluble extracellular domain of TRAIL for cancer treatment. Results have been mixed, with many cancer cell types displaying adaptive resistance to TRAIL via downregulation of pro-apoptosis signaling/effector proteins. In a screen for molecules able to sensitize apoptosis-resistant melanoma cells to TRAIL, the small molecule BMS-345541 was identified. As seen in Figure 11 melanoma cell lines are relatively insensitive to treatment with TRAIL or BMS-345541 alone, but exhibit a substantial loss in impedance signal when treated with both agents simultaneously. Subsequent studies demonstrated this robust effect to be the result of increased apoptosis due to BMS-345541's ability to activate the apoptosis stimulating protein Bax. In short, BMS-345541 places the apoptosis system on a hair trigger so that TRAIL-induced signaling effectively results in target cell death.

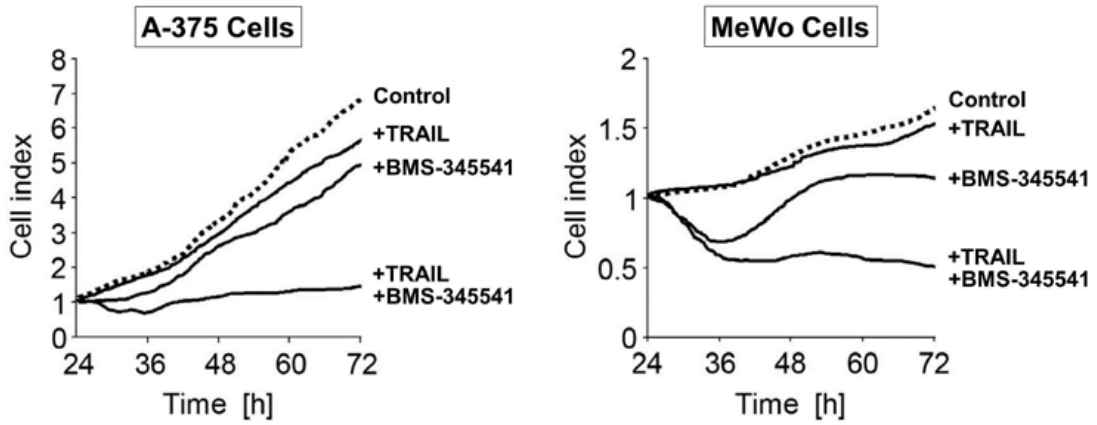

Figure 11. The small molecule BMS-345541 sensitizes melanoma cells to apoptotic signaling induced by TRAIL. See text for details. Figure adapted from Cell Death Dis. 2013 Jan 24;4:e477.

Select Publications:

1. **Inactivation of glycogen synthase kinase-3beta contributes to brain-derived neutrophic factor/TrkB-induced resistance to chemotherapy in neuroblastoma cells.** Li Z, Tan F, Thiele CJ. Mol Cancer Ther. 2007 Dec;6(12 Pt 1):3113-21.
2. **Sensitization of melanoma cells for death ligand-induced apoptosis by an indirubin derivative--Enhancement of both extrinsic and intrinsic apoptosis pathways.** Berger A, Quast SA, Plötz M, Hein M, Kunz M, Langer P, Eberle J. Biochem Pharmacol. 2011 Jan 1;81(1):71-81.

Hallmark #6: Replicative Immortality

Self-sufficiency in growth signaling, insensitivity to anti-growth signals, and evasion of apoptosis are three hallmarks of cancer that endow a cell with the ability to proliferate uncoupled from signals in its environment. It turns out, however, that by themselves these three properties are insufficient for expansive tumor growth because cells contain an intrinsic, autonomous replication "clock" that limits the number of times they can double. This clock is based on the finite length of telomeric DNA, which is progressively eroded with every cycle of genome replication. When its telomeres are sufficiently shortened a cell will enter the non-proliferative but viable state known as senescence. If genetic modifications to regulator proteins such as p53 enable the cell to escape this senescence blockade and continue replicating its telomeres will eventually become too short to function as protective "caps" and chromosome termini will undergo end-to-end fusions that lead to a crisis phase associated with cell death. In order for a cancer cell to attain replicative immortality it must first overcome the senescence checkpoint and ultimately evade crisis by re-expressing the telomere lengthening enzyme telomerase.

In a study of normal dermal fibroblast (NDF) aging it was found that overexpression of lamin B1 (LMNB1) causes senescence. Using xCELLigence real-time impedance monitoring, NDFs overexpressing LMNB1 were found to remain viable and continue adhering to the plate bottom but failed to proliferate (dark blue trace in Figure 12). However, coexpression of an activated form of the p53 oncogene enabled the NDF's to escape senescence and maintain replicative competence (light blue trace). This p53-mediated bypass of the senescence blockade is consistent with what must occur in order for cancer cells to attain replicative immortality.

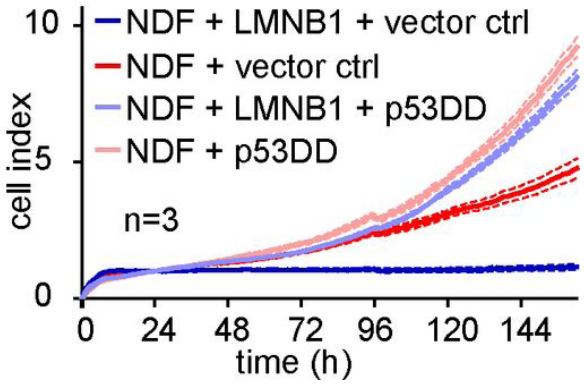

Figure 12. The senescence induced in normal dermal fibroblasts (NDFs) by LMNB1 overexpression can be overcome by coexpression of activated p53. See text for details. Figure adapted from J Cell Biol. 2013 Mar 4;200(5):605-17.

Select Publications:

1. **Kinetic tracking of therapy-induced senescence using the real-time cell analyzer single plate system.** Weiland T, Berger A, Essmann F, Lauer UM, Bitzer M, Venturelli S. Assay Drug Dev Technol. 2012 Jun;10(3):289-95.
2. **Lamin B1 fluctuations have differential effects on cellular proliferation and senescence.** Dreesen O, Chojnowski A, Ong PF, Zhao TY, Common JE, Lunny D, Lane EB, Lee SJ, Vardy LA, Stewart CL, Colman A. J Cell Biol. 2013 Mar 4;200(5):605-17.
3. **MicroRNA 299-3p modulates replicative senescence in endothelial cells.** Jong HL, Mustafa MR, Vanhoutte PM, AbuBakar S, Wong PF. Physiol Genomics. 2013 Apr 1;45(7):256-67.

Upon exposure to carcinogens, mice that are genetically engineered to be deficient for different components of the immune system develop tumors more frequently than immunocompetent controls. These and other data have established that for at least some types of cancer both the innate and adaptive branches of the immune system provide a substantial barrier to tumor formation and progression. Accordingly, in addition to evading extrinsically- and intrinsically-stimulated apoptotic death programs cancer cells must also evade destruction from the immune system.

In the below example the capacity for killing different types of cancer cells was evaluated using peripheral blood mononuclear cells (PBMCs) in combination with an EpCAM-specific bispecific T cell engager (BiTE). As seen in Figure 13A, based on immunofluorescence the EpCAM protein is present on the surface of both T47D breast cancer cells and primary lung cancer cells. Using xCELLigence real-time impedance monitoring T47D cells (pink trace) show a dramatic decrease in cell index upon simultaneous exposure to PBMCs and the EpCAM BiTE (Figure 13B; blue trace). In contrast, killing of primary lung cancer cells by PBMCs is not significantly different in the presence or absence of EpCAM BiTE. This inability for EpCAM-specific BiTE to mediate effective killing of the EpCAM-expressing lung cancer cells is indicative of one or more immune evasion strategies. Subsequent flow cytometry studies demonstrated that these primary lung cancer cells express programmed death ligand 1 (PDL-1), which inhibits activation of IL-2 production and T cell proliferation (not shown here). In contrast, T47D cells do not express PDL-1. While there may be other factors beyond PDL-1 expression at play here, this serves as an excellent example of the types of mechanisms that can enable cancer cells to evade immune destruction.

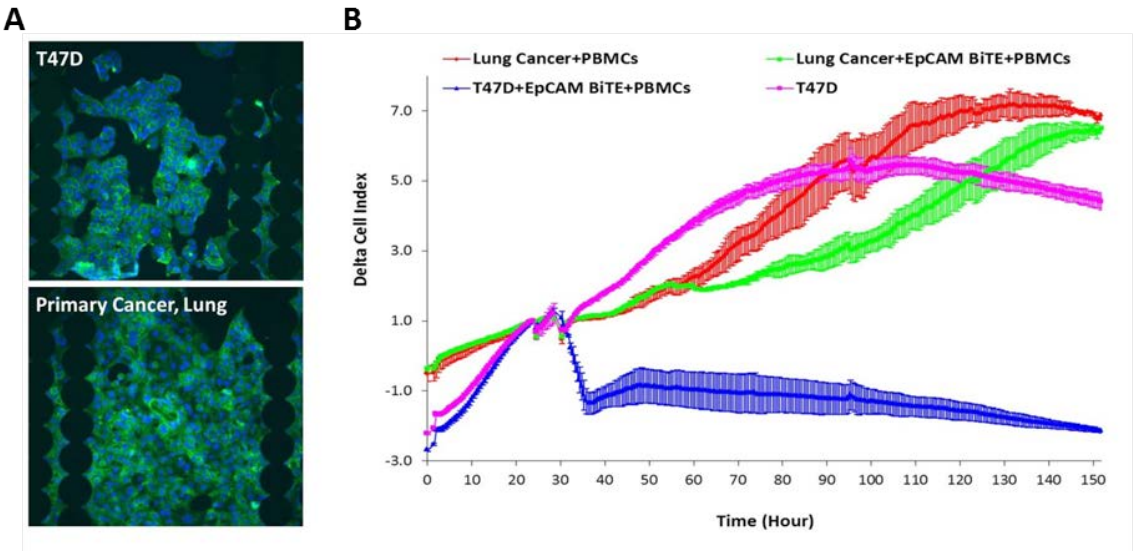

Figure 13. T47D breast cancer cells evade EpCAM BiTE-mediated destruction by PBMCs. (A) Immunofluorescence analysis of EpCAM expression (green) in the T47D cell line and primary lung cancer cells. Blue is Hoechst 33342 staining of nuclei. The large black circles are silhouetted microelectrodes. (B) Real-time impedance traces for T47D and primary lung cancer cells subjected to different treatments. Error bars represent standard deviation. See text for details. Figures are unpublished data from ACEA Biosciences. Primary lung cancer cells were obtained from Propagenix.

Select Publications:

1. **Mature cytotoxic CD56(bright)/CD16(+) natural killer cells can infiltrate lymph nodes adjacent to metastatic melanoma.** Messaoudene M, Fregni G, Fourmentraux-Neves E, Chanal J, Maubec E, Mazouz-Dorval S, Couturaud B, Girod A, Sastre-Garau X, Albert S, Guédon C, Deschamps L, Mitilian D, Cremer I, Jacquelot N, Rusakiewicz S, Zitvogel L, Avril MF, Caignard A. Cancer Res. 2014 Jan 1;74(1):81-92.
2. **Novel bispecific antibodies increase γδ T-cell cytotoxicity against pancreatic cancer cells.** Oberg HH, Peipp M, Kellner C, Sebens S, Krause S, Petrick D, Adam-Klages S, Röcken C, Becker T, Vogel I, Weisner D, Freitag-Wolf S, Gramatzki M, Kabelitz D, Wesch D. Cancer Res. 2014 Mar 1;74(5):1349-60.
3. **CAR-T Cells Inflict Sequential Killing of Multiple Tumor Target Cells.** Davenport AJ, Jenkins MR, Cross RS, Yong CS, Prince HM, Ritchie DS, Trapani JA, Kershaw MH, Darcy PK, Neeson PJ. Cancer Immunol Res. 2015 May;3(5):483-94.

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