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METHOD IN CELL SCIENCE

Dynamic assessment of cell viability, proliferation and migration using real time cell analyzer system (RTCA)

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Abstract Cell viability and cell migration capacities are critical parameters for cell culture-related studies. It is essential to monitor the dynamic changes of cell properties under various co-culture conditions to our better understanding of their behaviours and characteristics. The real time cell analyzer (RTCA, xCELLigence, Roche) is an impedance-based technology that can be used for label-free and real-time monitoring of cell properties, such as cell adherence, proliferation, migration and cytotoxicity. The practicality of this system has been proven in our recent cancer studies. In the present method, we intend to use co-cultures of pancreatic cancer cells (HP62) and mesenchymal stem cells to describe in detail, the procedures and benefits of RTCA.

Keywords RTCA · xCELLigence · Cytotoxicity assay · Migration assay

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Introduction

Cancer therapy still remains one of the challenges of today's medicine. Among different cancers, pancreatic cancer is one of the most lethal, with 2–4 % survival rate in 5 years, and the current therapies lack specificity to a large degree (Siegel et al. 2012). Current research is focused on the development of new therapeutic strategies for finding a novel approach to treatment of this debilitating disease. If not all, most current therapies are passive and symptomatic in nature leading to a pressing demand on scientists to improve the specificity of the current therapies.

Mesenchymal stem cells (MSCs) are a group of adult stem cells first identified from human bone marrow by Friedenstein and colleagues in 1960s (Friedenstein et al. 1966, 1968). They originate from the mesodermal germ layers and are capable of differentiating into tissues of muscle, the vascular system, and connective tissue. MSCs are known to have tumor tropism and homing capacity that provides a great therapeutic advantage in combination with their fast and easy acquisition (Dai et al. 2011; Moniri et al. 2012; Sasportas et al. 2009; Sun et al. 2011; Yang et al. 2009). Anticancer gene-engineered MSCs have been studied extensively and our recent studies demonstrate the ability of pancreas-derived engineered-MSCs to harness anti-tumor effect in pancreatic and hepatic cancer cell models (Moniri et al. 2012; Sun et al. 2011). For the purpose of this methodology, we will concentrate on the use of RTCA technology in the field of

pancreatic cancer therapy and its benefits over the conventional assays for monitoring cell cytotoxicity, proliferation, and the migration/invasion. We shall discuss the detailed procedure on how this technology can aid in studying the effects of MSCs on pancreatic cancer cells.

Conventional assays

Flow cytometry and microscopy along with many endpoint assays have been used widely to assist researchers in determining the cells reaction to specific treatments. For instance, in determining the cytotoxicity of a treatment, assays such as Annexin V staining and Live/Dead assays are commonly utilized and experimented (Schmid et al. 1992; Shimokawa et al. 1998). Similarly, for determining the proliferation rate of cells in accordance to a treatment, there are many cell cycle stage stainings that can aid in better understanding the states of the cells (Krishan 1975). However, these endpoint assays and similar ones have many disadvantages: (1) they can only determine the end physiological state of the cells, (2) due to various steps involved with the protocol, false positives are plausible, and (3) no information is provided during the course of the treatment, unless many time-points are performed, which by itself can be a source of error.

The scratch wound healing assay is one of the earliest in vitro methods used to measure and observe cell migration and was developed based on cell migration in vivo during the process of wound healing (Menon et al. 2009). In an in vivo environment, due to contact inhibition, proliferation is prohibited once a monolayer of endothelial cells has reached confluency (Lingen 2003). However, by scratching the confluent monolayer to produce a "wound" the cells at the edge of the wound, that were once contact inhibited, are now able to migrate back to "heal" the exposed region (Lingen 2003). The rate at which the exposed space is once again covered by a monolayer of cells can be measured and is considered a quantification of in vitro wound healing (Lingen 2003). The scratch wound healing assay is used in a variety of studies that look at cell polarization, migration and other activities associated with the various types of adherent cell lines (Menon et al. 2009). However, although results can be seen quickly, it is not an assay that will provide reliable information on directed migration in which, in vivo angiogenesis is a critical factor that needs to be considered (Lingen 2003). Furthermore, the quantification of migration is at most semi-quantitative, quite labor intensive (Menon et al. 2009), and may cause damage to the cells at the very edges of the wound, preventing proper migration of the cells behind them (Wang et al. 2008). The Boyden chamber is an additional method that is widely used for measuring chemotactic capacity of cells (Boyden 1962). This technique is a membrane-based assay in which cells are seeded on one side of the membrane and after migration period through the porous membrane, they are stained for further analysis via microscopy (Boyden 1962). As stated above, all such assays do not give an accurate depiction of cells during the migratory period but rather, measure end-points. Currently, the only machine that is capable of performing all of these tasks with ease and reproducible accuracy is the RTCA.

Real time cell analyzer (RTCA)

RTCA is a novel technique that uses real time cell monitoring to detect migration, cytotoxicity, and adherence/proliferation of cells during direct and indirect co-cultures. The RTCA system was developed by the partnership between Roche Applied Science (Quebec, Canada) and ACEA Biosciences Inc. (San Diego, CA, USA). This technology allows for uninterrupted, label free, and real time analysis of the cells over the course of the experiment. It is now widely used by researchers across the world in many different fields of research, and the full list of references to the device is available via the Roche website.

The RTCA is capable of carrying three plates and can be used for different assays: cell proliferation/cytotoxicity, migration/invasion, and co-culture studies. The functional mechanism of this instrument was summarized in previous reviews (Qiao et al. 2008; Reya et al. 2001). Briefly, this machine functions on the basis of electronic impedance reading from the gold plated sensor electrodes that are placed at the bottom of the plates in adhesion and cytotoxicity plates (E-16 plate), or the lower face in the migration plates (CIM plate). Electronic readings change as cells attach or detach from the surface electrodes, thus producing a change in impedance that is calculated via complex mathematical algorithms and plotted as cell index (CI) values. There is a direct correlation between the number of cells attaching and the CI

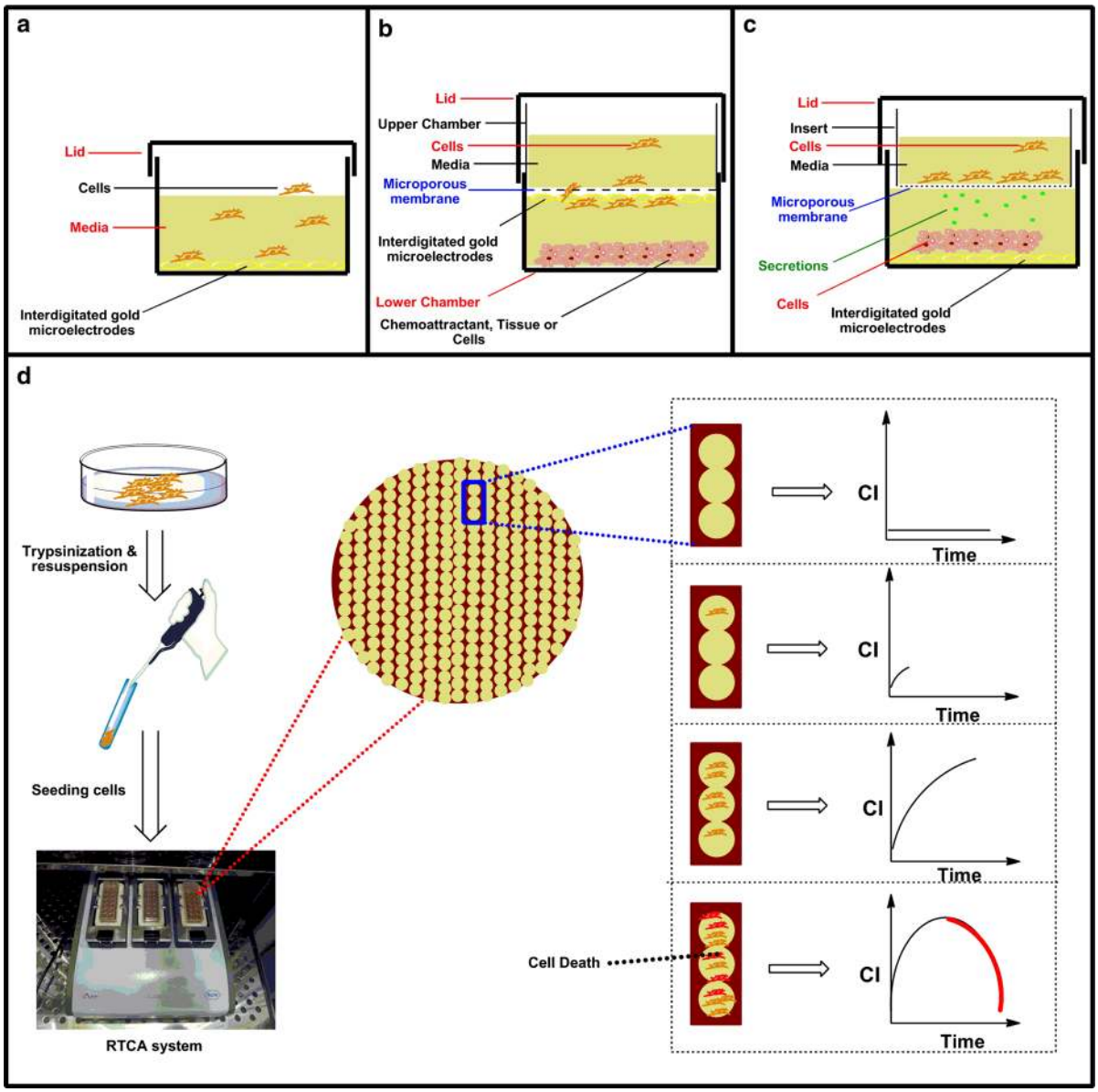

Fig. 1 Real time cell analyzer system: various plates and impedance technology. **a** Depicts the cytotoxicity/proliferation plate. **b** Displays the migration/invasion plate with their *upper* and *lower* chambers. **c** shows the insert used in conjunctions with the plate in "**a**" that is used for co-culture studies. **d** Demonstrates the relationship between cell adherence to the gold plated microelectrode and the CI readings by the machine

readout on the machine, and vice versa. This impedance reading could be affected by the quality of cell interactions and adherent properties between each cell and the electrodes. Figure 1a–c illustrates the differences seen within each plate system and in (d) the correlation between the CI readings with cell adherence to the plate is demonstrated. As the number of attached cells increases, a higher value of CI is recorded until the plate is 100 % confluent at the peak of the curves. On the other hand, the CI value decreases either by the addition of a cytotoxic inducing agent or over-confluency of the plate. This technology creates a label free environment for the cells and can realistically represent the influences upon cells during an experiment, without the use of toxic assays or end point assays that can lead to the

termination of the experiment. In the study that follows, the RTCA is used as a monitoring device to detect the cytotoxicity and migration capabilities of MSCs towards a pancreatic cancer cell line, HP62.

Experimental design

Cell culture conditions

Human pancreatic MSCs were isolated from ductal tissue of a human organ donor's pancreas and expanded ex vivo, as previously described (Duncan et al. 2005). HP62 cells were a gift from Dr. Ou (Department of Surgery, University of British Columbio, Vancouver, Canada). Both MSCs and HP62 cells were cultured in minimum essential medium (MEM) with 10 % fetal bovine serum (FBS), 2 mM L-glutamine and 1 % penicillin- streptomycin solution (all from Invitrogen, Carlsbad, CA, USA) and incubated at 37 °C in a humidified, 5 % CO_2 atmosphere. The cells used in this study were limited to fewer than nine passages upon thawing from the frozen stock. Cells were frozen upon initial harvest and propagation, and were stored in a −80 °C freezer in 1.5 ml cryopreservation vials. They were frozen in a total volume of 1 ml containing: 70 % cell suspension + 20 % FBS + 10 % dimethyl sulfoxide (DMSO) and kept in the −80 °C freezer for 1 week before being moved into liquid N_2 for long term storage. When thawing the cells, care was taken to transfer cells from the vial to fresh medium as soon as they were thawed in the 37 °C water bath under aseptic conditions. After one wash using fresh medium, the cell suspension was seeded into a 25 cm^2 culture flasks and incubated at 37 °C, in a 5 % CO_2 and 95 % air environment.

TRAIL-bearing vector transfection of MSCs

Details of design and transfections of the vectors can be found in our recent publications (Moniri et al. 2012; Sun et al. 2011).

Real time cell analyzer system

Proliferation and characterization

The RTCA instrument was used to assess the adherence property of MSC and HP62 cells during a titration assay. An initial titration of different cell densities (40, 20, 10, 5 and 2,500 cells/well) was performed and based on the results 20,000 cells was identified to be the ideal cell density for seeding. Cells were trypsinized briefly, counted using trypan blue dye exclusion and a haemacytometer, and were then re-suspended in culture medium. Background measurements were taken from the wells by adding 50 µl of the same medium to the E-16 plates. Subsequently, RTCA Software Package 1.2 was used to calibrate the plates. Cells were plated at a density of 20,000/well with fresh medium to a final volume of 200 µl. Cells were incubated for 4 min at 37 °C and 5 % CO_2 in the RTCA cradle. The impedance signals were recorded every 5 min for the first 25 scans (2 h) and every 10 min until the end of the experiment (up to 72 h).

Cytotoxicity assessment: indirect co-culture

HP62 cells were trypsinized and counted using trypan blue dye exclusion and a haemocytometer and resuspended in culture medium. Background measurements were taken from the wells by adding 50 µl of the same medium to the E-16 plates. Subsequently, RTCA Software Package 1.2 was used to calibrate the plates. Cells were plated at a density of 20,000/well in fresh medium to a final volume of 200 µl. Cells were incubated for 4 min at 37 °C and 5 % CO_2 in the RTCA cradle before the software schedule was initiated. The impedance signals were recorded every 5 min for the first 25 scans (2 h) and every 10 min until the end of the experiment (up to 72 h). After 20 h of impedance reading, 140 µl of medium was removed from each well and replaced with the appropriate volume of conditioned medium (CM). CM was obtained from a flask of TRAIL-engineered MSC cells grown in normal culture medium for 48 h. This medium was then prepared at 25, 50 and 100 % in normal culture medium and added to a final volume of 200 µl. The experiment was run for another 42 h and the impedance signals were recorded every 10 min until the end of the experiment.

Cytotoxicity assessment: direct co-culture

The procedure to the 20 h time point remains the same as described for the indirect co-culture above, with the exception of the additional step of the preparation of the direct culture insert that needs to be prepared a day in advance. Naive MSC cells were trypsin-digested and

counted by trypan blue dye exclusion. MSC cells were seeded into the insert at a density of 3×10^4/well in fresh medium to a total volume of 180 µl. The control consisted of a well with no cells seeded in the same insert(s). After ~24 h of incubation at 37 °C and 5 % CO_2, the inserts were taken to the RTCA unit where 140 µl of culture medium was removed and replaced with 130 µl of fresh medium before the inserts were slowly lowered into the E-16 plate (in the correct orientation). The impedance recording was then resumed on the machine for the duration of 72 h in 5 min intervals.

Migration/invasion assay

The RTCA system was also applied to monitor cell migration by using CIM-plates. CM was obtained from a flask of HP62 cells grown in normal culture medium for 48 h. This medium was then prepared at 25, 50 and 100 % in normal culture medium. Normal culture medium, SFM, or CM medium was then placed in the lower chamber. The plate was left to settle for 30 min at room temperature (RT) in sterile conditions. The upper chamber was then mounted and 25 µl of SFM medium was added to each well and left to equilibrate in the incubator for 1 h at 37 °C and 5 % CO_2. After the incubation, a background reading was taken for each well. MSCs were prepared in SFM, 40,000 cells were plated into each well of the upper chamber of the CIM-plates, and fresh medium was added to make up a total volume of 180 µl. Readings were recorded initially at 25 scans at 5 min intervals and then followed by scans at every 10 min intervals until the

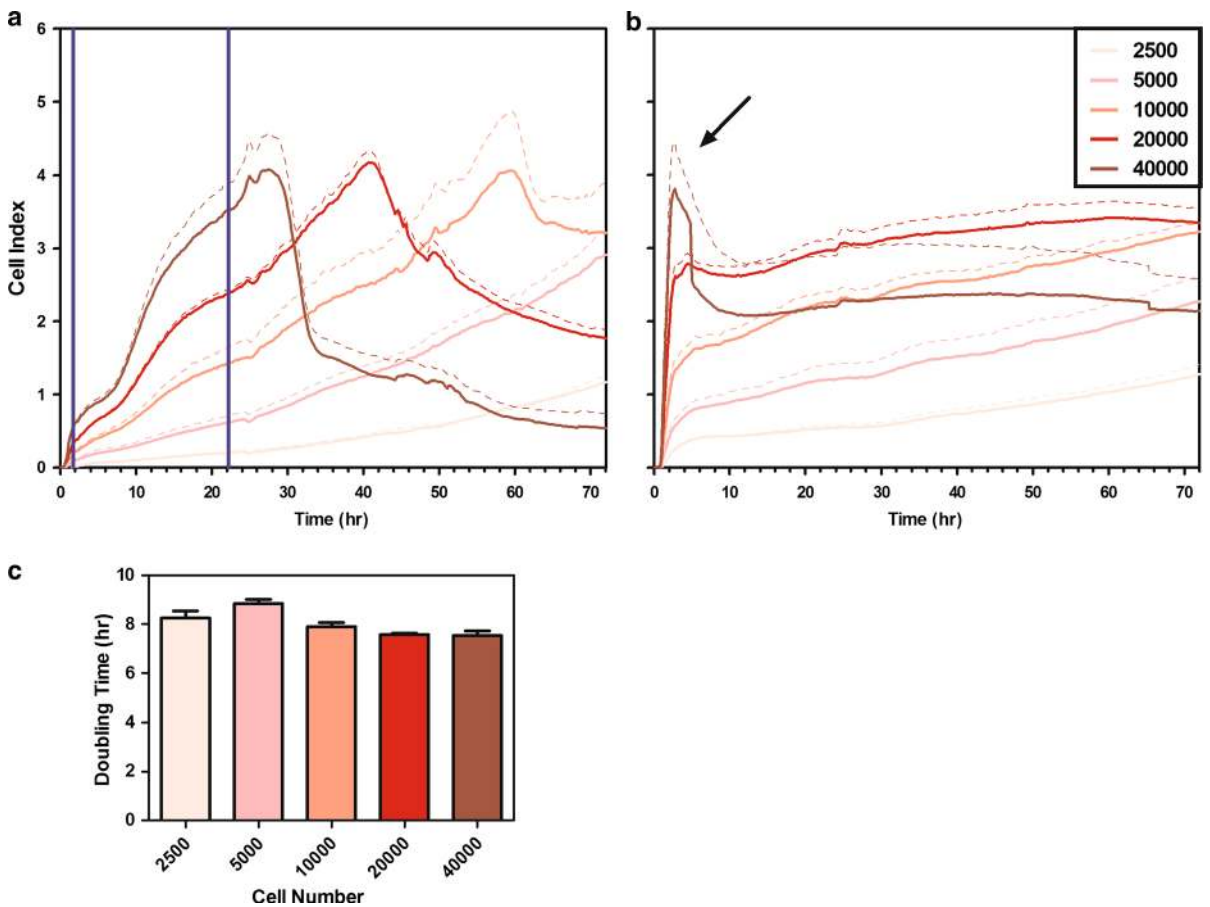

Fig. 2 Real time analysis of proliferation of HP62 and MSCs cells using E-16 plates. **a** Depicts the HP62 cell line proliferation profile during a cell titration from 2,500 to 40,000 cells per well. **b** MSC's proliferation profile. The *arrow* points to the peak that is uniquely of MSC's characterization. **c** Doubling time of Hp62 cell line with different initial cell seeding density between 2 and 22 h time points (*blue line* interval in **a**). All experiments were carried out and averaged above in the *solid lines* N = 3 ± SD (*the dotted lines*)

end of the experiment (up to 24 h). As an additional control, SFM was placed in the lower chamber and all other CI values were normalized to this baseline.

Statistical analysis

Cell index (CI) for real-time dynamic cytotoxicity assessment (N = 3) and slope calculations for the migration assessments were calculated automatically by the RTCA Software Package 1.2 of the RTCA system. Normalizations were performed using the RTCA Software Package 1.2. Numerical data were expressed as a mean ± standard deviation. Statistical differences between the means for the different groups were evaluated with Prism 5.0 (GraphPad software, La Jolla, CA, USA) using the Student's *t* test with the level of significance at $p < 0.05$. One way ANOVA with Bonferroni correction p values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in analysis of cytotoxicity measurements.

Results and discussion

This methodology paper describes the newest technique in real time monitoring of cells in vitro to detect proliferation, cytotoxicity, and migration. Here we demonstrate the proliferation profiles of two types of cell lines, HP62 and MSCs. HP62 cells reached their maximum CI at confluency after which the CI value declined as result of over confluency (Fig. 2a). Noteworthy, is the unique proliferation profile of MSCs (Fig. 2b), which show fast initial attachment to the plates followed by steady growth. These proliferation profiles can lead to calculation of doubling time (Fig. 2c), and can also be used to aid researchers to identify critical cell density/time points at which to perform transfection, cytotoxic treatments for the optimal response.

As has been observed previously, MSC's showed capacity to migrate at a higher rate towards the CM compared with NM and to the SFM controls (Moniri et al. 2012). This real time observation was not possible without RTCA technology and it confirmed the current understanding of the migratory capacity of these cells towards the site of cancer tissue or cells. As depicted in Fig. 3a, MSCs showed concentration dependent migratory capacity towards the conditioning medium obtained from HP62 cancer cells. Figure 3b is a representation of interval calculated slope that is determined by the RTCA software. Between 0 and 6 h, MSCs migrated at a statistically significant rate toward the CM obtained from cancer cells. These migration/invasion studies can be performed with much more efficiency than conventional methods, such as Boyden chamber, with the benefit of detecting migration under label free environment.

An indirect co-culture study of CM obtained from TRAIL-engineered MSC cells and HP62 cells was performed and demonstrates dose dependent cytotoxic

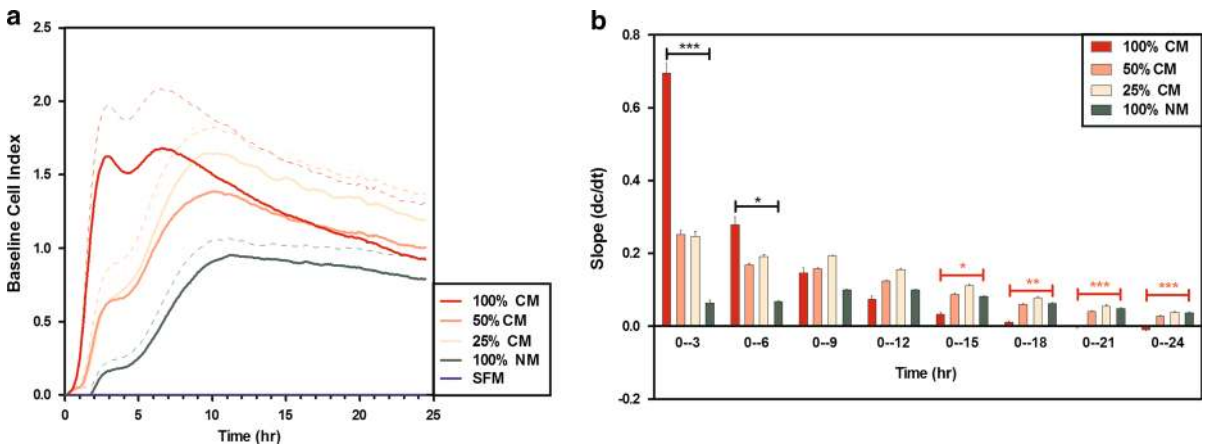

Fig. 3 Dynamic exploration of MSC's capacity of migrating towards cancer cells using CIM-plates. **a** Depicts the MSC's capacity to migrate towards cancer cell medium via a concentration dependant manner. Cell index values were normalized to SFM conditions. **b** The analysis of slope of the *lines* in different time intervals for **a**. All experiments were carried out and averaged above in the *solid lines* N = 3 ± SD (*the dotted lines*)

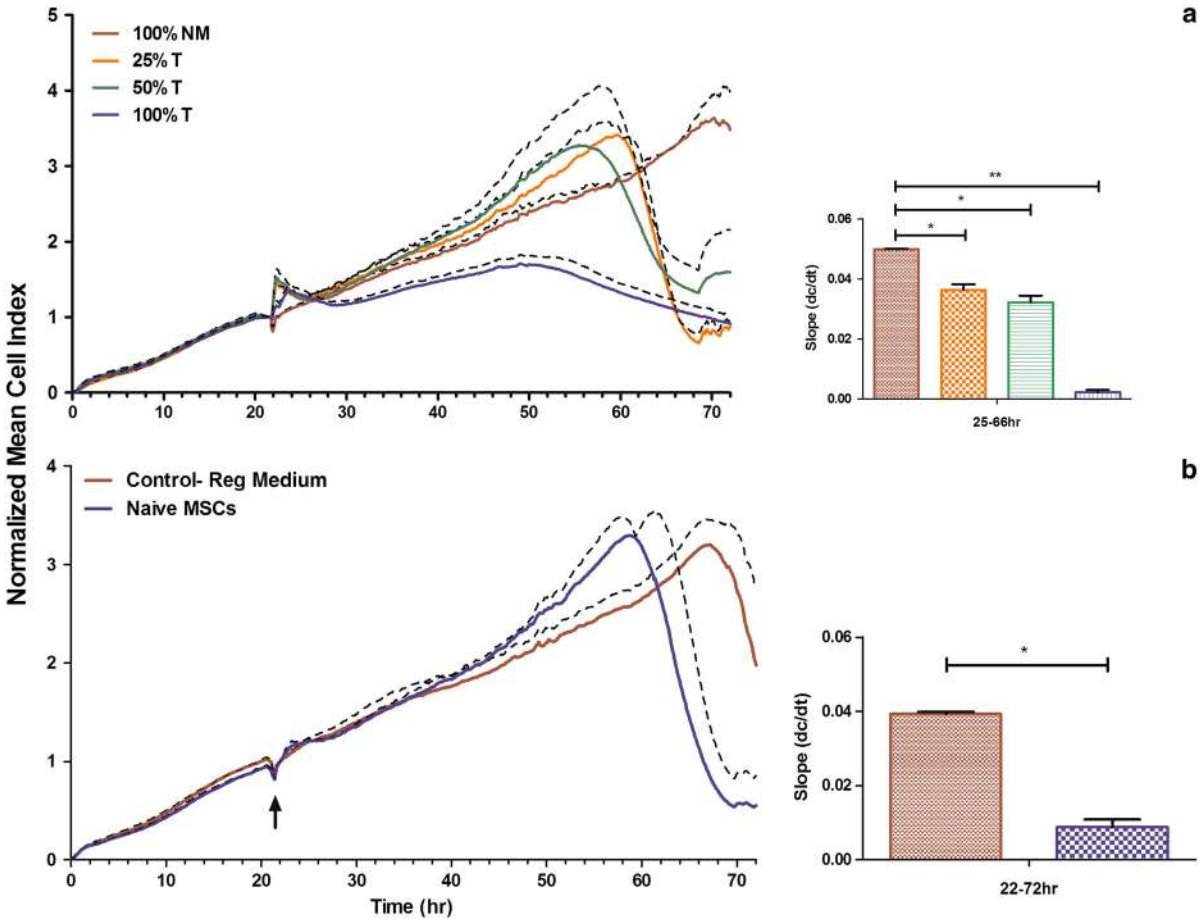

Fig. 4 Real time depiction of MSC's cytotoxic capacity in direct and indirect co-cultures using E-16 plates and E-16 inserts. **a** Represents the MSC-TRAIL concentration dependent cytotoxic conditioning medium (CM) when used on HP62 cells. The reduction in the slope of the real time curve in 100 %T (MSC-TRAIL CM) is apparent and more significant compared to other two concentrations. On the *right corner* there is a depiction of analysis of slope performed by the software. **b** Shows the naive-MSC capacity to induce cytotoxic effect on HP62 cells in a direct co-culture setting. The *blue line* (naive-MSC) apexes at much earlier time compared to the control *red line* indicating their cytotoxic capacity in direct cultures with cancer cells. All experiments were carried out and averaged above in the *solid lines* N = 3 ± SD (*the dotted lines*)

effect (Fig. 4a). To demonstrate the additional capacity of the RTCA, direct co-culture studies were conducted using E-16 plates and their corresponding inserts (as depicted in Fig. 1). Naïve MSCs that were seeded in the insert negatively affected the HP62 cell proliferation profile (Fig. 4b). This effect could be explained by mechanisms such as cell-to-cell contact between the MSC and HP62 cells or the paracrine function of MSCs. These results are very promising and confirm what is currently known in literature with regard to the innate ability of MSCs to induce the cell death of cancer cells. Furthermore, these inserts could thus be used to study the paracrine function of effector cells on their targets.

While the current study analyzed the data using slope calculation, the RTCA package provides other means of calculation depending on the desired experimental endpoint, these include: area under the curve calculations, IC50 and EC50. Overall, the protocols discussed in this study could be applied to drug/compound screening or other cell-to-cell interaction studies with high efficiency and reproducibility.

Conclusions

It is evident that by using the RTCA system, we can enhance our in vitro understanding of the characteristics

of a cell's response to a treatment. By using this approach we can monitor cells in real time and use the CI value as a measure to determine the proliferation rate, cytotoxicity effect, and migration capacity of the cells without any interruption in data collection or outside sources of error. Furthermore, by using RTCA there is the added advantage of performing endpoint assays such as FACS, microscopy and molecular analysis at any time interval during the experiment. Finally, as shown in this study using primary MSC cells, the RTCA system could be used as a monitoring platform in the clinic to determine the effectiveness of a drug treatment for an individual patient.

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Conflict of interest The authors declare no conflict of interest.

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