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A novel approach to quantify the wound closure dynamic

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ABSTRACT

The Wound Healing (WH) assay is widely used to investigate cell migration *in vitro*, in order to reach a better understanding of many physiological and pathological phenomena.

Several experimental factors, such as uneven cell density among different samples, can affect the reproducibility and reliability of this assay, leading to a discrepancy in the wound closure kinetics among data sets corresponding to the same cell sample. We observed a linear relationship between the wound closure velocity and cell density, and suggested a novel methodological approach, based on transport phenomena concepts, to overcome this source of error on the analysis of the Wound Healing assay. In particular, we propose a simple scaling of the experimental data, based on the interpretation of the wound closure as a diffusion-reaction process. We applied our methodology to the MDA-MB-231 breast cancer cells, whose motility was perturbed by silencing or over-expressing genes involved in the control of cell migration. Our methodological approach leads to a significant improvement in the reproducibility and reliability in the *in vitro* WH assay.

1. Introduction

Cell migration and proliferation play pivotal roles in a variety of physiological and pathological processes. These include morphogenesis, angiogenesis, inflammation, tissue repair, and tumor invasion [1,2]. Therefore, it is of great interest and potential therapeutic importance to understand the mechanisms driving cell dynamic behavior, which are still far from being fully understood [3,4].

The wide range of *in vitro* assays to quantitatively evaluate cell motility, invasion and chemotaxis include straightforward and economical ones, such as the Boyden Chamber assay [5], or more expensive, time-consuming and technically demanding as the cell random motility assay [6,7]. This last one enables the investigation of cell motility at the level of both individual cells and entire cell population. The choice of one or the other method depends on the specific research question and cell type under investigation [8].

One of the most popular, widely used, and straightforward methods to characterize and quantify cell dynamic behavior *in vitro* is the Wound Healing (WH) assay [8], because of its low cost and simplicity to set-up [9]. Recently, it was proved the results from WH assay can be used to estimate single cell motility parameters [10], such as random motility coefficient, typically measured by time consuming random motility assay. In the conventional WH assay, also known as scratch test, cells are seeded on a planar surface, allowed to attach, spread and proliferate until they reach confluency. An artificial “wound” is then made by dragging a sterile pipette tip or needle across the cell monolayer [11,12]. Then cells are washed with an appropriate medium to remove debris and floating cells. Recently, novel non-mechanical techniques have been developed to achieve wounding without mechanically disrupting the cell layer [13-19]. After the wounding, the cells on the edges of the newly created gap loose the cell-cell contact inhibition and, stimulated by the availability of free space, start to move and proliferate until new cell-cell contacts are re-established and the wound is closed [20]. Contact inhibition is a property of normal somatic cells and a key anticancer mechanism that arrests cell division when cells reach a high density. Cancerous cells typically lose this property and thus grow in an uncontrolled manner even when in contact with neighboring cells. Loss of contact-inhibition has important implications for cancer invasion and metastasis [21,22].

Two different experimental approaches can be followed to monitor the wound closure process; microscope images within the sample can

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be acquired at a limited number of defined time steps [23-26] or by time-lapse microscopy (TLM), that allows to iteratively image specific regions along the wound at a fixed time frequency [24], while controlling the environmental conditions to ensure cell viability [10,27]. This last approach is more reliable, produces a larger amount of data for each sample, and allows to reduce the error related to the random choice of sample position for each acquisition. The wound closure dynamic is typically quantified by counting cells that repopulate the wound region at each time step [28,29], or by measuring the change in the wound size over time [23-26].

The time required and mechanism involved in the healing of the artificial wound strictly depends on the type of cells. Specifically, epithelial-like cells cover the wound area in concert as two coherent cell sheets [30,31], whereas fibroblast-like cells repopulate the wound region moving as individual units [32] due to the lack of strong cell-cell contacts [8,11,32,33].

The wound healing process can be modeled according to the Fisher-Kolmogoroff equation [34-37] (Eq. (1)), which is a diffusion-reaction equation. Inspired by transport phenomena concepts, this model mathematically describes the evolution in time of the cell density special profile u(x,t) as the sum of two contributions, i.e., cell motility and proliferation, both involved in the spatial spreading of the cells in the wound region:

$$\frac{\partial u}{\partial t} = D\frac{\partial^2 u}{\partial x^2} + ku\left(1 - \frac{u}{\hat{u}}\right) \quad (1)$$

Here, u is cell density at time t at a given distance x, measured from the wound edge, D is the constant diffusivity (also referred as random motility coefficient), k is the proliferation rate and û is cell density at confluency [38]. Cell motility is modeled as a Fickian-like diffusion, and depends on the differences between cell density at the wound edge and the cell-free area. Cell proliferation is mathematically described as a logistic growth, and includes crowding effects by reducing the growth rate as the cell density approaches confluency. Cell density as a function of distance is predicted to have a “double s” shaped profile, being maximum far from the wound and decreasing down to 0 in the cell nude area. Overall, the Fisher-Kolmogoroff equation predicts that after a short transient phase, the invading cell front propagates at a constant speed perpendicularly to the wound edge reducing the size of the wound area [39].

Easy to perform, the WH assay is particularly suitable to study and compare cell dynamic behavior *in vitro* under different experimental conditions. However, mechanical injure to the cells on the wound edges [40], attachment of cell debris to the wound margins and damages to the migrating surface [41] may represent a limit in accomplishing reproducible and reliable quantitative results [42]. In particular, equal cell density among different samples is challenging to achieve and reproduce, because of possible inaccuracy in cell counting, partial cell seeding, and, in particular, because of lipofection, electroporation or other invasive treatments cells can be subjected to before *in vitro* WH assay [43]. These treatments can result in a poor control of the effective cell density of the samples. Altogether, the aforementioned drawbacks affect the direct comparison of WH experiments made in different experimental conditions.

Here, we propose a novel approach to quantify the wound closure dynamic. The proposed method is based on transport phenomena concepts and takes into account the differences in cell density, overcoming the difficulty to compare samples that have been subjected to different experimental procedures. As a case study, we have applied our method to analyze data obtained from WH assays performed with MDA-MB-231 breast cancer cells, whose motility was perturbed by depletion or over-expression of genes involved in the control of cell adhesion and migration.

2. Materials and methods

2.1. Cell culture

MDA-MB-231 cells derived from metastatic breast carcinoma were purchased from Cell Line Service (CLS, Germany) and cultured at 37 °C and 5% CO_2, in DMEM supplemented with 10% FBS.

2.2. Transfection and RNA interference

MDA-MB-231 breast cancer cells were seeded at a density of about 70% confluence and transfected with 2.0 μg of empty or ΔNp63α expression vector using Lipofectamine 2000 reagent (InVitrogen, Life Technologies. Inc.). The expression vector encoding ΔNp63α was previously described [44].

YB-1 transient silencing was carried out by RNA interference with RIBOXX (IBONI YB-1siRNA pool) and RNAiMAX reagent (Invitrogen), according to the manufacturer's recommendations. Briefly, cells were seeded at 70% confluence (1.5×10^6 cells) in 100-mm dishes and transiently silenced with IBONI YB-1-siRNA at 20 nm final concentration. YB-1 guide sequences:

UUUAUCUUCUUCAUUGCCGCCCCC;
UUAUUCUUCUUAUGGCAGCCCCC;
UUCAACAACAUCAAACUCCCCC;
UCAUAUUUCUUCUUGUUGGCCCCC.

“All Star Negative Control siRNA”, provided by Quiagen, was used as negative control.

2.3. Immunoblot and antibodies

Immunoblot was performed following standard methods [43,45]. Briefly, 30 μg of whole cell extracts were separated by SDS-PAGE, subjected to western blot and incubated overnight at 4 °C with antibodies. Antibodies against p63 (4A4) and YB-1 (ab12148) were from Abcam; actin (1-19) antibody was from Santa Cruz Biotechnology (Inc. CA, USA). Signal intensities of Western blot bands were quantified by Quantity One software (Biorad).

2.4. Cell proliferation rate

YB-1 silenced, ΔNp63α and Mock transfected MDA-MB-231 cells were plated onto 12-well culture plates at 1×10^4 cells/cm^2 with FBS. At 24, 48 and 72 h, cells were harvested by suspension in 0.025% trypsin in 0.02% EDTA solution. Cell counts were performed in triplicates using a Neubauer chamber, with trypan blue exclusion assay to identify viable cells. Growth curves were generated and the cell population doubling time estimated (PDT).

2.5. WH assays

To perform WH assays, MDA-MB-231 control cells, MDA-MB-231 cells silenced for YB-1 expression and MDA-MB-231 cells transfected with ΔNp63α were plated on 12-well culture dishes (Corning) at a density of 7.63×10^4 cells/cm^2 (80% confluence). The cells were allowed to adhere and grow for 24 h in order to obtain uniform monolayers. The samples were wounded manually with a p200 pipette tip to mechanically remove an area of cells. The culture medium was removed and the wounded monolayers were washed twice with phosphate-buffered saline (PBS) to remove cell debris and suspended cells. Before starting TLM image acquisition, cell samples were covered with fresh culture medium. Each assay was repeated in triplicate.

2.6. Fluorescence microscopy

MDA-MB-231 cells were plated in 35 mm dish (4.2×10^3 cells/cm^2)