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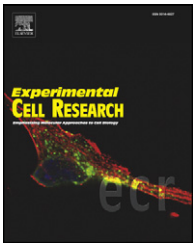

Research Article

Reprogramming of cell junction modules during stepwise epithelial to mesenchymal transition and accumulation of malignant features *in vitro* in a prostate cell model

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ABSTRACT

Epithelial to mesenchymal transition (EMT) is pivotal in tumor metastasis. Our previous work reported an EMT model based on primary prostate epithelial cells (EP156T) which gave rise to cells with mesenchymal phenotype (EPT1) without malignant transformation. To promote prostate cell transformation, cells were maintained in saturation density cultures to select for cells overriding quiescence. Foci formed repeatedly following around 8 weeks in confluent EPT1 monolayers. Only later passage EPT1, but not EP156T cells of any passage, could form foci. Cells isolated from the foci were named EPT2 and formed robust colonies in soft agar, a malignant feature present neither in EP156T nor in EPT1 cells. EPT2 cells showed additional malignant traits *in vitro*, including higher ability to proliferate following confluence, higher resistance to apoptosis and lower dependence on exogenous growth factors than EP156T and EPT1 cells. Microarray profiling identified gene sets, many of which belong to cell junction modules, that changed expression from EP156T to EPT1 cells and continued to change from EPT1 to EPT2 cells. Our findings provide a novel stepwise cell culture model in which EMT emerges independently of transformation and is associated with subsequent accumulation of malignant features in prostate cells. Reprogramming of cell junction modules is involved in both steps.

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Introduction

Carcinomas arise from normal epithelial tissues in a multistep process. The breakdown of epithelial cell homeostasis leading to aggressive cancer progression is associated with the loss of epithelial characteristics and the acquisition of a migratory phenotype, referred to as epithelial to mesenchymal transition (EMT). EMT is thought to be a crucial event in tumor progression in which EMT follows or accompanies malignant transformation and endows cancer cells with invasive and metastatic competence [1–7]. In a transformation attempt, however, we have observed complete EMT from primary prostate epithelial cells (EP156T) to cells with mesenchymal phenotype (EPT1) without malignant transformation [8]. EPT1 cells were found to exhibit mesenchymal morphological and functional attributes, E-cadherin to N-cadherin switching, as well as extensive reprogramming of cell junction modules, including tight junctions, gap junctions, desmosomes and hemidesmosomes [8]. Neither EP156T nor EPT1 cells, however, can grow anchorage-independently in soft agar, which constitutes a hallmark of malignant transformation, suggesting that EMT emerged before and independently of key features of malignant transformation [8].

In the present study, we asked whether EMT is an earlier step to malignant transformation and can promote subsequent acquisition of malignant features. One way to verify this hypothesis is by comparing the growth properties of the original epithelial cells (EP156T) with the properties of the progeny cells that underwent EMT (EPT1). Neoplastic cells exhibit a number of altered phenotypic characteristics as compared to non-transformed wild type cells, including altered cellular morphology, ability to form foci or growth to elevated cellular density levels before slowing or cessation of proliferation in confluent cultures, ability to grow without substrate anchorage and to grow in media containing low levels of serum or growth factors [9,10]. Extended incubation of cultured cells at confluence may induce neoplastic transformation and the generation of dense foci, which could reflect a physiological adaptation exploited by carcinogenesis [11]. In an attempt to transform prostate cells, both EP156T and EPT1 cells were kept growing at confluence in long-term cultures, and neoplastic features were examined by different assays.

Materials and methods

Cell lines and cell cultures

The prostate cell lines EP156T and EPT1 were grown in MCDB153 medium (Biological Ind. Ltd., Israel) with 1% and 5% fetal calf serum (FCS), respectively, and supplemented with growth factors and antibiotics as described [8,12]. The cells were grown in tissue culture plates in a humidified atmosphere containing 5% CO_2 at 37 °C. The medium was changed every third day. Cells were passaged or harvested for analysis when they reached 70% confluence.

Generation and cloning of EPT2 cells

EPT1 cells at passage 37 (p37) were kept growing at full confluence in 6-well plates in standard culture medium. The medium was changed every third day without trypsinization until foci formed 8 weeks later. Cells in the foci were picked to new plates using a P200 pipette tip under the microscope and were dissociated enzymatically (10 min in 0.05% trypsin, 0.53 mM EDTA) and mechanically, using a fire-polished Pasteur pipette. The dissociated cells were passed through a 40 μm sieve and analyzed microscopically for single cellularity. Single cells were seeded in soft agar cultures following standard procedures (see below). One week later, colonies in soft agar were recovered and transferred to 96-well plates to get one single colony per well. Cells from each well were considered as one clone and were expanded and recorded for further use.

Over-confluence proliferation assay

Each kind of cell in exponential phase was seeded in two 24-well plates with 5×10^4 cells per well. Cells from the three wells were harvested and counted using a hemocytometer every 2 days. Cells in remaining wells were kept growing without trypsinization. Medium was changed every second day.

Migration, invasion and apoptosis assay

Cell migration and invasion were examined using trans-well migration assay kits (Cell Biolabs Inc., San Diego, CA, USA) as described [8]. Apoptosis was determined using the Caspase-Glo 3/7 Assay (G8090, Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, cells were plated in 96-well plates and incubated for 12 h followed by staurosporine (Sigma, S6942) treatment at indicated concentrations for 6 h at 37°C to induce apoptosis. Equal volumes of Caspase-Glo 3/7 Reagent was added directly to each well and the plates were incubated at room temperature for 2 h before recording luminescence in a TECAN Infinite 200 (Tecan, Germany). Each cell line was tested in triplicates and values were expressed as mean ± standard deviation.

Growth factor independence assay

Cells in exponential phase were seeded into 96-well plates with 3500 cells per well. One group of cells were cultured in basic MCDB153 medium (Sigma M7403) without FCS and without extra growth factors. Control cells were grown in complete medium in parallel. Cells were split and transferred to new wells 1 week after seeding. The proliferation of each group was measured 1 week later using the CellTiter96®AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI, USA). MTS reagent was added directly to culture wells and, following incubation for 3 h at 37°C, absorbance was recorded at 490 nm using a 96-well plate reader (Powerwave spectrophotometer). The dependence of cells on serum and growth factors was represented as the relative proliferation of the test group compared with the control group.

Anchorage-independent growth assay

Anchorage-independent growth was examined in soft agar (Cell Biolabs; CytoSelect™ Cell, Colorimetric kit) as described [8]. Cells in the agar were stained with 0.005% crystal violet solution for 1 h (Sigma, C0775) before photographs. To quantitatively measure cell growth in soft agar, the agar matrix was solubilized, cells were stained and absorbance was recorded at 570 nm. Data show the quantification of proliferation of cells in the soft agar cultures.