Free Radical Biology and Medicine 76 (2014) 1–13

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Redox processes inform multivariate transdifferentiation trajectories associated with TGFβ-induced epithelial–mesenchymal transition

Adam F. Prasanphanich, C. Andrew Arencibia, Melissa L. Kemp *

The Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA 30332-0363, USA

ARTICLE INFO

Article history:
Received 8 January 2014
Received in revised form
16 July 2014
Accepted 24 July 2014
Available online 1 August 2014

Keywords:
Transforming growth factor β
Nucleophilic tone
Epithelial–mesenchymal transition
Transdifferentiation
Principal component analysis
Multivariate analysis
Multivariate phenotype
In-cell Western assay

ABSTRACT

Phenotype reprogramming during transforming growth factor β (TGFβ)-induced epithelial–mesenchymal transition (EMT) is an extensive and dynamic process, orchestrated by the integration of biological signaling across multiple time scales. As part of the numerous transcriptional changes necessary for EMT, TGFβ-initiated Smad3 signaling results in remodeling of the redox environment and decreased nucleophilic tone. Because Smad3 itself is susceptible to attenuated activity through antioxidants, the possibility of a positive feedback loop exists, albeit the time scales on which these mechanisms operate are quite different. We hypothesized that the decreased nucleophilic tone acquired during EMT promotes Smad3 signaling, enhancing acquisition and stabilization of the mesenchymal phenotype. Previous findings supporting such a mechanism were characterized independent of each other; we sought to investigate these relationships within a singular experimental context. In this study, we characterized multivariate representations of phenotype as they evolved over time, specifically measuring expression of epithelial/mesenchymal differentiation, redox regulators, and Smad transcription factors. In-cell Western (ICW) assays were developed to evaluate multivariate phenotype states as they developed during EMT. Principal component analysis (PCA) extracted anticorrelations between phospho-Smad3 (pSmad3) and Smad2/Smad4, which reflected a compensatory up-regulation of Smad2 and Smad4 following cessation of TGFβ signaling. Measuring transcript expression following EMT, we identified down-regulation of numerous antioxidant genes concomitant with up-regulation of NADPH oxidase 4 (NOX4) and multiple mesenchymal phenotype markers. TGFβ treatment increased CM-H_2DCF-DA oxidation, decreased H_2O_2 degradation rates, and increased glutathione redox potential. Our findings suggest that the decreased nucleophilic tone during EMT coincides with the acquisition of a mesenchymal phenotype over too long a time scale to enable enhanced Smad3 phosphorylation during initiation of EMT. We further challenged the mesenchymal phenotype following EMT through antioxidant and TGFβ inhibitor treatments, which failed to induce a mesenchymal-epithelial transition (MET). Our characterization of multivariate phenotype dynamics during EMT indicates that the decrease in nucleophilic tone occurs alongside EMT; however, maintenance of the mesenchymal phenotype following EMT is independent of both the nascent redox state and the continuous TGFβ signaling.

Introduction

More than seven decades since Waddington first introduced the concept of canalization, systems biology is still very focused on how to best characterize phenotype robustness, in which small perturbations from a developmental trajectory are protected against by a steep energetic landscape of descent toward a terminal state [1]. In the modern era, however, biological insight into the reversibility of differentiation processes through transdifferentiation and the reacquisition of pluripotency has expanded our view of how cells behave dynamically as they progress from one phenotype to another. The ability to measure many biomarkers in combination allows contemporary researchers to interrogate how cellular trajectories during transdifferentiation are driven or reinforced by various cellular programs. In particular, the influences of genetic reprogramming of a cellular state, such as redox potential, and external influences on this state can be investigated in parallel with traditional phenotype markers to

Abbreviations: TGFβ, transforming growth factor β; EMT, epithelial–mesenchymal transition; MET, mesenchymal-epithelial transition; PCA, principal component analysis; PC1, principal component 1; PC2, principal component 2; ICW, in-cell Western; qRT-PCR, quantitative real-time polymerase chain reaction; NOX4, NADPH oxidase 4; H_2O_2, hydrogen peroxide; H_2DCF, dichlorodihydrofluorescein; k_{deg}, H_2O_2 degradation rate; GSH, reduced glutathione; GSSG, oxidized glutathione; E_{GSH}, GSSG/2GSH half-cell reduction potential

* Corresponding author at: Petit Institute of Bioengineering and Bioscience, Georgia Institute of Technology, 315 Ferst Drive NW, Atlanta, GA 30332-0363.
E-mail address: melissa.kemp@bme.gatech.edu (M.L. Kemp).

http://dx.doi.org/10.1016/j.freeradbiomed.2014.07.032
0891-5849/

ask how cellular oxidation influences the progression from one cell type toward another.

One such transdifferentiation process, epithelial–mesenchymal transition (EMT), occurs when epithelial cells loose certain phenotypic qualities (such as apical–basal polarity and basement membrane interaction) and acquire mesenchymal characteristics (such as cell migration and production of extracellular matrix components) [2]. The cytokine transforming growth factor beta (TGFβ) induces EMT and has been implicated in increased invasiveness of cancers and in the formation of metastases [3–5]. TGFβ signaling is enhanced within the tumor microenvironment through interactions with cancer-associated fibroblasts, immune cells, and extracellular matrix [6,7]. Circulating tumor cells from breast cancer patients are enriched for mesenchymal markers and TGFβ signaling, implicating TGFβ-mediated EMT as a mechanism for entry into to the circulatory system [8]. On reentry into distant tissues, reversal of the mesenchymal-like phenotype into an epithelial phenotype (i.e., MET) may play a critical role in the establishment and progression of metastases [9]. A recent investigation of ovarian cancer, stratification of tumor phenotypes into epithelial, intermediate epithelial, intermediate mesenchymal, and mesenchymal states found increased 5-year progression-free survival in patients whose tumors were classified as epithelial or intermediate epithelial. Furthermore, of 43 classified cell lines, intermediate states were found to be more responsive to kinase inhibition [10]; thus, the timing of phenotype transition dynamics or characterization of intermediate phenotype states may have major implications for therapeutic strategies.

Numerous gene expression changes are initiated by TGFβ signaling as part of EMT. On binding of its cognate receptor, TGFβ quickly activates canonical and noncanonical signaling. Canonical signaling occurs through phosphorylation of Smad2 and Smad3 transcription factors, which bind Smad4 before translocation into the nucleus [11,12]. Smad3 phosphorylation and its transcriptional activity are critical steps in TGFβ-mediated EMT [13,14]. Smads have been linked to a number of critical steps involved in the formation of metastases. In one study, the formation of bone metastases by xenografted cancer cells relied on Smad4 [15]. In another, *in vitro* and *in vivo* metastatic processes were dependent on Smad3 and enhanced on Smad2 knockdown [16], while, in yet another study, Smad2 elevation enhanced *in vitro* and *in vivo* prometastatic processes [17]. Thus, the procarcinogenic mechanisms of TGFβ rely on Smad signaling to carry out transcriptional remodeling, though in ways that may be cancer or cell-type specific.

In addition to induction of EMT, TGFβ can transform the regulation of the intracellular redox environment through a variety of mechanisms, such as the up-regulation of NADPH oxidase 4 (NOX4), which constitutively produces hydrogen peroxide (H_2O_2) [18,19], increasing free intracellular iron, and the down-regulation of glutaredoxin-1 or reduced glutathione (GSH) levels [20–26]. The state of reduction equivalent capacity, capable of eliminating electrophiles such as ROS, has been defined as the nucleophilic tone of the cell and is determined by the expression of antioxidant system components [27]. As such, decreased nucleophilic tone would impair the cell's ability to clear ROS. Elevation of reactive oxygen species (ROS) during EMT can lead to direct activation or enhancement of a variety of redox-sensitive signal transduction pathways [28] and H_2O_2 treatment itself has been shown to induce EMT in a TGFβ-dependent manner [29]. Antioxidant attenuation of Smad2/3 phosphorylation and transcription has been observed in a variety of cell types [30–34] and is attributed to the prevention of TGFβ-mediated EMT [23,26,31,35]. Thus TGFβ signaling has the capacity to modify the redox environment and also be subject itself to regulation by the redox environment.

Despite extensive studies devoted to the contribution of cellular oxidation on many individual biochemical processes involved in TGFβ-induced EMT, inclusion of redox markers in the characterization of the multivariate phenotype trajectory has never been performed in a systematic manner. We hypothesized that the previously reported cellular oxidation during TGFβ-mediated EMT [21,23] reinforces TGFβ signaling in a feed-forward manner during EMT as well as contributes to maintenance of mesenchymal differentiation following EMT. Investigation of the aforementioned processes is fraught with the complexity arising from studying a transition that involves evaluating epithelial, mesenchymal, Smad signaling, and redox regulatory phenotypic characteristics as they evolve over time. To address the numerous interconnected regulators in this biological system, we developed a custom panel of markers for a multiwell in-cell Western (ICW) assay [36] that could generate time-dependent samples for numerous epithelial, mesenchymal, TGFβ-specific, and redox markers. These data were compiled with other available information for multivariate modeling, specifically principal component analysis (PCA), to collapse features of high-dimension, temporal dynamics during EMT into latent variable space. This novel experimental and analytical approach allowed us to investigate whether cellular redox features are informative metrics of EMT transdifferentiation in A549 lung carcinoma cells. Using PCA we extracted a multivariate description of phenotype during the time course of EMT that was capable of interrogating how the cellular redox state may influence and relate to transdifferentiation between epithelial and mesenchymal states.

Materials and methods

Cell culture and treatment conditions

A549 lung carcinoma cells were obtained from American Type Culture Collection (ATCC; CCL-185) and maintained in high glucose DMEM with L-glutamine (Sigma D5796), 10% FBS (Sigma F4135), and penicillin (50 IU/ml)–streptomycin (50 μg/ml) (Cellgro 30-001-CI). Cells were plated in 96-well plates at density of 5000 cells per well in growth media and maintained at 37 °C and supplemented with 5% CO_2. The following day, the cells were serum-starved with reduced serum (0.5% FBS) media for 24 h prior to treatment. Cells were maintained and treated in 175 μl media per well. Cells were treated with a bolus of 200 pM TGFβ for the EMT time course, qRT-PCR, CM-H_2DCF-DA, and H_2O_2 degradation studies. For the GSH study, cells were seeded in T-75 flasks at a density of 5000 cells/cm^2 and maintained in 15 ml of culture media. Culture media for the EMT intervention study was changed daily and consisted of either plain media, 100 pM TGFβ (Millipore, GF111), 10 μg/ml neutralizing anti-TGFβ antibody (R&D Systems, MAB240), 2 μM A8301 (Santa Cruz, sc-203791; mobilized in DMSO), 2 mM *N*-acetyl-L-cysteine (NAC; Sigma-Aldrich, A9165), 2 μM ebselen (Alfa Aesar, J63190; mobilized in DMSO), or 0.2% v/v DMSO (Fisher Scientific, BP231). All experiments were the result of three independent biological replicate experiments.

In-cell Western assay

Following treatment, cells were washed with PBS with Ca^{2+}/Mg^{2+} and fixed with 100 μl 4% paraformaldehyde per well for 20 min at ambient temperature. The cells were permeabilized by washing five times with 50 μl 0.1% Triton X-100 solution for 5 min with gentle rotation at ambient temperature. The plates were blocked with 100 μl of blocking buffer consisting of 0.5X Rockland Blocking Buffer for Fluorescent Western Blotting (MB-070) in Tris-buffered saline (TBS) for 1.5 h with gentle rocking at ambient