Methods xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Methods

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

Analysis of post-transcriptional regulation during cancer progression using a donor-derived isogenic model of tumorigenesis

Laura S. Bisogno, Jack D. Keene *

Department of Molecular Genetics & Microbiology, Duke University Medical Center, Durham, NC 27710, United States

ARTICLE INFO

Article history:
Received 8 April 2017
Accepted 16 May 2017
Available online xxxx

Keywords:
Post-transcriptional regulation
RNA binding proteins
RNA regulons
Tumorigenesis
Isogenic models

ABSTRACT

Post-transcriptional regulation of gene expression by RNA binding proteins (RBPs) and non-coding RNAs plays an important role in global gene expression. Many post-transcriptional regulators are misexpressed and misregulated in cancers, resulting in altered programs of protein biosynthesis that can drive tumor progression. While comparative studies of several RBPs and microRNAs expressed in various cancer types have been reported, a model system that can be used to quantify RBP regulation and functional outcomes during the initiation and early stages of tumorigenesis is lacking. It was previously demonstrated that oncogenic transformation of normal human cells can be induced by expressing hTERT, $p53^{DD}$, cyclin D1, $CDK4^{R24C}$, $C\text{-}MYC^{T58A}$ and $H\text{-}RAS^{G12V}$. Here we describe a user-friendly method for generating this genetically defined model of step-wise tumorigenesis beginning with normal donor-derived human cells. This method immortalizes a donor's normal cells in about a week, reducing the chances of senescence. The entire stable system can be established in less than 12 weeks. We then demonstrate the utility of such a system in elucidating the expression of multiple RBPs at an early step of tumor formation. We identify significant changes in the expression levels of transcripts encoding RBPs prior to transformation, suggesting that our described donor-derived isogenic system can provide insight about post-transcriptional regulation during the earliest stages of tumorigenesis in the context of diverse genetic backgrounds.

1. Introduction

Precise control of gene expression is achieved through regulation at both the transcriptional and post-transcriptional levels. While many studies focus on steady state mRNA levels to measure gene expression, it has become increasingly clear that this does not provide an accurate picture of the complex regulatory features of gene expression occurring within a cell. Post-transcriptional regulation (PTR) of mRNA expression is controlled and coordinated by *trans*-acting RNA binding proteins (RBPs), and also non-coding RNAs, that bind to *cis* regulatory elements contained in nascent transcripts [1]. RBPs coordinate functionally regulated mRNAs into RNA regulons, allowing for combinatorial changes in PTR expression in response to perturbations [1]. Tightly regulated PTR events include splicing, nuclear export, localization, mRNA stability and ultimately translation [1–4]. Large numbers of mRNAs undergo PTR, as evidenced by the fact that steady state protein levels do not always correlate with steady state mRNA levels [5–9]. RBPs in eukaryotes have been estimated to outnumber DNA binding proteins [10,11], are highly expressed when compared to any other class of genes [12], and are, as a class, more conserved than transcription factors in metazoans [13], highlighting the importance of PTR.

Many recent studies have shown that PTR is an important determinant of gene expression in both normal cellular processes and pathological states. It is now widely recognized that RBPs robustly influence cancer-related gene expression patterns, as they regulate many mRNAs encoding proto-oncogenes, growth factors, cytokines, and cell cycle regulators [14,15]. Cancer has traditionally been viewed as being driven by aberrant transcriptional regulation and signaling events, though, over the past several years, many RBPs have emerged as critical players in tumor development [14,16]. Global studies have identified many RBPs that are significantly misexpressed in tumors compared to normal tissues [12,17], and several studies have suggested that RBPs dynamically and differentially regulate target mRNAs in different states and contexts [18–22]. However, cancers are derived from normal cells that often evolve step-wise and progressively to a neoplastic state, and the involvement of PTR in this progression has not been looked at in the context of tumor initiation and step-wise progression. Thus,

* Corresponding author.
E-mail addresses: les36@duke.edu (L.S. Bisogno), jack.keene@dm.duke.edu (J.D. Keene).

http://dx.doi.org/10.1016/j.ymeth.2017.05.012
1046-2023/

more studies are needed in order to fully understand the PTR regulators and downstream genetic programs activated by cancer driver mutations that coordinate tumor origins, evolution and progression.

Studies of tumorigenesis typically involve the use of cancer cell lines. While cell line models have been informative and have demonstrated the importance of transcriptional and post-transcriptional control of cancer [18,19,23], they typically involve cell lines that are already immortalized, and thus they may already be well along the path to becoming a cancer cell. For example, it is common to set up an isogenic system of late stage tumor formation by adding an oncogene, such as H-RAS^{G12V}, to an already immortalized cell line, for example, MCF10A human mammary epithelial cell line. However, one major drawback of such an isogenic system is that it cannot be used to study the earliest stages of oncogenesis, the process by which a normal cell becomes progressively tumorigenic, since there are no normal isogenic primary cells to compare. Likewise, comparisons between patient tumor tissues and normal matched tissues do not provide information about the intermediate stages of transformation. In addition, certain cancer cell lines, such as the ever-popular MCF7 breast cancer cell line, have been demonstrated to evolve and adapt differently over time [24]. Thus, findings from one laboratory may not be replicable in the same cell line in another laboratory due to accumulated mutations in these lines. While mouse models can help to illuminate genetic alterations that lead to cancer, many alterations characteristic of human cancers do not yield the same cancers in mice [9,25,26].

Seminal work in Robert Weinberg's laboratory demonstrated that normal human epithelial and fibroblast cells are converted to a tumorigenic state through the combined ectopic expression of hTERT, oncogenic HRAS^{G12V}, and the Simian Virus 40 (SV40) large and small t tumor antigens [27]. Expression of these transgenes systematically establishes: 1) telomere maintenance, 2) unlimited replication potential and, 3) complete oncogenic transformation. Importantly, this work identified minimal intracellular pathways whose disruption is sufficient for creation of a human tumor. Subsequent work in Chris Counter's laboratory expanded on these findings, and identified a core set of proteins that together disrupt the same pathways as the Weinberg SV40 system to drive the process of tumorigenesis [8,28]. In this system, the introduction of hTERT, p53^{DD}, cyclin D1, CDK4^{R24C} and c-MYC^{T58A} immortalizes the cell, while subsequent expression of HRAS^{G12V} converts the cell to a fully tumorigenic state. The elimination of proteins of viral origin better recapitulates what happens in human tumors, since most cancers are not caused by viruses, and introduction of viral proteins into human cells has been shown to significantly alter other physiological and biological properties [29]. Using this system, well-defined genetic models of tumorigenesis can be established for most normal human cells, allowing for the investigation of the earliest stages of transformation in a variety of tumor types.

An important aspect of pharmacogenomics concerns differences in genotype and gene expression among individual patients that vary in drug responses. To this end, we believe that a donor derived, genetically defined, model offers an advantage for taking into consideration individual genetic differences in preclinical cancer studies. Thus, rather than using standard cancer cell lines as pre-clinical models, establishing multiple donor derived isogenic lines in which to conduct initial studies could be highly beneficial. Despite this benefit, relatively few laboratories have taken advantage of the cell systems developed by the Weinberg and Counter laboratories. Unfortunately, these cell lines were not available to be shared between laboratories due in part to the fact that primary cells have extremely limited passage numbers and are in short supply. While the immortalized and transformed lines could be shared if available, the main advantage of this system, the comparison to primary cells, would be lost. In addition, if one is to make comparisons between different individuals of various genetic backgrounds, multiple primary cells from several donors must be obtained and step-wise transformed. Therefore, the cell lines must be engineered by the laboratory wishing to use them.

The process of engineering a stable isogenic system in the absence of viral genes has been described [8,9]. We have simplified this method, as detailed in this paper, with a few useful modifications to streamline the numerous steps of transfection and infection involved in establishing this system. Additionally, we have determined that for cell system expansion, which is necessary for the application of many methods of global analysis, modifications of the cell culture media are necessary. Furthermore, we have cultured this system in the absence of selective pressures for over 20 passages, and demonstrated that cells still robustly express the essential transgenes. These modifications allow expansion and application of various published methods to study post-transcriptional regulation during the earliest stages of tumorigenesis. We demonstrate large changes in expression of RBPs during the immortalization step of normal human primary cells, many of which have been previously identified to play a role in cancer formation. It is important to note that we did not observe additional changes in expression levels of transcripts encoding RBPs during subsequent RAS transformation. Therefore, this isogenic model system can provide insights into how RBPs can affect global RNA regulation that are otherwise lost using standard immortalized cell lines.

2. Materials and methods

A workflow of the procedure for establishing an isogenic model is outlined in Fig. 1. Here we detail a user-friendly, step-by-step procedure for establishing and validating this model. This method makes a few minor modifications from the methods described by Chris Counter's laboratory for the sake of simplification [8,9]. This protocol is optimized for human mammary epithelial cells (HMECs); however this method can be adapted to virtually any available normal human cell. It is critical to start with cells that are not yet immortalized.

2.1. Materials

2.1.1. Cell lines and cell culture reagents

- HEK 293T/17 cells (ATCC)
- HEK 293 cells (ATCC); or any cell line that is not resistant to antibiotics listed
- Low passage primary cells of choice; in this paper, we use HMECs obtained at p9 (Lonza)
- DMEM (Thermo Fisher Scientific) +/− 10% FBS
- Primary cell specific media (for HMECs, MEGM (Lonza))
- Appropriate cell culture reagents (for HMECs, reagent pack (Lonza))
- 2× MEM (Thermo Fisher Scientific)

2.1.2. Plasmids

Plasmids to make this system are obtained from multiple published sources. As with all plasmids, we highly recommend sequence validating before use. Alternatively, all pBabe empty vector backbones with all appropriate selection markers are publicly available from Addgene, and one could easily clone vectors of interest

Please cite this article in press as: L.S. Bisogno, J.D. Keene, Methods (2017), http://dx.doi.org/10.1016/j.ymeth.2017.05.012