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Use of exogenous hTERT to immortalize primary human cells

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Abstract

A major obstacle to the immortalization of primary human cells and the establishment of human cell lines is telomere-controlled senescence. Telomere-controlled senescence is caused by the shortening of telomeres that occurs each time somatic human cells divide. The enzyme telomerase can prevent the erosion of telomeres and block the onset of telomere-controlled senescence, but its expression is restricted to the early stages of embryonic development, and in the adult, to rare cells of the blood, skin and digestive track. However, we and others have shown that the transfer of an exogenous hTERT cDNA, encoding the catalytic subunit of human telomerase, can be used to prevent telomere shortening, overcome telomere-controlled senescence, and immortalize primary human cells. Most importantly, hTERT alone can immortalize cells without causing cancer-associated changes or altering phenotypic properties. Primary human cells that have so far been established by the forced expression of hTERT alone include fibroblasts, retinal pigmented epithelial cells, endothelial cells, oesophageal squamous cells, mammary epithelial cells, keratinocytes, osteoblasts, and Nestin-positive cells of the pancreas. In this article, we discuss the use of hTERT to immortalize of human cells, the properties of hTERT-immortalized cells, and their applications to cancer research and tissue engineering.

Abbreviations: hTERT – human Telomerase Reverse Transcriptase; hTR – human Telomerase RNA.

Introduction

Primary human cells derived from non-cancerous tissues display a finite lifespan when cultivated in the laboratory. After a limited number of cell divisions, these cells enter a viable state of permanent quiescence, termed senescence (Hayflick and Moorhead 1961). Akin to terminal differentiation, senescence is accompanied by irreversible changes in both morphology and gene expression (Shay et al. 1992; Shelton et al. 1999). Because senescence limits the lifespan of laboratory cell samples, it has been a major obstacle to the analysis of primary human cells as well as their use as therapeutic agents. Recent studies have revealed the existence of distinct forms of senescence, each representing a separate obstacle to cellular immortality (Wright and Shay 1992; Ouellette and Lee 2001). Among these forms, telomere-controlled senescence (also known as M1; for Mortality stage 1) has emerged as a common obstacle to unlimited lifespan that most, if not all, types of primary human cells experience. Overcoming telomere-controlled senescence is an

absolute requirement to the immortalization of primary human cells and the establishment of human cell lines.

Telomere-controlled senescence: a universal barrier to cellular immortalization

The onset of telomere-controlled senescence is dictated by the shortening of the telomeres that occurs each time normal somatic cells divide (Bodnar et al. 1998; Vaziri and Benchimol 1998; Ouellette and Lee 2001). Telomeres are essential structures that cap and protect the ends of linear chromosomes. In humans, telomeres are made of the simple double-stranded DNA repeat (TTAGGG)n reiterated for over 2–30 kbp, and by a G-rich single-stranded 3′-overhang of 50–200 nucleotides in length (Blackburn 1994). Because of the inability of DNA polymerase α to fully replicate the ends of linear DNA molecules, telomeres shorten at each round of DNA replication. Current data suggest that senescence is induced when the shortest telomeres have become too short to perform their capping function (Allsopp and Harley 1995; de Lange 2002). The ends of chromosomes are natural interruption in the colinearity of genomic DNA, and a vital function of telomeres is to prevent these interruptions from being recognized by the DNA damage checkpoints as a processed ds-DNA breaks. As described in Dominique Broccoli's contribution to this issue of Cytotechnology, two features of telomeres contribute to this capping function: telomere-binding protein hTRF2 and a structure termed the T-loop. In support of this model is the observation that the presence of ds-DNA breaks and of uncapped telomeres leads to a mobilization of the same two anti-proliferative mechanisms, involving the $p16^{INK4a}$ /pRB and $p53/p21^{WAF}$ pathways (Shay et al. 1991; Di Leonardo et al. 1994; Robles and Adami 1998; Smogorzewska and de Lange 2002). Once activated, these two pathways act in concert to block cell cycle progression and inhibit cell division. Viral oncogenes that can inactivate both pRB and p53, such as the SV40 large T antigen and the E6/E7 proteins of the HPV16 virus, can overcome M1 and provide cells with an extended lifespan (Figure 1). However, cells expressing

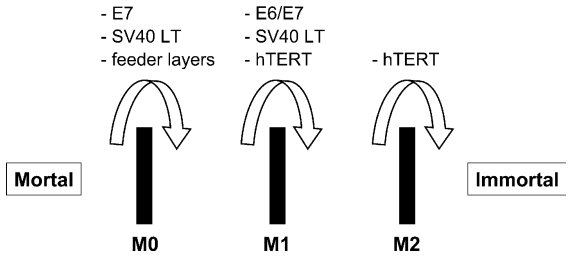

Figure 1. Multiple obstacles block the path to cellular immortality. At least three mortality stages (M0, M1, M2) have been described in the literature that can limit the lifespan of primary human cells. While M1 and M2 are caused by the shortening of telomeres, M0 appears to represent a delayed response to inadequate culture conditions. Feeder layers have successfully been used to overcome M0, while M1 and M2 are most efficiently bypassed by the expression of exogenous hTERT. Viral oncogenes, such as the SV40 large T antigen and the E6/E7 proteins of HPV16, can be employed to alleviate some of these obstacles, but this strategy invariably gives rise to cells that display cancer-associated changes. In conjunction with feeder layers, exogenous hTERT can bypass all three obstacles (M0, M1, M2) without causing significant changes in phenotypic properties.

these oncogenes are not yet immortal as telomeres continue to shorten with divisions (Shay and Wright 1996). Terminal telomere shortening eventually leads to M2 (for Mortality stage 2), an anti-proliferative state characterized by massive cell death. Immortal clones can sometimes emerge from M2 (at a frequency of 10^{-7}) that have gained the ability to maintain the size of their telomeres (Counter et al. 1992; Shay et al. 1993; Counter et al. 1994). In most instances, this stabilization of the telomeres is achieved through the aberrant induction of telomerase, an enzyme that can synthesize telomeric repeats and compensate for the 'end replication problem'. Without such compensation, telomeres shorten at each round of DNA replication and primary human cells enter M1 and/or M2 after a limited number of divisions.

In specialized cells of renewal tissues and at the early stages of embryonic development, a specialized DNA polymerase is present in human cells that can synthesize telomeric repeats, compensate for the 'end replication problem', and extend cellular lifespan. This enzyme, termed telomerase, is composed of two core components: the protein hTERT (*h*uman *T*elomerase *R*everse *T*ranscriptase) and the small nuclear RNA hTR (*h*uman

*T*elomerase *R*NA). The first provides catalytic activity and the second contains a short sequence (5′-CUAACCCUAAC-3′) that the enzyme utilizes as an internal template for the synthesis of telomeric repeats (Feng et al. 1995; Harrington et al. 1997; Kilian et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997). The enzyme functions as reverse transcriptase, as it creates a 6-bases telomeric repeat while using the RNA hTR as a template. Studies have shown that the presence of active telomerase is apparently determined by the expression of hTERT and not by the level of hTR, which abound in all cells (Meyerson et al. 1997). In most primary human cells, hTERT and telomerase activity are either absent or present at levels that are insufficient for telomere maintenance (Kim et al. 1994; Shay and Bacchetti 1997; Masutomi et al. 2003). The expression of exogenous hTERT in these cells suffices to reconstitute telomerase activity, maintain the size of telomeres, and prevent the onset of M1 and M2 (Figure 1; Bodnar et al. 1998; Counter et al. 1998).

In primary human cells where telomere-controlled senescence is the one and unique obstacle to unlimited lifespan, exogenous hTERT suffices to provide cells with immortality. Primary human cells that have thus far been immortalized by the use of hTERT alone include fibroblasts, retinal pigmented epithelial cells, endothelial cells, myometrial cells, oesophageal squamous cells, mammary epithelial cells, keratinocytes, osteoblasts, and Nestin-positive cells of the pancreas (Bodnar et al. 1998; Yang et al. 1999; Ouellette et al. 2000; Ramirez et al. 2001; Yudoh et al. 2001; Condon et al. 2002; Herbert et al. 2002; Lee et al. 2003; Morales et al. 2003). Because primary human cells are typically hard to transfect, exogenous hTERT is generally transferred through the use of retroviral vectors. In a typical experiment, the cells are transduced with vectors carrying no insert or carrying hTERT, and the antibiotic-selected cells are assayed for telomerase activity and lifespan extension. A successful immortalization of the hTERT-transduced cells should produce a more than 3-fold extension of cellular lifespan in the absence of a detectable crisis. It should be noted, however, that while bypassing telomere-controlled senescence is an absolute requirement for unlimited lifespan, hTERT alone does not always suffice to immortalize primary human cells.

Other forms of senescence: stress-induced artifacts or physiological barriers?

Additional forms of senescence have been described in the literature that are independent of telomere size and cannot be bypassed by hTERT alone. M0 (for Mortality stage 0) is a well-documented form of telomere-independent senescence. It limits the *in vitro* lifespan of certain types of primary human cells, including keratinocytes and mammary epithelial cells, and cannot be overcome by hTERT alone (Kiyono et al. 1998). M0 is accompanied by a mobilization of the $p16^{INK4a}$ / pRB pathway, and can be overcome by viral oncogenes, such as HPV16 E7, that can block pRB function (Figure 1; Brenner et al. 1998; Kiyono et al. 1998). Escape from M0 can occur spontaneously through clonal events, involving the loss of $p16^{INK4a}$ expression and the acquisition of aneuploidy, that are reminiscent of cancer development (Farwell et al. 2000; Romanov et al. 2001). Whether M0 is an artifact of *in vitro* cultivation or a true physiological process is still debated. Recent findings are most consistent with the notion of M0 representing a delayed reaction to inadequate culture conditions. Most significantly, M0 can be avoided when keratinocytes and mammary epithelial cells are cultured on feeder layers rather than plastic dishes (Ramirez et al. 2001). Moreover, these cells can now be immortalized by hTERT alone without any loss of $p16^{INK4a}$ expression or rampant aneuploidy (Herbert et al. 2002). Human cell types that require feeder layers for hTERT immortalization include mammary epithelial cells, keratinocytes, and oesophageal squamous cells (Ramirez et al. 2001; Herbert et al. 2002; Morales et al. 2003). These observations reiterate the need for an optimized environment capable of sustaining long-term cultivation as a prerequisite for the establishment of human cell lines. This need may especially be pertinent to the immortalization of primary human cells, as these cells tend to have growth requirements that are higher than those of cancer cells.

For many cell types, the main obstacle to immortality is their inability to proliferate *in vitro*. This failure to proliferate can be an intrinsic property of the cells, as in the case of post-mitotic terminally differentiated cells, which have lost all proliferative capacity upon differentiation (e.g. neurons). Alternatively, this failure can result from

our inability to emulate *in vivo* conditions that support growth. Indeed, there are several examples of cells with a capability for *in vivo* proliferation that are unable to divide in the artificial environment of the laboratory (e.g. hepatocytes). hTERT alone should not be expected to overcome these obstacles, as the enzyme does not appear to inhibit differentiation, alter phenotypic properties, or decrease growth requirements.

Properties of hTERT-immortalized cells and their use in research and medicine

A major advantage in using hTERT alone to immortalize primary human cells is that the enzyme telomerase can immortalize without causing cancer-associated changes or altering phenotypic properties (Jiang et al. 1999; Morales et al. 1999; Ouellette et al. 2000). Conventional methods from establishing human cell lines, such as the cultivation of tumor biopsies and the use of immortalizing oncogenes, have been used successfully to generate most of the human cell lines currently available to researchers, and these lines have been instrumental to the analysis of human biology. Unfortunately, these conventional methods have in common that they almost invariably give rise to cells that display cancer-associated changes. These aberrations may include a loss of contact inhibition, reduced growth factor requirements, inhibition of differentiation, genomic instability, aneuploidy, as well as disruptions of cell cycle checkpoints. These characteristics pose significant limitations to the analysis of many cellular functions, particularly those related to genomic integrity and cell cycle regulation. In contrast, primary human cells immortalized with hTERT alone tend to have a relatively 'normal' phenotype (Jiang et al. 1999; Morales et al. 1999; Yang et al. 1999; Ouellette et al. 2000; Ramirez et al. 2001; Yudoh et al. 2001; Herbert et al. 2002; Lee et al. 2003; Morales et al. 2003). Following their immortalization with hTERT, primary human cells remain diploid, differentiated, contact-inhibited, non-tumorigenic and anchorage dependent. They are genomically stable, possess functional cell cycle checkpoints, and express functional p53, pRB and $p16^{INK4a}$. Thus, aside from their unlimited lifespan, hTERT-immortalized cells have more in common with primary human cells than with cancer cells. This apparent 'normality' of hTERT-immortalized cells and their capacity for unlimited divisions make them ideal for applications in tissue engineering and as model systems for cancer research.

Cell-based therapies and tissue engineering share the promise of new cures for degenerative and age-related diseases. Through these approaches, replacement parts are created from cells taken from a patient, by the *in vitro* cultivation of these cells and their expansion to sufficient numbers to allow their genetic manipulation and/or rearrangement into a working tissue. Until recently, a major obstacle to rapid progress in this field had been the limited lifespan of normal human cells. This limitation can now be circumvented by the transient expression of hTERT. For a comprehensive overview of the novel possibilities offered by the use of hTERT in tissue engineering, please refer to Moustapha Kassem's contribution to this issue of the Journal.

Cancer development is driven by the acquisition of genetic and epigenetic alterations, which act in concert to override endogenous mechanisms regulating cell proliferation. The better definition of the role of these endogenous mechanisms in the physiology of primary human cells is vital to our understanding of cancer development. Until recently, two obstacles had limited the use of primary human cells in cancer research: their limited lifespan and their spectacular resistance to *in vitro* carcinogenesis. The stable expression of exogenous hTERT can alleviate both of these obstacles, as telomerase renders cells susceptible to oncogenic transformation without causing cancer-associated changes. Either as normal controls for comparative studies or as *in vitro* models for studies of carcinogenesis, hTERT-immortalized cells have become invaluable tools to cancer researchers. For a comprehensive overview of the use of hTERT-immortalized cells to study and recapitulate the various steps of cancer development, please refer to Boehm and Hahn's contribution to this special issue of the Journal.

Conclusion

For *ex vivo* applications, primary human cells offer the closest approximation of the biology of normal

human cells. Until recently, the finite lifespan of primary human cells had restricted their use to a limited number of research and clinical applications. Conventional methods for extending cellular lifespan almost invariably alter the phenotypic properties of cells, thereby reducing the value of the immortalized cells. While hTERT alone does not always suffice to immortalize primary human cells, this method can extend lifespan without causing cancer-associated changes or alter phenotypic properties. To researchers, hTERT-immortalized cells offer the prospect of being able to explore the 'normal' characteristics of primary human cells without the caveat of limited lifespan and the confounding effects of cellular senescence. hTERT-immortalized cells should more particularly be of value to the study the normal functioning of those processes that are commonly altered during cancer development, such cell cycle controls, motility, adherence, and regulation of gene expression. To clinicians, exogenous hTERT expression provides a first method of extending cellular lifespan that is both safe and effective, thereby removing senescence as a major obstacle to the advance of tissue engineering and cell-based therapies.

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