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# Establishment of murine cell lines by constitutive and conditional immortalization

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**Abstract**

Mouse cell lines were immortalized by introduction of specific immortalizing genes. Embryonic and adult animals and an embryonal stem cell line were used as a source of primary cells. The immortalizing genes were either introduced by DNA transfection or by ecotropic retrovirus transduction. Fibroblasts were obtained by expression of SV40 virus large T antigen (TAg). The properties of the resulting fibroblast cell lines were reproducible, independent of the donor mouse strains employed and the cells showed no transformed properties in vitro and did not form tumors in vivo. Endothelial cell lines were generated by Polyoma virus middle T antigen expression in primary embryonal cells. These cell lines consistently expressed relevant endothelial cell surface markers. Since the expression of the immortalizing genes was expected to strongly influence the cellular characteristics fibroblastoid cells were reversibly immortalized by using a vector that allows conditional expression of the TAg. Under inducing conditions, these cells exhibited properties that were highly similar to the properties of constitutively immortalized cells. In the absence of TAg expression, cell proliferation stops. Cell growth is resumed when TAg expression is restored. Gene expression profiling indicates that TAg influences the expression levels of more than 1000 genes that are involved in diverse cellular processes. The data show that conditionally immortalized cell lines have several advantageous properties over constitutively immortalized cells.


*Keywords:* Mouse cell lines; Immortalization; Oncogenic transformation; Controlled cell proliferation; Fibroblasts; Endothelial cells

*Abbreviations:* DMEM, Dulbecco's modified Eagle's medium; Dox, doxycycline; eGFP, enhanced green fluorescent protein; FCS, fetal calf serum; IRES, internal ribosomal binding site; LTR, long terminal repeat; MEF, murine embryonic fibroblast cell; neo, neomycin phosphotransferase; PymT, Polyoma virus middle T antigen; TAg, SV40 virus large T antigen; w.t., wild type

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## 1. Introduction

Cell lines are an essential standard tool for the elucidation of molecular process. They are used in fundamental research as well as in applied disciplines like pharmacology, high-through-put-screening and for the production of recombinant proteins. Mammalian cell lines can be generated from primary normal cells or from tumor cells by selection for continuous growth in vitro. Since even tumor cells usually do not show unlimited growth in vitro, additional mutations and/or epigenetic changes must occur. Alternatively, cell lines can be created by transduction of specific cellular or viral immortalizing genes. Nearly all cell lines obtained by these procedures are genetically instable and tend to acquire new mutations. Since most of the commonly used cell lines have been selected for rapid growth by cultivation for many generations the genomes are scrambled and there is a tendency to accumulate further mutations. In fact, most recombinant pharmaceutical proteins are produced by a limited number of such well-established mammalian cell lines. These cell lines are preferred since they meet multiple requirements like superior in vivo activity of the protein products in clinical applications, stability and human compatible post-translational modifications (Andersen and Krummen, 2002). However, the traditionally established cell lines do not reflect the differentiated nature of the cells in the tissue from which they were derived. These problems should be significantly reduced in freshly established lines when these are compared to cell lines that have been selected for permanent and rapid growth over hundreds of generations. Thus, specifically differentiated new cell lines gained by defined immortalization from recombinant mice are of great help at least for research purposes and use in biotechnology because of their defined and rather stable genetic background.

Cell lines that are derived from primary cells by mutations or by continuous expression of immortalizing genes have several limitations. The major disadvantage is that the immortalizing gene interferes with cellular processes. Reversible immortalization should overcome these problems. In addition, it should result in cell lines with regulatable cell growth (Noble et al., 1995; Obinata, 2001; Daniele et al., 2002). For the production of proteins from mammalian cell lines the control of cell growth could overcome the limitations of traditional fed-batch fermentations and allow the use of continuous fermentation processes. Here, the cells are first grown until an optimal cell density is reached, then further cell growth is restricted such that the cells can devote their resources to produce higher levels of recombinant protein. This procedure is expected to prolong the productive time period of a culture. Various attempts have been made to create proliferation-controlled producer cell lines from established cell lines. However, no such system has yet been developed to the stage of a commercial application (Bi et al., 2004; Ibarra et al., 2003; Chilov et al., 2003; Meents et al., 2002a,b; Watanabe et al., 2002; for reviews, see Fussenegger et al., 1999; Mueller et al., 1999).

In this article, we describe approaches to create new fibroblastoid and endothelial cell lines of murine origin. The transfer of non-oncogenic immortalizing genes allowed the reproducible isolation of cell lines with specific properties. Employment of a transcriptionally regulatable immortalization vector led to proliferation-controlled cell lines. The cell lines show properties that are of interest in fundamental studies as well as in industrial applications. The results suggest that it is worthwhile to exploit this methodology to create cell lines with specific differentiation properties.

## 2. Materials and methods

### 2.1. Establishment of fibroblastoid cell lines

All fibroblastoid cell lines were maintained at 37 °C in a humidified atmosphere with 5% $CO_2$ in DMEM (Gibco-BRL) with 10% fetal calf serum (Cytogen), 2 mM L-glutamine, penicillin (10 U/ml), streptomycin (100 μg/ml), 1 mM non-essential amino acids and 0.1 mM β-mercaptoethanol. If indicated doxycycline (Dox) was added to a concentration of 2 μg/ml and replenished by medium renewal every 4 days. The Balb/c 3T3 cell line was generated using the 3T3 protocol (Todaro and Green, 1963).

For immortalization of fibroblasts the early region of SV40 was used which encodes for the SV40 large T antigen (TAg), the SV40 small T antigen and the 17K T antigen. As TAg is most important for immortalization we use this name for simplification. Mouse embryo fibroblasts (MEFs) were isolated from days 13.5 embryos as previously described (May et al.,