METHODS IN MOLECULAR MEDICINE™

Tissue Engineering

Second Edition

Edited by

Hansjörg Hauser

Martin Fussenegger

HUMANA PRESS

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Editorial Overview

Classical tissue engineering is based on seeding cells into biodegradable polymer scaffolds or gels, culturing and expanding them in bioreactors, and finally implanting the resulting tissue into the recipient organism, where the maturation of the new organ takes place. Capitalizing on this basic concept, tissue engineering has rapidly evolved in the past decade into an integrating discipline in which every organ forms a science of tissue engineering: Each of these sciences are interfacing with different scientific communities, including biotechnology, biopharmaceutical manufacturing, chemical engineering, cell biology, developmental biology, gene therapy, medical sciences, and organic chemistry. With so many "tissue engineers" at work on this globe, the day of successful implantation of a fully functional artificial organ seems to be near. Yet, much knowledge on molecular crosstalk among cell communities is still missing as are technologies for precise multiscale control of vascularization, innervation, differentiation, and shape of multicellular organoid structures. Managing and covering all specialized methods implemented in current tissue engineering activities is a mission impossible. However, precise understanding of diverse technologies and methods used to drive tissue engineering into a clinical reality remains a key success factor. Like tissue engineering itself, this book is intended to gather experts of various disciplines to share recent advances in tissue engineering-related methodologies. Our goal is to provide a comprehensive volume that integrates a wide, but not all-inclusive, spectrum of methods required to implement current and future progress in tissue engineering. The knowledge collected in this volume defines the impressive progress made in many aspects of tissue engineering and also reminds us of how much remains to be overcome in this important field.

Hansjörg Hauser
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Contents

1

In Vitro Expansion of Tissue Cells by Conditional Proliferation

Tobias May, Hansjörg Hauser, and Dagmar Wirth

Summary

Cell therapies rely on the implantation of well-characterized functional cells with defined properties. Often, the cells of interest do not proliferate in vitro and thus cannot be expanded to the amount needed for characterization, purification, manipulation, or cloning. Here, we describe a method that allows the reversible expansion of cells by the introduction of a proliferator gene controlled by a regulatable expression module. The module is transferred by DNA transfer or by lentiviral transduction. The addition of a clinically accepted regulator [Doxycycline (Dox)] induces proliferator expression and expansion of the cells ad infinitum. Removal of the regulator eliminates the effect of the proliferator and leaves the cells in a non-proliferating status. The method has been applied to different mouse and human tissues. This chapter describes the method for the well-examined example of mouse embryonic fibroblast (MEF) expansion.

Key Words: Cell expansion; Tet-system; Autoregulated expression; Lentiviral transduction; Conditional immortalization.

1. Introduction

Immortalized cell lines represent a useful tool to study biological processes but are also indispensable for biotechnological applications. Among the advantages are their infinitive expansion capacity and the reproducible properties. Cell lines exist from various species and tissues. Many properties of these immortalized cells reflect those of the primary cells they have been derived from. However, the fact that immortalized cells have an infinitive life span and thus significantly changed proliferation properties makes them different from primary cells.

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Infinitive growth is a consequence of the immortalization process that is achieved by selection for random mutations or by stable expression of certain oncogenes.

Because of the in vitro cultivation, many cells rapidly lose specific differentiation properties. This is thought to be because of the alteration of environmental factors (soluble and cell bound) as well as the loss of a 3D architecture. Many cell lines show increased genetic instability, because of either the expression of the immortalizing oncogene or the random mutations due to long-term cultivation and selection for rapid proliferation.

Several cell types can be expanded by growth in specific media and conditions that allow them to keep their differentiation potential. However, this is not possible to the extent needed for experimental approaches or therapies for most cell types. One alternative might be to express genes that lead to the required expansion. The expression of these proliferator genes would have to be obliterated after the expansion period without leaving disturbing remainders. This approach has two critical components: one concerns the proliferator gene. It should specifically induce proliferation in a timely restricted manner and should not have influence on other cellular properties. In particular, it should not induce permanent (epi)genetic changes. Ideal candidates are genes that naturally control the growth of a given cell population. Such candidates have been successfully used for expansion. One example concerns the expansion of hematopoietic stem cells, which was accomplished by the constitutive expression of the intracellular domain of Notch *(1)*. As such proliferator genes are usually highly specific for a given cell type, a more general approach for experimental evaluation is to use broadly active oncogenes such as the SV40 virus-derived T antigen (TAg).

The second critical component concerns the way in which conditional expression of the proliferator gene is achieved. Several approaches have been undertaken to restrict the expression of the gene to the period of cell expansion. One of the first strategies for a controlled expansion used a temperature-sensitive mutant of the TAg (*2*, *3*). This approach has several drawbacks—among them are (i) high clonal variability concerning the proliferation control *(4)*, (ii) it is restricted to TAg, and (iii) the regulatory switch being suited only for in vitro use. A strategy that circumvents these problems employs recombinase-mediated excision by the Cre/loxP or the Flp/FRT system to eliminate the immortalizing gene *(5–9)*. The disadvantage of these systems is the need for expression of the recombinase, the completeness of its action, and the risk of recombinase gene integration. An alternative approach is to make use of transcriptionally regulated expression of the immortalizing gene(s) by the Tet system *(10–12)*.

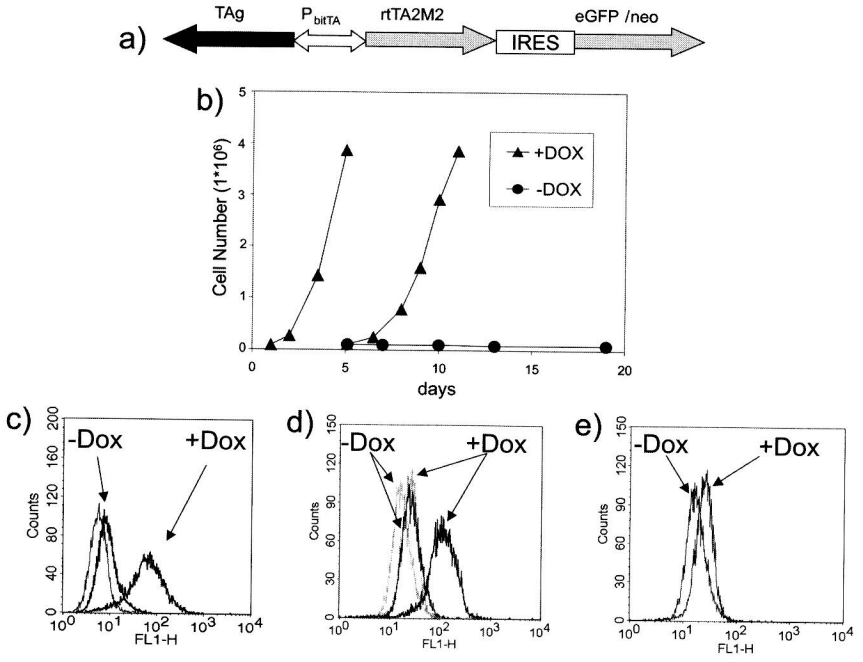

Fig. 1. Regulation potential of the autoregulatory immortalization vector. (a) Schematic presentation of the plasmid vector pRITA. The bidirectional Tet-dependent promoter P_{bitTA} drives the expression of two mRNAs. One encodes the TAg, and the other encodes the reverse transactivator rtTA2^{S}M2 ***(16)*** and a fusion protein of eGFP and neomycin phosphotransferase (eGFP/neo). (b) Cells are usually cultivated with Dox (triangles) that results in an exponential growth behavior. For monitoring Dox-dependent proliferation, the cells were split and cultivated in parallel with (triangles) or without (circles) Dox, respectively. Three independent cultures were cultivated and counted in duplicate. Cell numbers are scaled to 1×10^6. (c) For GFP analysis, the cells were washed and trypsinized followed by flow cytometry analysis. The arrows indicate the cultivation conditions. Balb/c 3T3 cells served as a control (light gray). (d) For determination of TAg levels, the cells were permeabilized after trypsinization. Subsequently, indirect immunostaining of intracellular TAg was performed, and cells were analyzed using flow cytometry. As controls, Balb/c 3T3 cells (not shown) and the secondary antibody alone with both activated and repressed cells (dotted lines) were analyzed. (e) The controls are shown in a separate histogram. (Reproduced from **ref. 11** with permission from Oxford University Press.)

The method described here concerns a recently developed transcriptional control system. The strategy is based on a transcriptionally regulated cassette that allows the autoregulated doxycycline (Dox)-dependent expression of a proliferator gene, the reverse transactivator (rtTA2), and a fusion protein of eGFP and neomycin phosphotransferase (eGFP/neo) (*see* **Fig. 1**) (***11***). In the presence of Dox, the inducible promoter is active, and all genes are expressed. This cassette allows the selection of immortalized cell clones in the presence of G418. Although cells can be immortalized by physical transduction ($DNA/Ca_3(PO_4)_2$ coprecipitation, lipofection-based protocols), the efficiency of these approaches is frequently not sufficient, mostly because of the cellular refractoriness toward physical transduction protocols. We therefore describe the integration of the autoregulated expression cassette into lentiviral particles. This results in highly efficient transduction of the fully regulatable cassette in a broad spectrum of mammalian cells and tissues.

To use this system for a broad range of cell types, we have used TAg as a proliferator gene. TAg has been used successfully for the establishment of cell lines of different origin (e.g., epithelial, endothelial) and of different species including mouse and rat (human cells can also be immortalized with TAg but less efficient) (***13***, ***14***). The vector system was designed in a way that permits easy replacement of TAg by tissue- and species-specific proliferators at wish.

Cells that were conditionally immortalized using the autoregulated cassettes depicted in **Fig. 1** above show a strict regulation of growth, although a basal expression of TAg can be monitored. Interestingly, these cells can be repeatedly switched on and off, with all Dox-induced changes being fully reversible as monitored by gene-expression profiles (***11***, ***15***). Thus, the transcriptional control provides a reliable system for fully reverting the proliferating phenotype to a stationary mode, and this represents a new tool for the establishment of cell lines with improved properties.

2. Materials

2.1. Cell Culture

1. A standard cell-culture equipment is required. For the lentiviral transduction, the respective safety requirements have to be considered. Cells are handled according to standard protocols. In this chapter, all cell-culture media and solutions, which are required for the methods described in the Materials, are listed Section in alphabetical order.
2. $CaCl_2$: 2.5 M solution in H_2O, sterile filtered, stored at −20° C.
3. Crystal violet solution: 5 g crystal violet, 8.5 g NaCl, 143 ml formaldehyde, 500 ml ethanol adjusted to 1000 ml with H_2O, stored at room temperature.

4. Dulbecco's modified Eagle's medium (DMEM): Sigma no. D-7777, high glucose with sodium pyruvate dissolved in aqua dest, sterile filtered, aliquoted and stored at 4°C.
5. DMEM-F: DMEM supplemented with 2 mM glutamine, 100 U penicillin, 100 µg/ml streptomycin, 1 mM non-essential amino acids, 0.1 mM ß-mercaptoethanol, and 10% fetal calf serum (FCS), stored at 4°C.
6. DMEM-V: DMEM supplemented with 2 mM glutamine, 100 U penicillin, 100 µg/ml streptomycin, and 10% FCS, stored at 4°C.
7. Dox: 2 mg/ml stock solution in 70% ethanol, sterile filtered. The stocks are wrapped with aluminium foil and stored at −20°C.
8. Fixation solution (SA-ß-Gal): 2% formaldehyde, 0.1% glutaraldehyde in phosphate-buffered saline (PBS) pH 7, freshly prepared.
9. Freezing solution: FCS with 5% dimethylsulfoxide (DMSO), stored at 4°C.
10. G418: 100 mg/ml stock solution in H_2O, sterile filtered, stored at −20°C.
11. Glutamine stock solution: 0.2 M glutamine in H_2O, aliquoted, sterile filtered, stored at −20°C.
12. HEBS: 280 mM NaCl, 50 mM 4-(2-hydroxyethyl)-piperazine-1-ethane sulfonic acid (HEPES), 1.5 mM Na_2HPO_4, pH 7.1; aliquoted and stored at −20°C.
13. HEPES stock solution: 1 M pH 7.12, sterile filtered, stored at 4°C.
14. Lysis buffer (intracellular staining): 0.5% Triton-X100, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1% bovine serum albumin (BSA) dissolved in PBS pH 7.
15. PBS: 140 mM NaCl, 27 mM KCl, 7.2 mM Na_2HPO_4, 14.7 mM KH_2PO_4, pH 6.8–7; autoclaved and stored at 4°C.
16. PBS*: PBS supplemented with 2% FCS, filtered with 0.45-µm filter, stored at 4°C.
17. Penicillin stock solution: 10,000 U/ml penicillin in H_2O, aliquoted, sterile filtered, stored at −20°C.
18. Polybrene stock solution: 4 mg/ml sterile filtered, stored at −20°C.
19. Propidium iodide stock solution: 5 mg/ml in PBS, sterile filtered, stored at 4°C.
20. Staining solution (SA-ß-Gal): 5 mM potassium hexacyanoferrate (II) ($K_4[Fe(CN)6]$), 5 mM potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$), 2 mM $MgCl_2$, 150 mM NaCl, 40 mM NaH_2PO_4, 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (dissolved in dimethylformamid), and 40 mM citric acid pH 6.0.
21. Streptomycin stock solution: 10 mg/ml streptomycin in H_2O, aliquoted, sterile filtered, stored at −20°C.
22. TEP: 6 mM EDTA in PBS, 0.1–0.2% trypsin, sterile filtered, stored at 4°C.
23. Virus production medium: DMEM-V supplemented with 20 mM HEPES pH 7.12, stored at 4°C.

2.2. Preparation and Maintenance of Mouse Embryonic Fibroblasts

1. A pregnant mouse (days 13–14 post fertilization).
2. 70% ethanol for disinfection.