CULTURE OF CELLS FOR TISSUE ENGINEERING

Editors

Gordana Vunjak-Novakovic, PhD
Department of Biomedical Engineering
Columbia University
New York, NY

R. Ian Freshney, PhD
Center for Oncology and Applied Pharmacology
University of Glasgow
Scotland, UK

WILEY-LISS

A JOHN WILEY & SONS, INC., PUBLICATION

CULTURE OF CELLS FOR TISSUE ENGINEERING

Culture of Specialized Cells

Series Editor

R. Ian Freshney

CULTURE OF CELLS FOR TISSUE ENGINEERING
Gordana Vunjak-Novakovic and R. Ian Freshney, Editors

CULTURE OF EPITHELIAL CELLS, SECOND EDITION
R. Ian Freshney and Mary G. Freshney, Editors

CULTURE OF HEMATOPOIETIC CELLS
R. Ian Freshney, Ian B. Pragnell and Mary G. Freshney, Editors

CULTURE OF HUMAN TUMOR CELLS
R. Pfragner and R. Ian Freshney, Editors

CULTURE OF IMMORTALIZED CELLS
R. Ian Freshney and Mary G. Freshney, Editors

DNA TRANSFER TO CULTURED CELLS
Katya Ravid and R. Ian Freshney, Editors

CULTURE OF CELLS FOR TISSUE ENGINEERING

Editors

Gordana Vunjak-Novakovic, PhD
Department of Biomedical Engineering
Columbia University
New York, NY

R. Ian Freshney, PhD
Center for Oncology and Applied Pharmacology
University of Glasgow
Scotland, UK

WILEY-LISS

A JOHN WILEY & SONS, INC., PUBLICATION

Published by John Wiley & Sons, Inc., Hoboken, New Jersey.
Published simultaneously in Canada.

Library of Congress Cataloging-in-Publication Data is available.

ISBN-13 978-0-471-62935-1
ISBN-10 0-471-62935-9

Printed in the United States of America.

10 9 8 7 6 5 4 3 2 1

Contents

Preface

Culture of Cells for Tissue Engineering is a new volume in the John Wiley series *Culture of Specialized Cells*, with focus on procedures for obtaining, manipulating, and using cell sources for tissue engineering. The book has been designed to follow the successful tradition of other Wiley books from the same series, by selecting a limited number of diverse, important, and successful tissue engineering systems and providing both the general background and the detailed protocols for each tissue engineering system. It addresses a long-standing need to describe the procedures for cell sourcing and utilization for tissue engineering in one single book that combines key principles with detailed step-to-step procedures in a manner most useful to students, scientists, engineers, and clinicians. Examples are used to the maximum possible extent, and case studies are provided whenever appropriate. We first talked about the possible outline of this book in 2002, at the World Congress of in vitro Biology, encouraged by the keen interest of John Wiley and inspired by discussions with our colleagues.

We made every effort to provide a user-friendly reference for sourcing, characterization, and use of cells for tissue engineering, for researchers with a variety of backgrounds (including basic science, engineering, medical and veterinary sciences). We hope that this volume can also be a convenient textbook or supplementary reading for regular and advanced courses of cell culture and tissue engineering. To limit the volume of the book, we selected a limited number of cells and tissues that are representative of the state of the art in the field and can serve as paradigms for engineering clinically useful tissues. To offer an in-depth approach, each cell type or tissue engineering system is covered by a combination of the key principles, step-by-step protocols for representative established methods, and extensions to other cell types and tissue engineering applications. To make the book easy to use and internally consistent, all chapters are edited to follow the same format, have complementary contents and be written in a single voice.

The book is divided into two parts and contains fifteen chapters, all of which are written by leading experts in the field. ***Part I*** describes procedures currently

used for the in vitro cultivation of selected major types of cells used for tissue engineering, and contains five chapters. *Chapter 1* (by Ian Freshney) reviews basic considerations of cell culture relevant to all cell types under consideration in this book. This chapter also provides a link to the Wiley classic *Culture of Animal Cells*, now in its *Fifth Edition. Chapter 2* (by Donald Lennon and Arnold Caplan) covers mesenchymal stem cells and their current use in tissue engineering. *Chapter 3* (by Shulamit Levenberg, Ali Khademhosseini, Mara Macdonald, Jason Fuller, and Robert Langer) covers another important source of cells: embryonic human stem cells. *Chapter 4* (by Brian Johnstone, Jung Yoo, and Matthew Stewart) deals with various cell sources for tissue engineering of cartilage. *Chapter 5* (by Henning Madry) discusses the methods of gene transfer, using chondrocytes and cartilage tissue engineering as a specific example of application.

Part II deals with selected tissue engineering applications by first describing key methods and then focusing on selected case studies. *Chapter 6* (by Gordana Vunjak-Novakovic) reviews basic principles of tissue engineering, and provides a link to tissue engineering literature. *Chapter 7* (by Koichi Masuda and Robert Sah) reviews tissue engineering of articular cartilage, by using cells cultured on biomaterial scaffolds. *Chapter 8* (by Jingsong Chen, Gregory H. Altman, Jodie Moreau, Rebecca Horan, Adam Collette, Diah Bramano, Vladimir Volloch, John Richmond, Gordana Vunjak-Novakovic, and David L. Kaplan) reviews tissue engineering of ligaments, by biophysical regulation of cells cultured on scaffolds in bioreactors. *Chapter 9* (by Jennifer Elisseeff, Melanie Ruffner, Tae-Gyun Kim, and Christopher Williams) reviews microencapsulation of differentiated and stem cells in photopolymerizing hydrogels. *Chapter 10* (by Janet Shansky, Paulette Ferland, Sharon McGuire, Courtney Powell, Michael DelTatto, Martin Nackman, James Hennessey, and Herman Vandenburgh) focuses on tissue engineering of human skeletal muscle, an example of clinically useful tissue obtained by a combination of cell culture and gene transfer methods. *Chapter 11* (by Thomas Eschenhagen and Wolfgang H. Zimmermann) describes tissue engineering of functional heart tissue and its multidimensional characterization, in vitro and in vivo. *Chapter 12* (by Rebecca Y. Klinger and Laura Niklason) describes tissue engineering of functional blood vessels and their characterization in vitro and in vivo. *Chapter 13* (by Sandra Hofmann, David Kaplan, GordanaVunjak-Novakovic, and Lorenz Meinel) describes in vitro cultivation of engineered bone, starting from human mesenchymal stem cells and protein scaffolds. *Chapter 14* (by Peter I. Lelkes, Brian R. Unsworth, Samuel Saporta, Don F. Cameron, and Gianluca Gallo) reviews tissue engineering based on neuroendocrinal and neuronal cells. *Chapter 15* (by Gregory H. Underhill, Jennifer Felix, Jared W. Allen, Valerie Liu Tsang, Salman R. Khetani, and Sangeeta N. Bhatia) reviews tissue engineering of the liver in the overall context of micropatterned cell culture.

We expect that the combination of key concepts, well-established methods described in detail, and case studies, brought together for a limited number of interesting

and distinctly different tissue engineering applications, will be of interest for the further growth of the exciting field of tissue engineering. We also hope that the book will be equally useful to a well-established scientist and a novice to a field. We greatly look forward to further advances in the scientific basis and clinical application of tissue engineering.

Gordana Vunjak-Novakovic
R. Ian Freshney

List of Abbreviations

AAF	athymic animal facility
ACL	anterior cruciate ligament
ACLF	human ACL fibroblasts
AIM	adipogenic induction medium
AMP	2-amino-2-methylpropanol
ARC	alginate-recovered-chondrocyte
BAMs	bioartificial muscles
BDM	2,3-butanedione monoxime
bFGF	basic fibroblast growth factor (FGF-1)
BPG	β-glycerophosphate
BDNF	brain-derived growth factor
BSA	bovine serum albumin
BSS	balanced salt solution
CAT	chloramphenicol-acetyl transferase
CBFHH	calcium and bicarbonate-free Hanks' BSS with HEPES
CLSM	confocal light scanning microscopy
CM	cell-associated matrix
CMPM	cardiomyocyte-populated matrices
DA	dopamine
DBH	dopamine-β-hydroxylase
Dex	dexamethasone
DMEM	Dulbecco's modification of Eagle's medium
DMEM-10FB	DMEM with 10% fetal bovine serum
DMEM-HG	DMEM with high glucose, 4.5 g/L
DMEM-LG	DMEM with low glucose, 1 g/L
DMMB	dimethylmethylene blue
DMSO	dimethyl sulfoxide
%dw	percentage by dry weight
E	epinephrine (adrenaline)
EB	embryoid bodies

EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EHT	engineered heart tissue
ELISA	enzyme-linked immunosorbent assay
EMA	ethidium monoazide bromide
ES	embryonal stem (cells)
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FM	freezing medium
FRM	further removed matrix
GAG	glycosaminoglycan
GRGDS	glycine-arginine-glycine-aspartate-serine
HARV	high aspect ratio vessel
HBAMs	human bioartificial muscles
HBSS	Hanks' balanced salt solution
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
hES	human embryonal stem (cells)
HIV	human immunodeficiency virus
HMEC	human microvascular endothelial cell
hMSC	human mesenchymal stem cell
HPLC	high-performance liquid chromatography
HUVEC	human umbilical vein endothelial cell
IBMX	isobutylmethylxanthine
ID	internal diameter
IM	incubation medium
IP	intraperitoneal
LAD	ligament augmentation devices
L_{max}	length at which EHTs develop maximal active force
MEF	mouse embryo fibroblasts
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
MSCGM	mesenchymal stem cell growth medium
NASA	National Aeronautics and Space Administration
NE	norepinephrine (noradrenaline)
NGF	nerve growth factor
NT2	NTera-2/clone D1 teratocarcinoma cell line
NT2M	NT2 medium
NT2N	Terminally differentiated NT2
OD	optical density
OD	outer or external diameter
OP-1	osteogenic protein 1 (BMP-7)
PAEC	porcine aortic endothelial cells

PBSA	Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+}
PECAM	platelet endothelial cell adhesion molecule (CD31)
PEG	polyethylene glycol
PEGDA	polyethylene glycol diacrylate
Pen/strep	penicillin-streptomycin mixture, usually stocked at 10,000 U and 10 mg/ml, respectively
PEO	polyethylene oxide
PET	polyethylene terephthalate
PGA	polyglycolic acid
PITC	phenylisothiocyanate
PLA	poly-L-lactic acid
PLGA	polylactic-co-glycolic acid
PNMT	phenylethanolamine-*N*-methyl-transferase
RCCS	Rotatory Cell Culture Systems™
RGD	arginine-glycine-aspartic acid
RWV	rotating wall vessel bioreactors
SA	sympathoadrenal
SC	Sertoli cells
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecyl (lauryl) sulfate
SMC	smooth muscle cell
SNAC	Sertoli-NT2N-aggregated-cell
SR	sarcoplasmic reticulum
SSEA-3 and 4	stage-specific embryonic antigens 3 and 4
STLV	slow turning lateral vessel (NASA derived)
SZP	superficial zone protein
TBSS	Tyrode's balanced salt solution
TH	tyrosine hydroxylase
TJA	total joint arthroplasty
TRITC	tetramethylrhodamine isothiocyanate
TT	twitch tension
Tween 20	polyoxyethylene-sorbitan mono-laurate
UTS	ultimate tensile strength

Part I

Cell Culture

1

Basic Principles of Cell Culture

R. Ian Freshney

Centre for Oncology and Applied Pharmacology, Cancer Research UK Beatson Laboratories, Garscube Estate, Bearsden, Glasgow G61 1BD, Scotland, UK, i.freshney@ntlworld.com

Culture of Cells for Tissue Engineering, edited by Gordana Vunjak-Novakovic and R. Ian Freshney

1. INTRODUCTION

The bulk of the material presented in this book assumes background knowledge of the principles and basic procedures of cell and tissue culture. However, it is recognized that people enter a specialized field, such as tissue engineering, from many different disciplines and, for this reason, may not have had any formal training in cell culture. The objective of this chapter is to highlight those principles and procedures that have particular relevance to the use of cell culture in tissue engineering. Detailed protocols for most of these basic procedures are already published [Freshney, 2005] and will not be presented here; the emphasis will be more on underlying principles and their application to three-dimensional culture. Protocols specific to individual tissue types will be presented in subsequent chapters.

2. TYPES OF CELL CULTURE

2.1. Primary Explantation Versus Disaggregation

When cells are isolated from donor tissue, they may be maintained in a number of different ways. A simple small fragment of tissue that adheres to the growth surface, either spontaneously or aided by mechanical means, a plasma clot, or an extracellular matrix constituent, such as collagen, will usually give rise to an outgrowth of cells. This type of culture is known as a *primary explant*, and the cells migrating out are known as the *outgrowth* (Figs. 1.1, 1.2, See Color Plate 1). Cells in the outgrowth are selected, in the first instance, by their ability to migrate from the explant and subsequently, if subcultured, by their ability to proliferate. When a tissue sample is disaggregated, either mechanically or enzymatically (See Fig. 1.1), the suspension of cells and small aggregates that is generated will contain a proportion of cells capable of attachment to a solid substrate, forming a *monolayer*. Those cells within the monolayer that are capable of proliferation will then be selected at the first subculture and, as with the outgrowth from a primary explant, may give rise to a *cell line*. Tissue disaggregation is capable of generating larger cultures more rapidly than explant culture, but explant culture may still be preferable where only small fragments of tissue are available or the fragility of the cells precludes survival after disaggregation.,

2.2. Proliferation Versus Differentiation

Generally, the differentiated cells in a tissue have limited ability to proliferate. Therefore, differentiated cells do not contribute to the formation of a primary culture, unless special conditions are used to promote their attachment and preserve their differentiated status. Usually it is the proliferating committed precursor compartment of a tissue (Fig. 1.3), such as fibroblasts of the dermis or the basal epithelial layer of the epidermis, that gives rise to the bulk of the cells in a

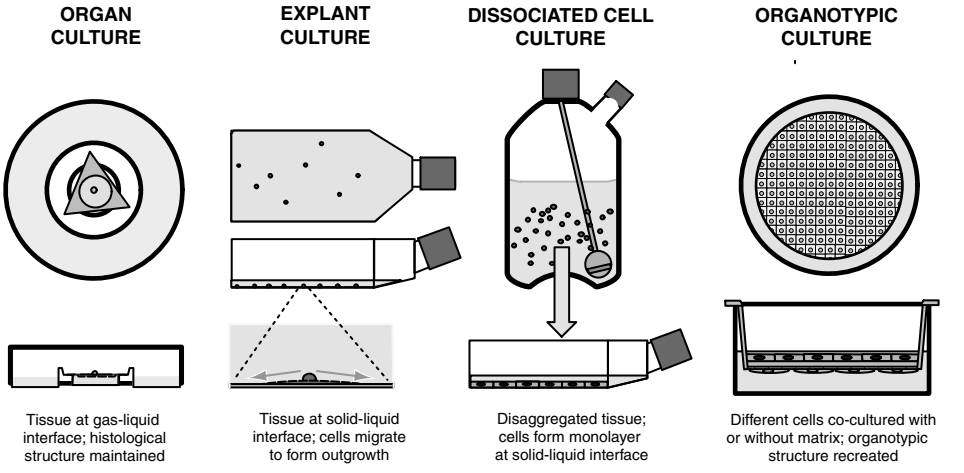

Figure 1.1. Types of culture. Different modes of culture are represented from left to right. First, an organ culture on a filter disk on a triangular stainless steel grid over a well of medium, seen in section in the lower diagram. Second, explant cultures in a flask, with section below and with an enlarged detail in section in the lowest diagram, showing the explant and radial outgrowth under the arrows. Third, a stirred vessel with an enzymatic disaggregation generating a cell suspension seeded as a monolayer in the lower diagram. Fourth, a filter well showing an array of cells, seen in section in the lower diagram, combined with matrix and stromal cells. [From Freshney, 2005.]

Figure 1.2. Primary explant and outgrowth. Microphotographs of a Giemsa-stained primary explant from human non-small cell lung carcinoma. a) Low-power (4× objective) photograph of explant (top left) and radial outgrowth. b) Higher-power detail (10× objective) showing the center of the explant to the right and the outgrowth to the left. (See Color Plate 1.)

primary culture, as, numerically, these cells represent the largest compartment of proliferating, or potentially proliferating, cells. However, it is now clear that many tissues contain a small population of regenerative cells which, given the correct selective conditions, will also provide a satisfactory primary culture, which may be propagated as stem cells or mature down one of several pathways toward

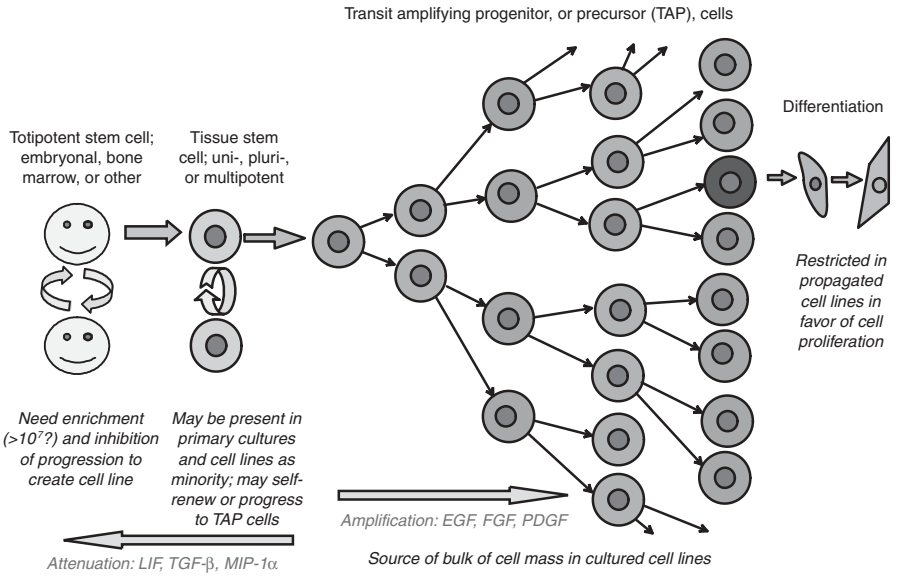

Figure 1.3. Origin of cell lines. Diagrammatic representation of progression from totipotent stem cell, through tissue stem cell (single or multiple lineage committed) to transit amplifying progenitor cell compartment. Exit from this compartment to the differentiated cell pool (far right) is limited by the pressure on the progenitor compartment to proliferate. Italicized text suggests fate of cells in culture and indicates that the bulk of cultured cells probably derive from the progenitor cell compartment, because of their capacity to replicate, but accepts that stem cells may be present but will need a favorable growth factor environment to become a significant proportion of the cells in the culture. [From Freshney, 2005.]

differentiation. This implies that not only must the correct population of cells be isolated, but the correct conditions must be defined to maintain the cells at an appropriate stage in maturation to retain their proliferative capacity if expansion of the population is required. This was achieved fortuitously in early culture of fibroblasts by the inclusion of serum that contained growth factors, such as platelet-derived growth factor (PDGF), that helped to maintain the proliferative precursor phenotype. However, this was not true of epithelial cells in general, where serum growth factors such as transforming growth factor β (TGF-β) inhibited epithelial proliferation and favored differentiation. It was not until serum-free media were developed [Ham and McKeehan, 1978, Mather, 1998, Karmiol, 2000] that this effect could be minimized and factors positive to epithelial proliferation, such as epidermal growth factor and cholera toxin, used to maximum effect.

Although undifferentiated precursors may give the best opportunity for expansion in vitro, transplantation may require that the cells be differentiated or carry the potential to differentiate. Hence, two sets of conditions may need to be used, one for expansion and one for differentiation. The factors required to induce differentiation will be discussed later in this chapter (See Section 7.4) and in later chapters. In general, it can be said that differentiation will probably require a selective medium for the cell type, supplemented with factors that favor differentiation, such as

retinoids, hydrocortisone, and planar-polar compounds, such as sodium butyrate (NaBt). In addition, the correct matrix interaction, homotypic and heterotypic cell interaction, and, for epithelial cells, the correct cellular polarity will need to be established, usually by using an organotypic culture. This assumes, of course, that tissue replacement will require the graft to be completely or almost completely differentiated, as is likely to be the case where extensive tissue repair is carried out. However, there is also the option that cell culture will only be required to expand a precursor cell type and the process of implantation itself will then induce differentiation, as appears to be the case with stem cell transplantation [Greco and Lecht, 2003].

2.3. Organotypic Culture

Dispersed cell cultures clearly lose their histologic characteristics after disaggregation and, although cells within a primary explant may retain some of the histology of the tissue, this will soon be lost because of flattening of the explant with cell migration and some degree of central necrosis due to poor oxygenation. Retention of histologic structure, and its associated differentiated properties, may be enhanced at the air/medium interface, where gas exchange is optimized and cell migration minimized, as distinct from the substrate/medium interface, where dispersed cell cultures and primary outgrowths are maintained. This so-called *organ culture* (See Fig. 1.1) will survive for up to 3 weeks, normally, but cannot be propagated. An alternative approach, with particular relevance to tissue engineering, is the amplification of the cell stock by generation of cell lines from specific cell types and their subsequent recombination in *organotypic culture*. This allows the synthesis of a tissue equivalent or construct on demand for basic studies on cell-cell and cell-matrix interaction and for in vivo implantation. The fidelity of the construct in terms of its real tissue equivalence naturally depends on identification of all the participating cell types in the tissue in vivo and the ability to culture and recombine them in the correct proportions with the correct matrix and juxtaposition. So far this has worked best for skin [Michel et al., 1999, Schaller et al., 2002], but even then, melanocytes have only recently been added to the construct, and islet of Langerhans cells are still absent, as are sweat glands and hair follicles, although some progress has been made in this area [Regnier et al., 1997; Laning et al., 1999].

There are a great many ways in which cells have been recombined to try to simulate tissue, ranging from simply allowing the cells to multilayer by perfusing a monolayer [Kruse et al., 1970] to highly complex perfused membrane (Membroferm [Klement et al., 1987]) or capillary beds [Knazek et al., 1972]. These are termed *histotypic cultures* and aim to attain the density of cells found in the tissue from which the cells were derived (Fig. 1.4). It is possible, using selective media, cloning, or physical separation methods (See Section 3.4), to isolate purified cell strains from disaggregated tissue or primary culture or at first subculture. These purified cell populations can then be combined in organotypic culture to recreate both the tissue cell density and, hopefully, the cell interactions and

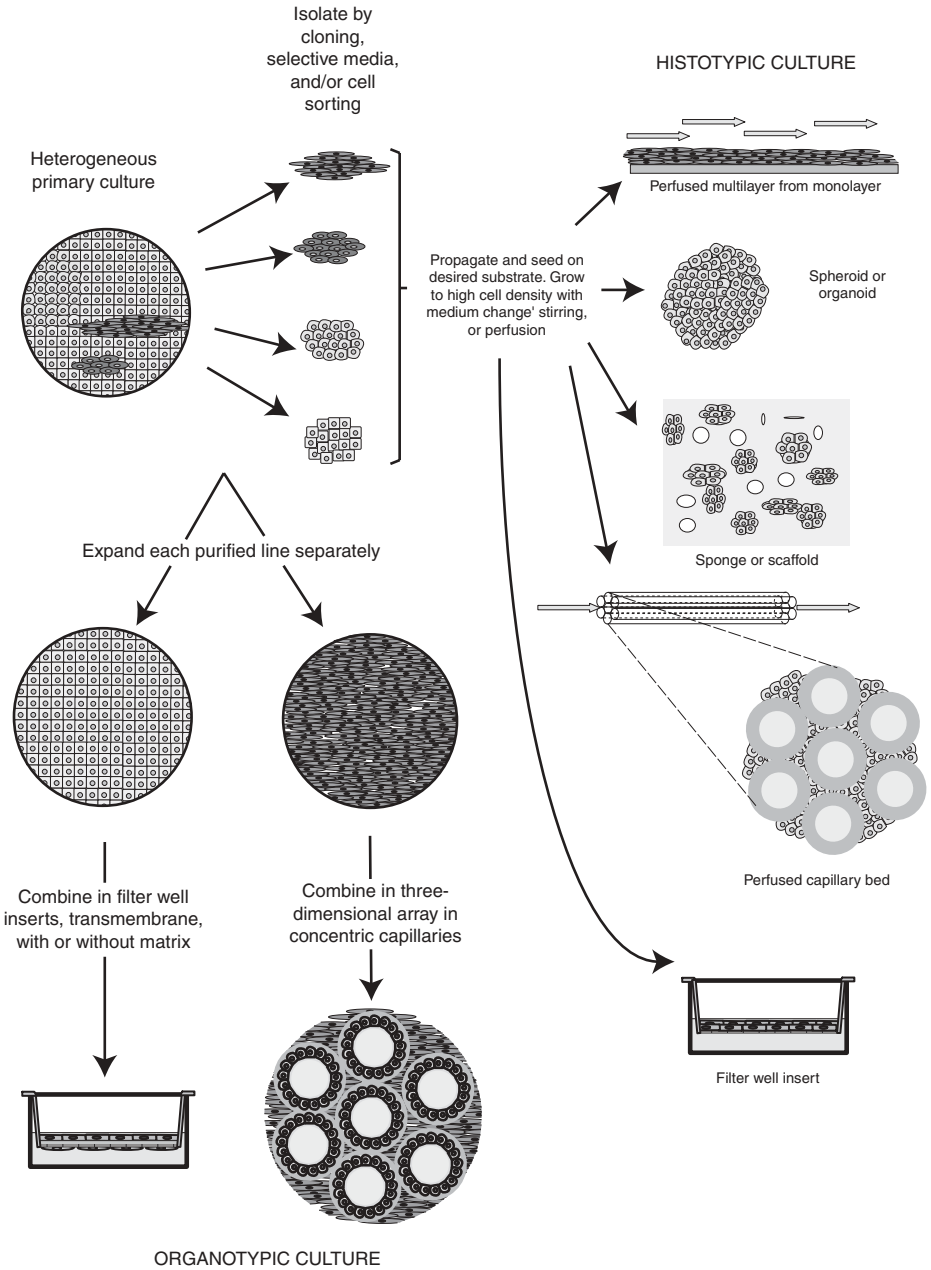

Figure 1.4. Histotypic and organotypic culture. Indicates the heterogeneity of a primary culture (top left), how this might be purified to give defined cell populations, which, if expanded and seeded into appropriate conditions can give high-density cultures of one cell type in perfused multilayers (top right), spheroids or organoids in stirred suspension (second top right), three-dimensional multilayers in perfused capillaries (third top right), or monolayers or multilayers in filter well inserts (bottom right). Expansion of purified populations and recombination can generate organotypic cultures, in filter well inserts (bottom left) or on concentric microcapillaries (bottom center). This last seems to be suggested by the architecture of the device (CellGro Triac), but the author has no knowledge of its use in this capacity.

matrix generation found in the tissue (See Fig. 1.4). Filter well inserts provide the simplest model system to test such recombinants, but there are many other possibilities including porous matrices, perfused membranes, and concentric double microcapillaries (Triac hollow fiber modules, [www.spectrapor.com/1/1/9.html]).

2.4. Substrates and Matrices

Initially, cultures were prepared on glass for ease of observation, but cells may be made to grow on many different charged surfaces including metals and many polymers. Traditionally, a net negative charge was preferred, such as found on acid-washed glass or polystyrene treated by electric ion discharge, but some plastics are also available with a net positive charge (e.g., Falcon Primaria), which is claimed to add some cell selectivity. In either case, it is unlikely that the cell attaches directly to synthetic substrates and more likely that the cell secretes matrix products that adhere to the substrate and provide ligands for the interaction of matrix receptors such as integrins. Hence it is a logical step to treat the substrate with a matrix product, such as collagen type IV, fibronectin, or laminin, to promote the adhesion of cells that would otherwise not attach.

The subject of scaffolds will be dealt with in detail in later chapters (See Part II). Suffice it to say at this stage that scaffolds have the same requirements as conventional substrates in terms of low toxicity and ability to promote cell adhesion, often with the additional requirement of a three-dimensional geometry. If the polymer or other material does not have these properties, derivatization and/or matrix coating will be required.

Most studies suggest that cell cultivation on a three-dimensional scaffold is essential for promoting orderly regeneration of engineered tissues in vivo and in vitro. Scaffolds investigated to date vary with respect to material chemistry (e.g., collagen, synthetic polymers), geometry (e.g., gels, fibrous meshes, porous sponges, tubes), structure (e.g., porosity, distribution, orientation, and connectivity of the pores), physical properties (e.g., compressive stiffness, elasticity, conductivity, hydraulic permeability), and degradation (rate, pattern, products).

In general, scaffolds should be made of biocompatible materials, preferentially those already approved for clinical use. Scaffold structure determines the transport of nutrients, metabolites, and regulatory molecules to and from the cells, whereas the scaffold chemistry may have an important role in cell attachment and differentiation. The scaffold should biodegrade at the same rate as the rate of tissue assembly and without toxic or inhibitory products. Mechanical properties of the scaffold should ideally match those of the native tissue being replaced, and the mechanical integrity should be maintained as long as necessary for the new tissue to mature and integrate.

3. ISOLATION OF CELLS FOR CULTURE

3.1. Tissue Collection and Transportation

The first, and most important, element in the collection of tissue is the cooperation and collaboration of the clinical staff. This is best achieved if a member of

the surgical team is also a member of the culture project, but even in the absence of this, time and care must be spent to ensure the sympathy and understanding of those who will provide the clinical material. It is worth preparing a short handout explaining the objectives of the project and spending some time with the person likely to be most closely involved with obtaining samples. This may be the chief surgeon (who will need to be informed anyway), or it may be a more junior member of the team willing to set up a collaboration, one of the nursing staff, or the pathologist, who may also require part of the tissue. Whoever fulfils this role should be identified and provided with labeled containers of culture medium containing antibiotics, bearing a contact name and phone number for the cell culture laboratory. A refrigerator should be identified where the containers can be stored, and the label should also state clearly **DO NOT FREEZE!** The next step is best carried out by someone from the laboratory collecting the sample personally, but it is also possible to leave instructions for transportation by taxi or courier. If a third party is involved, it is important to ensure that the container is well protected [See, for example, www.ehs.ucsf.edu/Safety%20Updates/Bsu/Bsu5.pdf], preferably double wrapped in a sealed polythene bag and an outer padded envelope provided with the name, address, and phone number of the recipient at the laboratory. Refrigeration during transport is not usually necessary, as long as the sample is not allowed to get too warm, but if delivery will take more than an hour or two, then one or two refrigeration packs, such as used in picnic chillers, should be included but kept out of direct contact.

If the tissue sample is quite small, a further tissue sample (any tissue) or a blood sample should be obtained for freezing. This will be used ultimately to corroborate the origin of any cell line that is derived from the sample by DNA profiling. A cell line is the culture that is produced from subculture of the primary, and every additional subculture after this increases the possibility of cross-contamination, so verification of origin is important (See Section 6). In addition, the possibility of misidentification arises during routine subculture and after recovery from cryopreservation (See Section 5).

3.2. Biosafety and Ethics

All procedures involved in the collection of human material for culture must be passed by the relevant hospital ethics committee. A form will be required for the patient to sign authorizing research use of the tissue, and preferably disclaiming any ownership of any materials derived from the tissue [Freshney, 2002, 2005]. The form should have a brief layman's description of the objectives of the work and the name of the lead scientist on the project. The donor should be provided with a copy.

All human material should be regarded as potentially infected and treated with caution. Samples should be transported securely in double-wrapped waterproof containers; they and derived cultures should be handled in a Class II biosafety cabinet and all discarded materials autoclaved, incinerated, or chemically disinfected. Each laboratory will its own biosafety regulations that should be adhered to, and anyone in any doubt about handling procedures should contact the local

safety committee (and if there is not one, create it!). Rules and regulations vary among institutions and countries, so it is difficult to generalize, but a good review can be obtained in Caputo [1996].

3.3. Record Keeping

When the sample arrives at the laboratory, it should be entered into a record system and assigned a number. This record should contain the details of the donor, identified by hospital number rather than by name, tissue site, and all information regarding collection medium, time in transit, treatment on arrival, primary disaggregation, and culture details, etc. [Freshney, 2002, 2005]. This information will be important in the comparison of the success of individual cultures, and if a long-term cell line is derived from the culture, this will be the first element in the cell line's provenance, which will be supplemented with each successive manipulation or experimental procedure. Such records are best maintained in a computer database where each record can be derived from duplication of the previous record with appropriate modifications. There may be issues of data protection and patient confidentiality to be dealt with when obtaining ethical consent.

3.4. Disaggregation and Primary Culture

Detailed information on disaggregation as a method for obtaining cells is provided in the appropriate chapters. Briefly, the tissue will go through stages of rinsing, dissection, and either mechanical disaggregation or enzymatic digestion in trypsin and/or collagenase. It is often desirable not to have a complete single-cell suspension, and many primary cells survive better in small clusters. Disaggregated tissue will contain a variety of different cell types, and it may be necessary to go through a separation technique [See Chapter 15, Freshney 2005], such as density gradient separation [Pretlow and Pretlow, 1989] or immunosorting by magnetizable beads (MACS), using a positive sort to select cells of interest [Carr et al., 1999] or a negative sort to eliminate those that are not required [Saalbach et al., 1997], or by using fluorescence-activated cell sorting (FACS) [See, e.g. Swope et al., 1997]. The cell population can then be further enriched by selection of the correct medium (e.g., keratinocyte growth medium (KGM) or MCDB 153 for keratinocytes [Peehl and Ham, 1980]), many of which are now available commercially (See Sources of Materials), and supplementing this with growth factors. Survival and enrichment may be improved in some cases by coating the substrate with gelatin, collagen, laminin, or fibronectin [Freshney, 2005].

4. SUBCULTURE

Frequently, the number of cells obtained at primary culture may be insufficient to create constructs suitable for grafting. Subculture gives the opportunity to expand the cell population, apply further selective pressure with a selective medium, and achieve a higher growth fraction and allows the generation of replicate cultures for

characterization, preservation by freezing, and experimentation. Briefly, subculture involves the dissociation of the cells from each other and the substrate to generate a single-cell suspension that can be quantified. Reseeding this cell suspension at a reduced concentration into a flask or dish generates a secondary culture, which can be grown up and subcultured again to give a tertiary culture, and so on. In most cases, cultures dedifferentiate during serial passaging but can be induced to redifferentiate by cultivation on a 3D scaffold in the presence of tissue-specific differentiation factors (e.g., growth factors, physical stimuli). However, the cell's ability to redifferentiate decreases with passaging. It is thus essential to determine, for each cell type, source, and application, a suitable number of passages during subculture. Protocols for subculture of specific cell types are given in later chapters, and a more general protocol is available in Chapter 13, Freshney [2005].

4.1. Life Span

Most normal cell lines will undergo a limited number of subcultures, or passages, and are referred to as *finite cell lines*. The limit is determined by the number of doublings that the cell population can go through before it stops growing because of senescence. Senescence is determined by a number of intrinsic factors regulating cell cycle, such as Rb and p53 [Munger and Howley, 2002], and is accompanied by shortening of the telomeres on the chromosomes [Wright and Shay, 2002]. Once the telomeres reach a critical minimum length, the cell can no longer divide. Telomere length is maintained by telomerase, which is downregulated in most normal cells except germ cells. It can also be higher in stem cells, allowing them to go through a much greater number of doublings and avoid senescence. Transfection of the telomerase gene hTRT into normal cells with a finite life span allows a small proportion of the cells to become immortal [Bodnar et al., 1998; Protocol 18.2, Freshney, 2005], although this probably involves deletion or inactivation of other genes such as p53 and *myc* [Cerni, 2000].

4.2. Growth Cycle

Each time that a cell line is subcultured it will grow back to the cell density that existed before subculture (within the limits of its finite life span). This process can be described by plotting a growth curve from samples taken at intervals throughout the growth cycle (Fig. 1.5), which shows that the cells enter a latent period of no growth, called the *lag period*, immediately after reseeding. This period lasts from a few hours up to 48 h, but is usually around 12–24 h, and allows the cells to recover from trypsinization, reconstruct their cytoskeleton, secrete matrix to aid attachment, and spread out on the substrate, enabling them to reenter cell cycle. They then enter exponential growth in what is known as the *log phase*, during which the cell population doubles over a definable period, known as the *doubling time* and characteristic for each cell line. As the cell population becomes crowded when all of the substrate is occupied, the cells become packed, spread less on the substrate, and eventually withdraw from the cell cycle. They then enter the *plateau* or *stationary phase*, where the growth fraction drops to close to zero. Some cells may

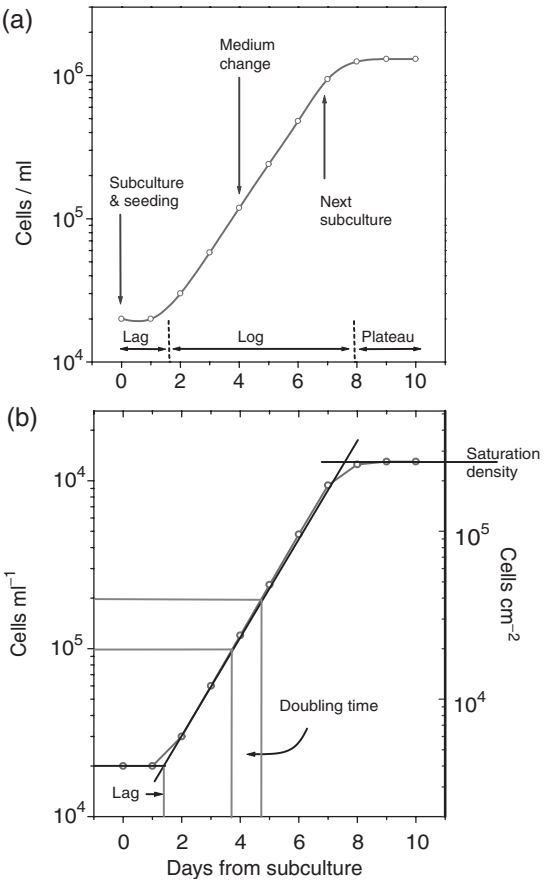

Figure 1.5. Growth curve. Increase in cell number on a log scale plotted against days from subculture. a) Defines the lag, log (exponential), and plateau phases, and when culture should be fed and subcultured after the indicated seeding time. b) Shows the kinetic parameters that can be derived from the growth curve: *lag* from the intercept between a line drawn through the points on the exponential phase and the horizontal from the seeding concentration; *doubling time* from the time taken, in the middle of the exponential phase, for the cell population to double; *saturation density* from the maximum (stable) cell density achieved by the culture, under the prevailing culture conditions. This is determined in cells/cm^2 (cell density rather than cell concentration) and is not absolute, as it will vary with culture conditions. It is best determined (as characteristic of the cell type) in conditions that are nonlimiting for medium, e.g., a small area of high-density cells in a large reservoir of medium (such as a coverslip, or a filter well insert, in a non-tissue culture-grade dish) or under continuous perfusion of medium. [Adapted from Freshney, 2005.]

differentiate in this phase; others simply exit the cell cycle into G_0 but retain viability. Cells may be subcultured from plateau, but it is preferable to subculture before plateau is reached, as the growth fraction will be higher and the recovery time (lag period) will be shorter if the cells are harvested from the top end of the log phase.

Reduced proliferation in the stationary phase is due partly to reduced spreading at high *cell density* and partly to exhaustion of growth factors in the medium at high *cell concentration*. These two terms are not interchangeable. Density implies that the cells are attached, and may relate to monolayer density (two-dimensional)

or multilayer density (three-dimensional). In each case there are major changes in cell shape, cell surface, and extracellular matrix, all of which will have significant effects on cell proliferation and differentiation. A high density will also limit nutrient perfusion and create local exhaustion of peptide growth factors [Stoker, 1973; Westermark and Wasteson, 1975]. In normal cell populations this leads to a withdrawal from the cycle, whereas in transformed cells, cell cycle arrest is much less effective and the cells tend to enter apoptosis.

Cell concentration, as opposed to cell density, will exert its main effect through nutrient and growth factor depletion, but in stirred suspensions cell contact-mediated effects are minimal, except where cells are grown as aggregates. Cell concentration per se, without cell interaction, will not influence proliferation, other than by the effect of nutrient and growth factor depletion. High cell concentrations can also lead to apoptosis in transformed cells in suspension, notably in myelomas and hybridomas, but in the absence of cell contact signaling this is presumably a reflection of nutrient deprivation.

4.3. Serial Subculture

Each time the culture is subcultured the growth cycle is repeated. The number of doublings should be recorded (Fig. 1.6) with each subculture, simplified by reducing the cell concentration at subculture by a power or two, the so-called *split ratio*. A split ratio of two allows one doubling per passage, four, two doublings, eight, three doublings, and so on (See Fig. 1.6). The number of elapsed doublings should be recorded so that the time to senescence (See Section 4.1) can be predicted and new stock prepared from the freezer before the senescence of the existing culture occurs.

5. CRYOPRESERVATION

If a cell line can be expanded sufficiently, preservation of cells by freezing will allow secure stocks to be maintained without aging and protect them from problems of contamination, incubator failure, or medium and serum crises. Ideally, $1 \times 10^6 - 1 \times 10^7$ cells should be frozen in 10 ampoules, but smaller stocks can be used if a surplus is not available. The normal procedure is to freeze a token stock of one to three ampoules as soon as surplus cells are available, then to expand remaining cultures to confirm the identity of the cells and absence of contamination, and freeze down a seed stock of 10–20 ampoules. One ampoule, thawed from this stock, can then be used to generate a using stock. In many cases, there may not be sufficient doublings available to expand the stock as much as this, but it is worth saving some as frozen stock, no matter how little, although survival will tend to decrease below 1×10^6 cells/ml and may not be possible below 1×10^5 cells/ml.

Factors favoring good survival after freezing and thawing are:

(i) High cell density at freezing ($1 \times 10^6 - 1 \times 10^7$ cells/ml).

(ii) Presence of a preservative, such as glycerol or dimethyl sulfoxide (DMSO) at 5–10%.