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Proliferating Cells versus Differentiated Cells in Tissue Engineering

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ABSTRACT

The efficiency of cell or tissue cultures is usually judged by how quickly confluence is reached within a Petri dish or on a scaffold. Growth factors and fetal bovine serum are employed to drive cultured cells from one mitosis to the next as quickly as possible. The tissue specific interphase is extremely short under these conditions, so that the degree of differentiation desired in tissue engineering cannot be achieved. To reach the goal of functional differentiation *in vitro* mitosis and interphase must be separated experimentally and tailored to the specific requirements of the cell-type used. This could be achieved by a three step concept for tissue-engineering in vitro as we present here. The expansion phase is followed by a phase in which tissue differentiation is initiated. The final phase serves to express and maintain histotypical differentiation of the generated tissue.

INTRODUCTION

TISSUE ENGINEERING, biomaterial and drug-release studies are impossible without functional tissues.[1–3] As a starting material for tissue-generation pluripotent stem cells as well as cells from developing or adult tissue can be used.[4–6] As variable the building plans of tissues may be, all tissues are basically made up from a cellular and an extracellular component. In analogy cells are cultured on an artificial extracellular matrix in tissue engineering.[7–9] *In vitro* development of functional tissues as used for cartilage repair or for the development of liver modules can only be expected when both components interact in an optimal manner. The goal in tissue engineering is to generate tissue specific features while avoiding atypical protein expression, caused by suboptimal culture conditions and cellular dedifferentiation. Experimental data shows that it is not yet possible to create functional tissues under *in vitro* conditions.[10–13] The major methodical obstacle appears to be the belief that cellular proliferation is always desirable.

MITOSIS VERSUS INTERPHASE

The primary difference between embryonic and adult tissue is the frequency of cell divisions. In growing tissue proliferation is necessary to increase tissue mass and volume. In adult tissue, proliferation is only found during tissue repair and to compensate mechanical and physiological load (Table 1). Compared to

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Table 1. Proliferation within Different Tissues, According to F.D. Bertalanffy[16]

Tissue	*Daily mitoses (%)*	*Life span (days)*
Nervous tissue	0	—
Parenchyma		
Liver	0.2–0.7	—
Kidney	0.3–0.4	—
Thyroid	0.3	—
Surface epithelia		
Urinary bladder (basal cells)	2	64
Trachea	2.1	47.6
Skin (Stratum germinativum)	5.2	19.2
Stomach (Corpus)	35.4	2.8
Stomach (Regio pylorica)	56.4	1.8
Small intestines (Jejunum)	79	1.3

adult tissue growing embryonic tissue shows only a minimum amount of functional differentiation. It is not before terminal differentiation close to the end of the growth phase that specific functions are fully expressed.

The life cycle of a cell basically consists of an interphase that varies in length depending on the cell-type and a relatively constant mitosis and cytokinesis phase.[14] By using immunological and metabolical mitosis markers the varying degree of proliferation within tissues can be demonstrated (Table 1).[15,16] Neuronal structures, cartilage as well as heart muscle show a very limited degree of proliferation (interphase ∞). Low proliferation and long interphases are also found in bone, liver and kidney parenchyma, and intestinal glands. High rates of proliferation on the other hand are found in skin, mucous membranes, and hematopoetic cells as well as tumors and experimental tumor cell lines (interphase only 1–2 days). Morphological and functional data show that proliferation is not only regulated on an organ level but can be regulated on tissue and cellular level. It is unknown though how being exposed to the obviously identical environment of an organ—for example, the villous epithelium of small intestine displays a high proliferative capacity while EC and Paneth cell populations within the crypts remain in the interphase. Chondroblasts and osteoblasts proliferate rapidly,[6,8,9] while matrix-producing osteocytes do not divide.

SWITCH OFF MITOSIS, SWITCH ON DIFFERENTIATION

Natural regulatory mechanisms to control cellular proliferation must be taken into consideration when engineering artificial tissue under *in vitro* conditions. As a first experimental step, the cell number usually needs to be expanded. The necessary cell proliferation can be achieved by adding fetal serum or growth factors to the culture medium. Most cell types used in tissue engineering today can be multiplied under these conditions. If these culture conditions are maintained during the whole experiment though, cells are continuously driven from one mitosis to the next without remaining in interphase. The point that is often neglected in these experiments is that parenchymal cells within an intact organ can only express specific functions while they are arrested in interphase. Depending on species and organ a number of differentiated cell types can reenter the cell cycle. This can be observed when primary cultures of these cells are prepared. Once these cells have progressed to mitosis they begin to lose specific characteristics. Mitosis and interphase are not parallel but subsequent events within a cell (Fig. 1). A dividing cell can only maintain a minimal degree of tissue specific differentiation. That is why the length of the interphase is specific for each tissue. To artificially generate differentiated tissue a phase of mitotic stimulation must be followed by a switch from proliferation to differentiation and a phase in which differentiation is maintained (Fig. 2).

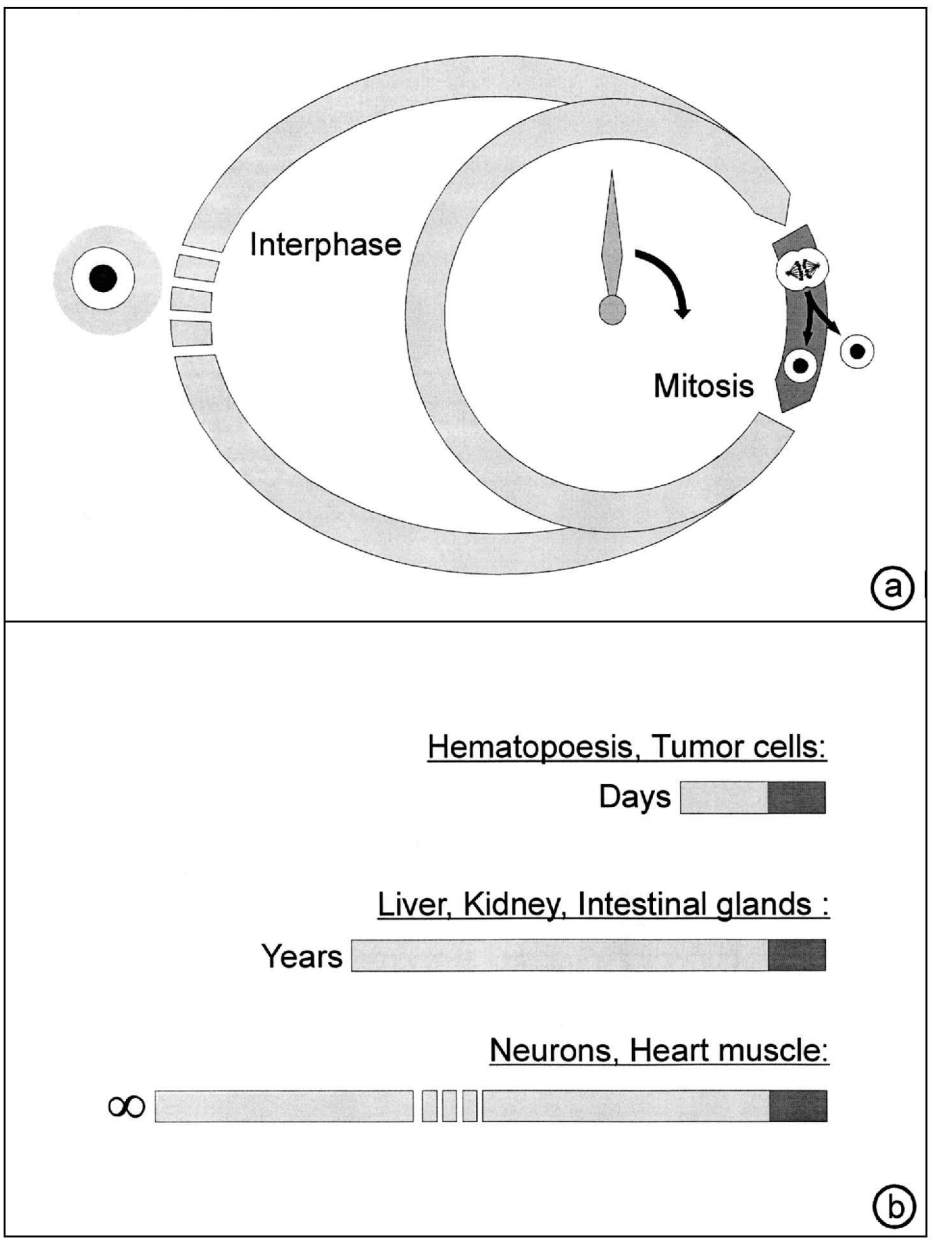


FIG. 1. Mitosis versus interphase. Cells show a maximum of differentiation during interphase. For that reason, mitosis and interphase must be regulated individually in tissue engineering.

STATIC CULTURES TEND TOWARDS PROLIFERATION

According to our experience, tissue generation can be accomplished using a three step protocol. In phase 1, the cell number is expanded in conventional culture media containing growth factors, fetal bovine serum or adult human serum.[17,18] Step 2 aims at reducing proliferation and inducing tissue-specific differentiation. In this phase, the tissue is not exposed to the static environment of a conventional culture dish, but is continuously supplied with fresh culture medium in a perfusion culture setup.[10] Preferably, serum and growth-factor–free media are used. Where complete omission of serum is not possible, depleted adult serum is used at low concentrations to reduce the concentration of mitogenic factors while retaining other nutri-

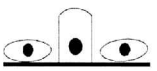
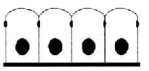
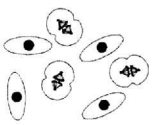
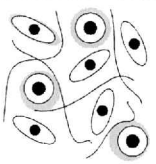
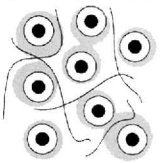
	Step 1	Step 2	Step 3
Goal	Expansion of cells	Initiation of differentiation	Maintenance of differentiation
Epithelia			
Connective tissue			
Culture technique	Static culture	Perfusion culture	Perfusion culture
Stimulus	Growth factors FCS in medium	Morphogens Serum-free media	Electrolyte-adapted, Serum-free media
Tissue reaction	Rapid cell division cycle	Reduced cell division cycle	Postmitotic phase Arrest in interphase
Mitotic stress	High	Low	Low
Differentiation	Low	Upregulated	High

FIG. 2. Controlling mitosis and interphase during *in vitro* differentiation.

tional properties. During step 3, tissue differentiation is stabilized in order to maintain tissue-specific characteristics. A three-step approach as proposed here has been successfully applied to modulate the differentiation of renal epithelial cells.[19] Epithelial cells expanded on a natural support were brought to a postmitotic state by perfusion culture under serum-free conditions. In this state of growth arrest, the upregulation of differentiation features could be demonstrated.

WHY PROLIFERATION RATES DIFFER

There is little information on tissue maturation *in vitro* and *in vivo.* A central aspect in functional tissue development is the mutual interaction of control over mitosis and the onset of terminal differentiation. Recent data show that mitogen-activated protein kinases (MAP)[20,21] and protein phosphatases[22] play a central role. Proliferation can be regulated by the extracellular matrix,[23] by morphogenic factors,[24] and by acute physiological parameters.[25,26] The morphogenic influences that initiate differentiation by halting mitosis as well as the subsequent mechanisms that control functional tissue development are not known so far.[27]

Functional tissue regeneration must be reevaluated—for example, why can fractured bone quickly regain its function by repair while neighboring cartilage can only produce fibrous repair tissue and remains functionally crippled? When engineering functional tissue constructs under *in vitro* conditions, many aspects of tissue development and regeneration can be examined experimentally. From a cell biological and technical perspective, we are still at a very early point in development. Compared to cell expansion, the initiation and maintenance of functional differentiation is a much more complex task only accomplishable through years of development. This is due to methodical difficulties and due to the lack of appropriate culture techniques. Although there is growing awareness of the need for functional tissue constructs, there are few efforts aimed towards experimental control of differentiation *in vitro.*

NEW MEDIA

There is a number of specially designed media and culture systems for the upscale culture of hybridoma cells at maximum antibody yield.[28] Most culture media developed to date were optimized for maximum cell proliferation and maximal synthesis within a short period of time. This is accomplished through an altered osmolarity and electrolyte composition of the medium. The resulting media have little in common with the interstitial fluid environment that cells are exposed to *in vivo*.[10] As pointed out above, cells arrested in interphase should not be exposed to mitotic stress. There is a need for specially designed media adapted to the needs of differentiated cells. It can be shown experimentally that perfusion culture in combination with electrolyte-adapted media can be used to maintain kidney epithelial tissue in a functional interphase state. Minute changes in NaCl concentration ($\pm$6 mmol/L) within the culture medium can modulate differentiation.[10,15,29]

FACIT

Proliferating cells do not represent functional tissue culture, not to mention organ culture. The focus of future scientific and technological development must be on culture tools and media that allow the generation of differentiated tissue and its maintenance *in vitro* for weeks and months. Regulatory control over mitosis and interphase will play a central role in the attempt to produce and maintain functionally differentiated tissue.

REFERENCES

1. Kaur, S., Cortiella, J., and Vacanti, C.A. Identifying a site for maximum delivery of oxygen to transplanted cells. Tissue Eng. **6,** 229, 2000.
2. Konig, C., Ruffieux, K., Wintermantel, E., et al. Autosterilization of biodegradable implants by injection molding process. J. Biomed. Mater. Res. **38,** 115, 1997.
3. Gautier, S., Boustta, M., and Vert, M. Alkylated poly(L-lysine citramine) as models to investigate the ability of amphilic macromolecular drug carriers to physically entrap lipophilic compounds in aqueous media. J. Control. Release **60,** 235, 1999.
4. Boheler, K.R., and Fiszman, M.Y. Can exogenous stem cells be used in transplantation? Cells Tissues Organs **165,** 237, 1999.
5. Miki, H., Ando, N., Ozawa, S., et al. An artificial esophagus constructed of cultured human esophageal epithelial cells, fibroblasts, polyglycolic acid mesh, and collagen. ASAIO J. **45,** 502, 1999.
6. Perka, C., Spitzer, R.S., Lindenhayn, K., et al. Matrix mixed culture: new methodology for chondrocyte culture and preparation of cartilage transplants. J. Biomed. Mater. Res. **49,** 305, 2000.
7. Mayer, J., Karamuk, E., Akaike, T., et al. Matrices for tissue engineering scaffold structure for a bioartificial liver support system. J. Control. Release **64,** 81, 2000.
8. Kreklau, B., Sittinger, M., Mensing, M.B., et al. Tissue engineering of biphasic joint cartilage transplants. Biomaterials **20,** 1743, 2000.
9. Rotter, N., Aigner, J., Naumann, A., et al. Cartilage reconstruction in head and neck surgery: comparison of resorbable polymer scaffolds for tissue engineering of human septal cartilage. J. Biomater. Res. **42,** 347, 1998.
10. Minuth, W.W., Schumacher, K., Strehl, R., et al. Physiological and cell biological aspects of perfusion culture technique employed to generate differentiated tissues for long term biomaterial testing and tissue engineering. J. Biomater. Sci. Polym. **11,** 495, 2000.
11. Papas, K.K., Long, R.C., Sambanis, A., et al. Development of a bioartificial pancreas: effect of oxygen on long-term entrapped βTC3 cell cultures. Biotechnol. Bioeng. **66,** 231, 1999.
12. Bader, A., Frühauf, N., Tiedge, M., et al. Enhanced oxygen delivery reverses anaerobic metabolic states in prolonged sandwich rat hepatocyte culture. Exp. Cell Res. **246,** 221, 1999.
13. Humes, H.D., Buffington, D.A., MacKay, S.M., et al. Replacement of renal function in uremic animals with a tissue-engineered kidney. Nat. Biotechnol. **17,** 451, 1999.
14. Weiss, L., and Greep, R.O. Histology, 4th ed. New York: McGraw Hill, 1977.
15. Minuth, W.W., Steiner, P., Strehl, R., et al. Modulation of cell differentiation in perfusion culture. Exp. Nephrol. **7,** 394, 1999.

16. Bertalanffy, F.D. Comparison of mitotic rates in normal renewing and neoplastic cell populations. Proc. Can. Cancer Conf. **7,** 65, 1967.
17. dos Santos, A.R., and Wada, M.L. Foetal calf serum and dexamethosone effects on Vero cell growth and differentiation. Cytobios **99,** 159, 1999.
18. Weiser, L., Bhargava, M., Attia, E., et al. Effect of serum and platelet-derived growth factor on chondrocytes grown in collagen gels. Tissue Eng. **5,** 533, 1999.
19. Minuth, W.W., Steiner, P., Strehl, R., et al. Modulation of cell differentiation in perfusion culture. Exp. Nephrol. **7,** 394, 1999.
20. Bokemeyer, D., Sorkin, A., and Dunn, M.J. Multiple intracellular MAP kinase signalling cascades. Kidney Int. **49,** 1187, 1996.
21. Omori, S., Hida, M., Ishikura, K., et al. Expression of mitogen activated kinase family in renal development. Kidney Int. **58,** 27, 2000.
22. Taylor, B.K., Stoops, T.D., and Everett, A.D. Protein phosphatase inhibitors arrest cell cycle and reduce branching morphogenesis in fetal rat lung cultures. Am. J. Physiol. Lung Cell Mol. Physiol. **278,** L1062, 2000.
23. Tajima, S., Akagi, A., Tanaka, N., et al. Expression of type XVI collagen in cultured skin fibroblasts is related to cell growth arrest. FEBS Lett. **469,** 1, 2000.
24. Sangfelt, O., Erickson, S., and Grander, D. Mechanism of interferon-induced cell cycle arrest. Front. Biosci. **1,** D479, 2000.
25. Lin, K., Hsu, P.P., Chen, S., et al. Molecular mechanism of endothelial growth arrest by laminar shear stress. Proc. Natl. Acad. Sci. U.S.A. **97,** 9385, 2000.
26. Bell, L.M., Leong, M.L., Kim, B., et al. Hyperosmotic stress stimulates promotor activity and regulates cellular utilization of the serum- and glucocorticoid-inducible protein kinase (Sgk) by a p38 MAPK-dependent pathway. J. Biol. Chem. **275,** 25262, 2000.
27. Whitmarsh, A.J., and Davis, R.J. A central control for cell growth. Nature **403,** 255, 2000.
28. Little, M., Kipriyanov, S.M., Le Gall, F., et al. Of mice and men: hybridoma and recombinant antibodies. Immunol. Today **21,** 364, 2000.
29. Schumacher, K., Strehl, R., Kloth, S., et al. The influence of culture media on embryonic collecting duct cell differentiation. In Vitro Cell. Dev. Biol. Anim. **35,** 465, 1999.

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