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Developing Cellular Systems *In Vitro* to Simulate Regeneration

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In the past two decades, cellular systems *in vitro* have progressed from predominantly monocellular testing models to study the toxic effects of new biomaterials for replacement to relevant human coculture systems for regeneration, often a combination of progenitor cells with novel biomaterials. Considerable progress has been made in understanding cellular cross talk and its contribution to the vascularization of bone. Future challenges include using the established physiological, that is, nonactivated, stem cell niches as a platform to develop coculture models, which will enable the true *in situ* regenerative niche to be investigated. Hypoxia and a changing inflammatory status are factors that need to be incorporated. Major advances in polymer synthesis permitting the incorporation of specific biologically relevant signals in hydrogels will help make this a reality.

Introduction

Understanding regenerative processes in the adult human organism is the prerequisite for a rational approach to tissue engineering (TE). Cell culture systems are useful models with which individual mechanisms can be elucidated, but their ultimate biological significance must be tested in a suitable holistic animal model and eventually confirmed in *homo sapiens sapiens*. However, despite their model character, culture techniques of higher complexity, such as cocultures, are regarded by the author as a valid instrument in TE development, acting in concert with innovations in responsive biomaterials. This brief perspective article will highlight some of the milestones already reached as well as challenges to be met in the future. The multidisciplinary approach is regarded as essential in achieving the desired goals. In the past two decades, major advances in stem cell biology, coculture techniques, and the development of responsive biomaterials, especially polymers, have led to meaningful three-dimensional (3D) *in vitro* systems as models to interrogate regenerative processes.

Historical Background

The literature shows that coculture systems have been used for some decades as models to study developmental biology[1,2] and cancer pathobiology.[3] However, in the biomaterial field, cocultures came at a much later stage, early investigations concentrating mostly on monoculture systems for cell compatibility to rule out toxic effects.[4,5]

The greatest initial impact of cocultures is undoubtedly in the field of skin regeneration as shown by Bell *et al.* in 1981 using fibroblast–keratinocyte cocultures and corroborated by numerous subsequent publications.[6–8] Further (nonexhaustive) examples of cocultures for TE are chondrocytes and smooth muscle cells on poly(glycolic acid) for soft tissue,[9] hepatocytes and embryonic stem cells on patterned ionic biopolymers,[10] smooth muscle and urothelial cells on bladder acellular matrix,[11] and lung epithelial and mesenchymal cells on temperature-sensitive poly(N-isopropylacrylamide) surfaces.[12]

Progress in Regeneration

In vitro models for regeneration have come of age during the past 20 years and have developed in two principal directions. First, cell cultures have served to help understand the mechanisms of cell interactions with biomaterials and cell–cell interactions in regenerative processes. Second, the abovementioned interactions have been advanced *in vitro* with a view to preseeding of a biomaterial for TE applications. It is evident that for both purposes, cells of human origin are a prerequisite. Moreover, advances in stem cell biology have elevated the field to a research area of eminent translational significance.

Our own coculture work has been in three main fields. In 2004, we succeeded in establishing the first human model of the alveolocapillary barrier,[13] which we have since used to study nanoparticle interaction and uptake,[14,15] as the inhalational pathway could be a means to target regeneration in the lung or, through the lung, to other organs. More recently, we have successfully cocultured the basal epithelial layer of the upper respiratory tract with lung fibroblasts to achieve a fully differentiated, ciliated respiratory mucosa, which also contains mucous cells.[16] This is currently being tested on new

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bioresorbable biomaterial candidates as well as decellularized tracheal matrix for upper respiratory tract regeneration.

The major thrust of our *in vitro* work in the TE field, however, has concerned bone vascularization using cocultures of human osteoblasts and either human outgrowth endothelial cells (OEC from peripheral blood progenitors) or human dermal microvascular endothelial cells (EC). Wenger *et al.*[17] and Rouwkema *et al.*[18] had successfully used 3D spheroid cultures of human umbilical vein EC with human osteoblasts and mesenchymal stem cells, respectively, to demonstrate the formation of prevascular networks. Our starting point for the cocultures was the addition of a suspension of each cell type to tissue culture dishes or on 3D open porous microfiber networks of biomaterials, such as fibroin or a blend of starch-poly(caprolactone). Surprisingly, from this random mixture, a marked vascular sprout formation occurred, with rapid development from week 3 to 5. Histology showed that a tissue-like self-assembly had occurred, all this happening in the absence of exogenous proangiogenic growth factors.[19–21] Proof-of-principle studies using subcutaneous implantation in rodent models showed that these preformed vascular structures could be rapidly inosculated *in vivo*.[22–24]

Despite the progress made in coculture models, there are many challenges remaining. These include the adequate simulation of cellular complexity in our *in vitro* systems.[25] Stem cell biology has made major contributions to our understanding of regeneration, and the major challenge at present is describing for each tissue and organ system the nature of the regenerative unit, the stem cell niche. However, *in situ*, the regenerative niche differs markedly from the physiological stem cell niche, in that there is generally hypoxia as well as a constantly changing inflammatory state in the former.[26] This cellular complexity is accompanied by the necessity to monitor and simulate temporospatial changes in bioactive molecules. It is expected that further refinement of the -omics platforms and algorithms in systems biology will greatly assist continuous monitoring of this complex biochemical microenvironment.[27] As if these challenges were not enough, there are other relevant conditions that are still scarcely addressed in cell culture approaches. Of special significance is the consideration of age-related influences on regeneration, the problems of multimorbidity, and the influence of medication.[28] As all cells in the body are subject to biomechanical stimulation, *in vitro* models of regenerative processes must also incorporate these factors as in a bioreactor. Major progress in micro- (and now) nanofluidics, as well as microchip systems, has led to the development of high-throughput models with the advantage of requiring few cells and small volumes of medium.[29–32]

The choice of cell types is a further area of development of great relevance. In this study, it is expected that induced pluripotent stem cells will be increasingly used to model disease states.[33–35] Tissue microarchitecture and simulation of true 3D in complex cellular systems are additional challenges, as a frequently used constellation is a (co)culture on a 3D open porous biomaterial scaffold. However, this scenario is not necessarily 3D, as cells tend to adhere to the scaffold fibers and grow as a monolayer on them. Despite the fact that the scaffold itself is 3D, the cellular growth pattern is often only two-dimensional. On tissue implantation of such a scaffold, the interstices would be filled by a protein hydrogel, and thus be a 3D situation for invading cells. Thus, for cellular models *in vitro*, such fibrous scaffolds could be filled by a relevant hydrogel, such as collagen and/or fibrin, to simulate more closely the regenerative niche.

Finally, there is the challenge of *in vivo* proof-of-principle and the choice of suitable animal models before clinical translation can be contemplated. The anatomical site, biomechanical status, and regenerative mechanisms in the chosen *in vivo* model should, if possible, be a good reflection of the human situation. Unfortunately, this is difficult to achieve in many forms of regeneration, for example in the nervous system.

Future Perspectives

I believe that polymer chemistry will play an increasing role in developing cellular systems to understand and thus direct tissue regeneration. This view is based on the fact that hydrogels can be self-assembled from monomer units in such a way that biologically active ligands can be exposed to embedded cells or those that can migrate into the hydrogel and set desired biological responses in motion or inhibit undesirable ones.[36–38] Thus, biodegradable, tunable and instructive biomaterials need to be studied in relevant culture systems, which recapitulate the status of the respective regenerative niche.

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