TISSUE ENGINEERING: Part B
Volume 15, Number 4, 2009

DOI: 10.1089/ten.teb.2008.0575

Developmental Engineering: A New Paradigm for the Design and Manufacturing of Cell-Based Products. Part I: From Three-Dimensional Cell Growth to Biomimetics of *In Vivo* Development

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Recent advances in developmental biology, systems biology, and network science are converging to poise the heretofore largely empirical field of tissue engineering on the brink of a metamorphosis into a rigorous discipline based on universally accepted engineering principles of quality by design. Failure of more simplistic approaches to the manufacture of cell-based therapies has led to increasing appreciation of the need to imitate, at least to some degree, natural mechanisms that control cell fate and differentiation. The identification of many of these mechanisms, which in general are based on cell signaling pathways, is an important step in this direction. Some well-accepted empirical concepts of developmental biology, such as path-dependence, robustness, modularity, and semiautonomy of intermediate tissue forms, that appear sequentially during tissue development are starting to be incorporated in process design.

Introduction

Tissue engineering is undergoing a major conceptual and methodological transformation in an effort to implement *in vitro* processes that mimic *in vivo* tissue development. Tissue engineering was introduced a few decades ago as a distinct research field with the aim of generating bioartificial tissues to meet a wide variety of therapeutic needs. According to the concepts developed by science historian and philosopher Thomas Kuhn, research practice in each scientific field is determined by a set of theories, beliefs, values, instruments, and methods called a "paradigm."[1] The first paradigm of tissue engineering is that of three-dimensional cell growth. This paradigm originated from the achievement of the groups of Langer and Vacanti, who succeeded in culturing cells in three dimensions using porous biomaterials (scaffolds) as cell supports.[2] Their work, described in the above-mentioned article, has been characterized as probably the most influential for the development of tissue engineering.[3] It has therefore identified, according to Kuhn's theory, the eligible problems and accepted solutions, determining consequently the objectives and methods of research practice in making bioartificial tissues.

According to the three-dimensional cell growth paradigm, the *in vitro* generation of bioartificial tissues involves the development and appropriate selection of the technical tools used in the *in vitro* process, such as cell types, growth/differentiation factors, scaffolds with different chemical and physical properties, bioreactor types, and modes of operation, that are provided by different fields such as biology, chemical engineering, and material science (Fig. 1).

How the cells optimally will survive and multiply in three dimensions, and how they will differentiate to cells of specific tissues is investigated empirically, by trying various combinations of the tools mentioned above. This method of research practice has been characterized as "Edisonian," with the different fields involved focusing on the solution of practical problems within their expertise instead of devoting efforts to develop a general unifying methodology for the design of an *in vitro* process.[3] As a result of this situation, tissue engineering means different things to researchers from

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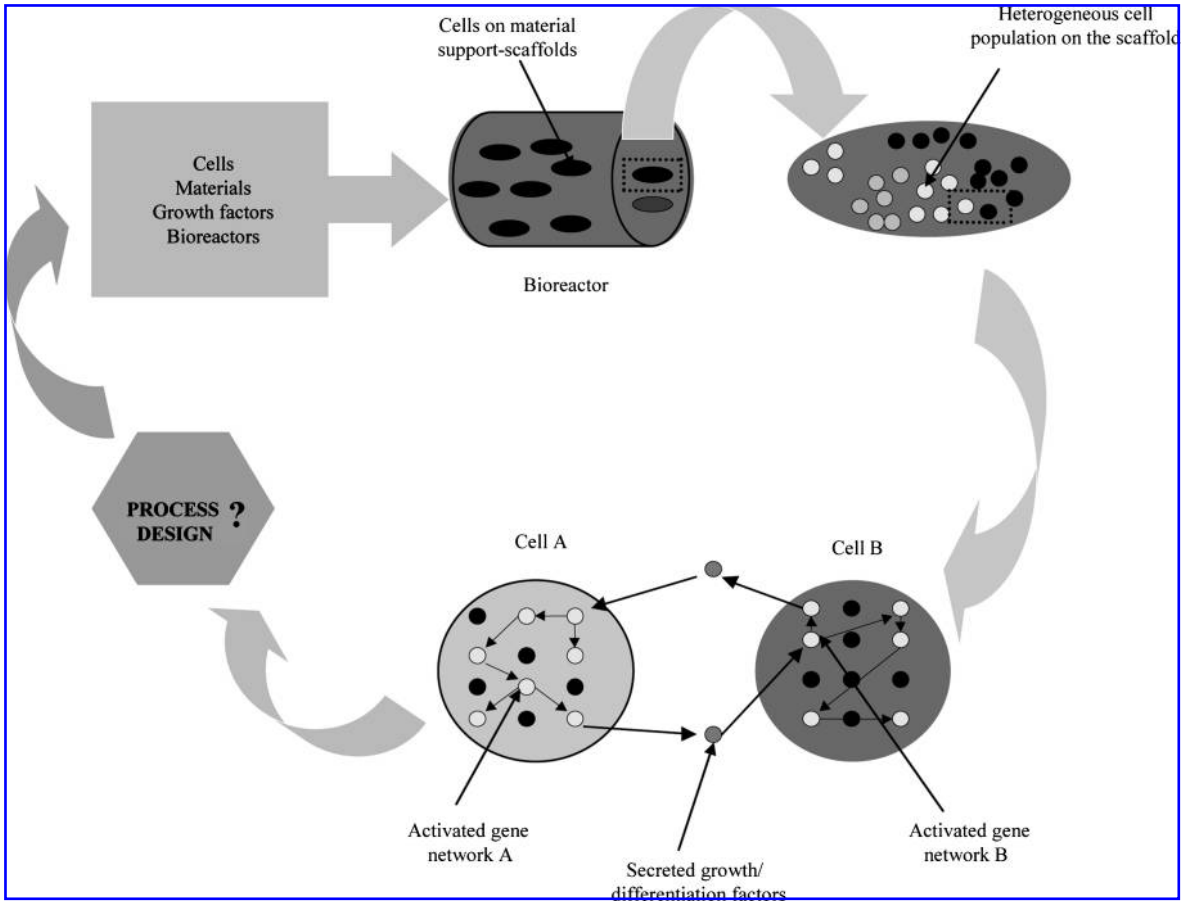

FIG. 1. The current methodology in tissue engineering under the three-dimensional cell growth/differentiation paradigm deals with the culture of the cells in three-dimensional material supports, scaffolds, and bioreactor systems. A factor, however, necessary for the formation of functional tissues is the proper communication of the heterogeneous population of tissue cells that organize them in semiautonomous entities, modules, during development. The cell communication depends on the organization of their expressed genes to similarly semiautonomous subsets, modules. A connection of the gene modules to the multicellular developmental ones is attempted here for the rational design of *in vivo*–like *in vitro* process under the new paradigm of "biomimetics of *in vivo* tissue development."

different fields specializing in different aspects of the *in vitro* process.[4] A methodology that will combine all the relevant fields as well as all the sequential procedures from cell sourcing to implantation, and which could lead to a cost-effective, quality-validated, and clinically oriented process, is still missing.[5] Thus, it is not easy to determine which of the numerous factors involved must be modified and how. For example, an increase in the scaffold porosity that enhances cell attachment, nutrition, and oxygenation leads to very slow structural recovery after unloading or to permanent destruction when this scaffold is used for bioartificial articular cartilage.[6] These undesirable consequences unavoidably necessitate a modification of other scaffold properties that are related to mechanical stability. However, these changes may affect the cell–material interactions adversely, which in turn may require additional modifications to be optimized, leading finally to a perpetual cycle of trial-and-error attempts.

A rational methodology to design an *in vitro* process could be developed from a fundamental understanding of what makes a tissue different from a three-dimensional cell construct. The necessity for such basic understanding and further basic science studies in tissue engineering systems was recommended explicitly at the Symposium of Reparative Medicine at the National Institutes of Health (NIH, United States Department of Health and Human Services) so that inherently limited trial-and-error approaches can be replaced by rational ones.[7] The lack of research related to the fundamental building block areas of science has also been mentioned in the recent U.S. Department of Health and Human Services report.[8]

Several reasons have been proposed for the premature focus of tissue engineering toward developing biological substitutes instead of basic research on the fundamental understanding of structure–function relationships in normal and pathological tissues (definition of tissue engineering given at a workshop of the U.S. National Science Foundation in 1988[9]). Among them is the urgent unmet medical need for tissues for transplantation due to donor scarcity. Equally important is the late involvement of governmental grant organizations in supporting tissue engineering research (as of 2001, private sector R&D investment was more than $4 billion,[10] whereas federal spending was $250 million[11]). The consequence has been that most development has occurred

in the corporate sector, where efforts are often driven by short-term practical goals that do not allow time for basic research addressing the fundamental scientific questions that must be answered to provide a rational foundation for tissue engineering practice.[10]

In this review and perspectives article, consisting of two parts, we discuss the newly evolving paradigm in tissue engineering based on biomimetics of developmental events in tissue formation and advances in systems biology and network science. In part I, concepts of developmental biology, such as path-dependence, robustness, modularity, and semiautonomy of intermediate tissue forms, are highlighted, and their utility for incorporation in process design is explained. In part II, we outline how recent progress in systems biology and network science provides the opportunity to express complex developmental processes in concrete mathematical terms. This in turn will allow more precise description of manufacturing processes. The net result of these advances is an emerging transition of tissue engineering from a trial-and-error approach into a more rigorous discipline, comparable with other branches of engineering.

Biomimetic *In Vivo* Development: A New Paradigm for Tissue Engineering

In vivo tissue development is not restricted to cell growth and differentiation. Even if these biological phenomena take place *in vitro* in three-dimensional cell supports, by themselves and without any effort to synchronize them in space and time, they are not adequate to form bioartificial tissues with natural structure and function. Tissue development *in vivo* involves a multitude of interrelated and orchestrated intercellular and intracellular processes that guide tissue morphogenesis. The most crucial phenomenon of tissue morphogenesis is the spatio-temporal organization of a heterogeneous cell population of differentiating cells to tissue structures from which the proper tissue functions arise. This organization is exquisitely regulated, unfolding gradually during development through both cell-autonomous processes and cell–cell interactions. These mechanisms are governed by a combination of maternal prepatterning, imprinting, gene expression, and modulation of transcription-independent signaling pathways (e.g., protein phosphorylation) in an elaborate network of feedback and feed-forward processes (Fig. 1). A rational approach for *in vitro* process design should therefore be modeled on the crucial parameters controlling tissue development that are being identified by developmental biology investigations. To the extent that the mechanisms of *in vivo* tissue development are known, similar *in vitro* processes can be designed and external process controls can be optimized so that the cells will self-assemble with appropriate structural and functional organization. Confirmation that the correct parameters have been identified will ultimately require verification at the level of global gene expression, protein phosphorylation, or other appropriate measures that determine cell behavior and consequently cell-to-cell communication.

The need for a transition from Edisonian empirically derived cell differentiation schemes to biomimetic approaches based on concepts developed from developmental biology was articulated explicitly at least a decade ago.[12] However, only recently has the tissue engineering research community started to consider incorporating these principles, based on newly initiated discussions between tissue engineers and developmental biologists.[13] Nevertheless, despite the fact that substantial information from developmental biology is available that could be incorporated in the design of *in vitro* processes, it is still not widely used in tissue engineering.[14] According to Ingber *et al.*,[13] this is because this information, though available, has typically not been expressed in terms useful for process design. Although much is now known about the molecular components and signaling pathways involved in various developmental process, little is known about how these components are organized into higher order functional networks,[15] and it is the latter type of information that is helpful to a tissue engineer who wants to determine design criteria.[13] What is needed are concepts developed on a more global scale that describe the mechanics of tissue formation in three dimensions in terms of the collective behavior of interacting cells during tissue morphogenesis, rather than from the standpoint of a single cell or gene.

In any event, growing awareness of tissue engineers and related disciplines of the necessity for such an approach is developing. Indeed, recent work has indicated that unless developmental processes are recapitulated *in vitro*, the quality of stem cell–derived products is likely to be uncertain. The equivalence of cell populations generated *in vitro* to those arising *in vivo* has been already questioned, and differences appear predominantly due to the lack of extensive *in vivo* cellular interactions (also called microenvironment) that determine cellular characteristics.[16] This suggests that approaches not based on critical developmental phenomena are unlikely to be successful.

A clear practical example in which the advantages of recapitulating *in vivo* developmental processes instead of relying on ill-defined differentiation pathways has already been described. The pancreas originates during *in vivo* development from the endodermal gut epithelium,[17] suggesting that endodermal cells should be used in a biomimetic *in vitro* process for the generation of β-cells, as had been proposed previously by Sipione *et al.*[18] D'Amour *et al.* exploited this concept by using endodermal instead of neuroectodermal cells to generate β-cells,[19] from human embryonic stem cells in a multistage *in vitro* process[19] clearly based on differentiation pathways known to occur during normal *in vivo* pancreatic organogenesis. This accomplishment was described as "remarkable progress" by Madsen and Serup[20] in terms of the efficiency of generation of β-cells and insulin release capacity compared to other protocols. In contrast, selection of nestin-positive cells from a neuro-ectodermal population does not correspond to a well-documented *in vivo* process for β-cell differentiation and appears to yield cells that are not true β-cells.[18] Although these cells produce a low amount of insulin, their secreted insulin is probably acting as a growth factor in nervous system development and not as a systemic metabolic regulator.[21]

Empirical Concepts of Developmental Biology and Their Significance in Engineering Terms

We start with an analysis of the empirical concepts of developmental biology and their significance in process design. A common ground should exist in the design rules. Indeed, as we will show below, empirical concepts established from the

studies of developmental phenomena are strikingly related to engineering concepts of robust process design, so that notwithstanding the difficulties described above, emergence of a common vocabulary between the two fields is in fact a natural development.

Robustness leads to process stability and product reproducibility

Developmental biology cannot yet provide a detailed theoretical basis for the foundation of methodological approaches in tissue engineering to the extent that physical or chemical laws have provided for the other engineering fields. Nevertheless, the concept of regulative development—extreme ability to compensate for a wide variety of perturbations—was already well appreciated by 19th century developmental biologists. Thus, a developing embryo meets the definition of robust system, for which the output is unchanged (within some tolerance) by a range of external perturbations. In an *in vitro* manufacturing scheme modeled on such a developmental process, robustness will be reflected in resistance to external perturbations that are unavoidable in an artificial environment. This is a highly desirable characteristic in pharmaceutical manufacture because such a process, even if temporarily disturbed, will return to within acceptable limits, providing cells or tissues with reproducible properties. This type of stable process can be transferred to commercial-scale manufacturing, where culture conditions are more difficult to control, much more easily and reliably than a nonrobust process. To achieve the goal of a validated manufacturing process consistent with successful commercialization and regulatory requirements, various often underappreciated deficits in automation and control will need to be addressed.[22] Engineered tissues, which commonly consist of one or more biological components and one or more synthetic biomaterials, may have complex requirements (Lysaght in Hunziker *et al.*[23]). These include validated, well-controlled manufacturing processes, relevant in-process controls, and product release specifications that provide reasonable assurance of consistent product safety and effectiveness.

The multistage character of in vivo *processes allows high observability and controllability*

Another empirical concept that facilitates process design is the multistage character of developmental processes. *In vitro* processes composed of distinct sequential subprocesses corresponding to different developmental stages will have high observability and controllability. Based on these two properties, the process and product can be optimized. The clear distinction of the stages of tissue development that take place in different sequential *in vitro* subprocesses improves the observability of the process. Observability, by analogy to the meaning of this term in control engineering, denotes that variables referring to the output of a stage of the process, for example, gene markers expressed in a developing tissue, provide information about its developmental state. A direct assessment of the tissue state can exploit information already existing in developmental biology for the same *in vivo* stage, thereby minimizing the need for extensive analysis. Controllability is also improved because interventions, such as addition of growth and differentiation factors to guide tissue development along the desired natural developmental pathway, can be directed at the appropriate stages when the cells are competent to respond to them.

This concept is apparent in the previously mentioned process designed by D'Amour *et al.* for the generation of β-cells from endodermal cells.[19] As the authors put it, their strategy combines an informed approach based on developmental biology and an empirical approach. The authors guided cell differentiation through a series of five stages of endodermal intermediates that are similar to those appearing *in vivo*: definitive endoderm, primitive gut tube, posterior foregut, pancreatic endoderm, endocrine precursors and hormone expressing endocrine cells. The informed approach refers to recapitulation of successive stages of pancreas development and use of information from developmental biology, using process controls based on detailed qualification studies, characterizing the cells appearing at each stage, and identifying appropriate patterns of marker expression. The empirical approach refers to the testing of various combinations and application schedules of growth factors for each step of the protocol. The observability of the process was high since the cells were characterized at each stage and compared to those of the corresponding *in vivo* stage. Controllability was also high because optimization of the combination of growth and differentiation factors that were used for each stage could be done separately for each stage and their effect could be compared directly with existing information on pancreatic development. This would be difficult or impossible for a process that is not divided into distinct developmental stages, since no assessment can be done as to which developmental stage has to be optimized and how. New data will be provided by ongoing studies of developmental biology. For example, large-scale gene expression analysis of *in vivo* pancreas development[24] will allow further refinements. Important remaining challenges in designing such processes for clinical applications will include the identification of in-process controls, suitable for implementation in a commercial manufacturing environment that will be most useful in ensuring each particular stage is achieved as *in vivo*, leading to a product that will be safe and effective.

Path-dependence makes processes semiautonomous

Another concept of developmental biology that facilitates optimization of *in vitro* developmental processes is path-dependence, which means that each developmental stage depends on the previous ones.[25] Consequently, optimal conditions are self-established by the process. If it progresses naturally, these conditions do not need to be incorporated explicitly in process design, making the process more semi-autonomous as it proceeds. An example is endochondral ossification, the developmental process for the formation of bone through gradual replacement of a cartilage template by bone. While tissue engineering studies usually favor intramembranous ossification, the one-stage direct osteogenic differentiation of mesenchymal stem cells, endochondral ossification has recently gained the attention of tissue engineers.[26] The reason for this is that optimal conditions for bone formation are established by the developing cartilage itself, which could be considered as a self-designed scaffold that is simultaneously osteoinductive[27] and angiogenic.[28]

It therefore controls osteoblast recruitment and differentiation as well as improves cell viability through enhanced vascularization, the lack of which leads to considerable loss of implanted osteoblasts.[29]

For similar reasons Nakaoka *et al.* proposed that signaling between chondrocytes and osteoblasts must be established for the generation of osteo-chondral tissues.[30] This implies that instead of dividing the *in vitro* process in cartilage and bone formation by using separate scaffolds placed in different bioreactors and subsequently joining them by inefficient artificial means, the division should be modeled after what occurs *in vivo*: bone formation in a cartilage template. As the osteoblasts replace the hypertrophic chondrocytes undergoing apoptosis, bone fingers will interdigitate inside the cartilage mass following the parallel columns of chondrocytes that are separated by columns of mineralized cartilage matrix,[31] solving one of the challenges in composite scaffolds, integration of the two tissues.[32] In this case too, the previous stages of the process provide optimal conditions for the integration of the two tissues.

Besides the self-establishment of optimal conditions, path-dependence allows high predictability with respect to the outcome of the process. Divergence from the *in vivo* situation in early stages of an *in vitro* process is a strong indication that the later stages will deviate further from the natural developmental pathway. In such a case it may not be productive to proceed until the stage in question is optimized. D'Amour *et al.*[19] started their study for the design of their process to generate β-cells only after optimizing the first stage, generation of definitive endodermal cells from human embryonic stem cells.[33] In addition, further optimization of the first stage was performed, since the authors observed that if this stage was suboptimal, β-cell productivity was low. For example, when low activin A was used, the quantity and the anterior pattern of the definitive endodermal cells produced was reduced, which in turn substantially diminished the production of endodermal intermediates and endocrine cells.[19]

Interdependent tissue variables control cell organization

The growth plate: An example of tissue variable interdependency. Cell organization into tissue structures constitutes a major challenge for tissue engineering. In contrast, cell organization to tissue structures is a natural characteristic of multistage *in vivo* developmental processes. *In vivo*, a carefully orchestrated and concerted progression of tissue size and cell differentiation takes place, allowing synchronization of the various processes required for cell organization. The interdependence of cell differentiation state, organization, and size of the structure in question can be observed easily in the growth plate because it can be traced at the cell and gene interaction level. The growth plate consists of parallel columns composed of chondrocytes in gradually advancing differentiation stages—resting, proliferating, prehypertrophic, and hypertrophic chondrocytes[31]—that are closely linked to their location in the developing growth plate (Fig. 2a).

Chondrocytes are almost randomly distributed in early fetal long bones.[34] They are arranged later in groups or clones in presumptive growth plates and even later are stacked in columns as long as the tissue size increases.[35,36] The size of the developing growth plate increases continuously during the process and is compatible with all the

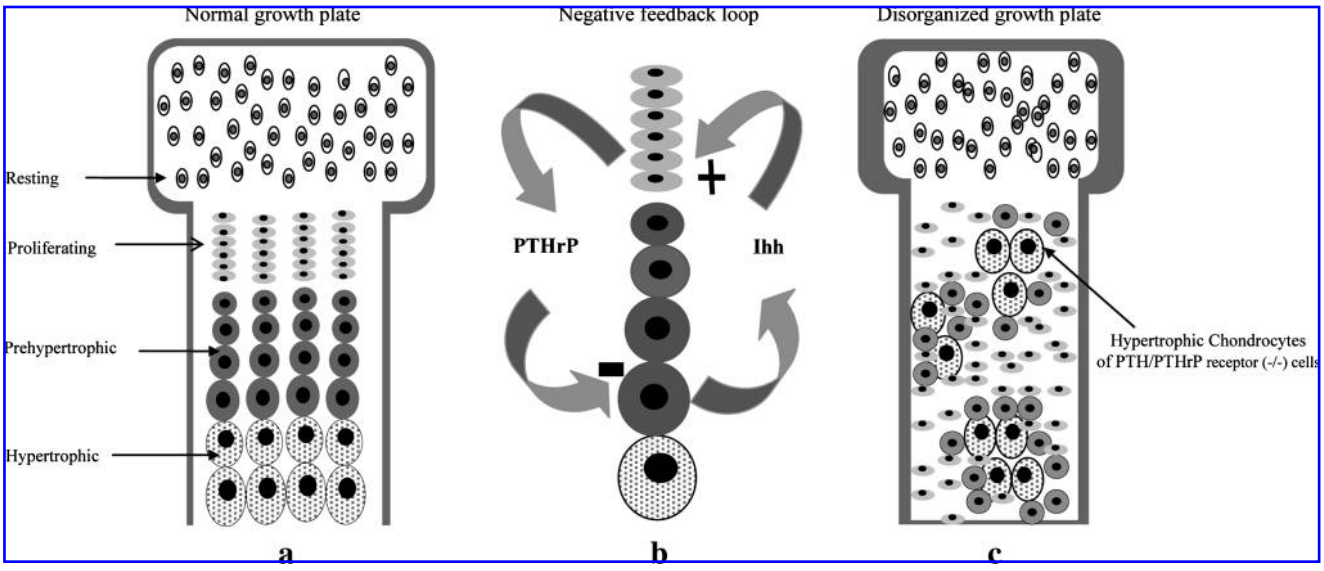

FIG. 2. (**a**) The growth plate consisting of parallel columns composed of chondrocytes in different and gradually advanced differentiation stages, from resting to hypertrophic chondrocytes. (**b**) The organization of the growth plate is the result of a signal exchange between chondrocytes achieved through a negative feedback loop of Indian Hedgehog (Ihh) and parathyroid hormone–related protein (PTHrP) that are exchanged between proliferating and prehypertrophic chondrocytes located at distinct heights along the column and retards the progression of the differentiating chondrocytes to the hypertrophic stage preserving the integrity of the columnar structure. (**c**) In chimeric mice containing both wild-type and PTH/PTHrP receptor (−/−) cells the mutant cells prematurely differentiated into hypertrophic chondrocytes leading to the disorganization of the growth plate.

phenomena taking place. For example, protein signal secretion by chondrocytes is adjusted to the increasing size to circumvent diffusional limitations. The organization of the growth plate is the result of such a signal exchange between chondrocytes, achieved through a negative feedback loop of Indian Hedgehog (Ihh) and parathyroid hormone–related protein (PTHrP). These signals are exchanged between proliferating and prehypertrophic chondrocytes located at distinct heights along the column[37,38] (Fig. 2b). PTHrP retards the progression of the differentiating chondrocytes to the hypertrophic stage and thereby delays column elongation, synchronizing it with other processes taking place in parallel, such as secretion and organization of the extracellular matrix, both of which are needed for columnar structural integrity. Disrupting the negative feedback loop by inhibiting or inducing the proteins involved disturbs chondrocyte communication and consequently their rate of differentiation. As a result, the rate of column elongation also changes because column elongation depends on differentiation of new chondrocytes from the proliferating stage and their movement along the columns. If the column elongation rate is altered to the point where it becomes incompatible with the processes involved in column stabilization, the columnar structure will become disorganized.[39,40] In addition, the structural organization of the developing joint must remain coordinated with cell differentiation and cell–cell communication throughout limb morphogenesis.

Rigid geometrical constraints prevent cell communication and organization. Restriction of chondrocytes and consequently their communication by the physical boundaries of a scaffold disperses the negative feedback loop signaling into several unconnected local areas of variable size inside the scaffold where the cells reside. Since differentiation rate depends on cell signaling, it will progress at different rates in different areas of the scaffold, so that appropriate overall architectural organization in the scaffold will not occur. Thus, artificial geometrical constraints suppress the dynamics of tissue development.[41]

For these reasons, disorganized bioartificial cartilage tissue having hypertrophic chondrocytes dispersed in its mass due to a high differentiation rate in areas where local conditions disrupted cell communication is unlikely to support optimal cellular architecture. It is also possible that bone may be formed inside the cartilage mass where hypertrophic chondrocytes reside as that microenvironment carries the appropriate factors for recruitment of osteoblasts from bone marrow and their differentiation to osteocytes.[27,42] *In vivo*, however, in an organized growth plate, bone formation is directed solely at a zone located at the end of growth plate where the hypertrophic chondrocytes of the parallel columns reside.

Experimental confirmation for this comes from experiments by Chung *et al.* in chimeric mice containing wild-type and PTH/PTHrP receptor (−/−) cells. The mutant cells differentiated prematurely into hypertrophic chondrocytes because their differentiation was not retarded by PTHrP while surrounded by wild-type proliferating cells.[43] The disturbance of growth plate organization in this case led to ectopic mineralization at the sites of hypertrophy and to ectopic bone at sites of ectopic hypertrophic chondrocytes (Fig. 2c).

Cell organization in bioartificial tissue of an increasing size. It is important to mention that in the study of Alsberg *et al.*,[44] the size of the tissue was a variable that increased during tissue development, not a fixed parameter; this was required for appropriate cell organization to take place. The authors developed a method for enhancing cell multiplication with a stimulus provided by the scaffold to assure cell growth inside the scaffold after implantation, so that an increase in the size of the bioartificial tissue could take place according to the needs of the organism (e.g., adjusted to the growth of a child). Chondrocytes and osteoblasts have been used inside biomaterials whose polymer chains were covalently linked to synthetic peptides containing the arginine–glycine–aspartic acid cell adhesion sequence, which was shown by the same group to promote cell proliferation.[45,46] When the cell-populated biomaterial was implanted in mice, the cells self-organized into structures resembling the growth plate. This provides evidence that conditions that allow for gradual increase of tissue size facilitate optimal cell organization. A similar study with bioartificial tissue developing *in vitro* might yield more detailed information for the design of *in vitro* processes.

Modular assemblies in sequential subprocesses. It thus becomes apparent that the need to emulate the gradual and concerted progression of tissue variables such as tissue size, cell differentiation, and cell organization that occurs *in vivo* suggests that obtaining tissue structures with the characteristics desired is most likely if modeled on natural mechanisms. Processes that self-establish without the need for explicit design directed specifically to the details of cell organization, allowing the tissue variables to evolve naturally and permitting cells to exchange their signals in a natural way, are much more likely to be successful. This does not mean that cell organization is a phenomenon that can take place automatically *in vitro*. Instead, the lesson is that special consideration should be given to the design of *in vitro* systems with the appropriate technological choices of bioreactor–biomaterial systems so that no inappropriate artificial constraints will be imposed. As discussed above, one-stage processes in which cells are seeded in a scaffold at the final bioartificial tissue size (step a1 in Fig. 3) impose this sort of artificial constraint. A likely result might be the inability to follow step a3 toward a structurally organized bioartificial tissue, leading finally to randomly distributed differentiated cells inside the scaffold after step a2. In keeping with the principle of progressive growth, coordinated with cell differentiation and signaling of the bioartificial construct as outlined above, we should implement the whole manufacturing scheme as a series of subprocesses as depicts in b1–b2–b3 of Figure 3. Such a process could start with the placement of stem cells in small scaffolds (b1), increasing the scaffold size at an intermediate stage when the cells have started but not yet completed their differentiation (b2). In a final stage (b3), the scaffold is increased to its final size.

For example, to implement path b1–b2–b3 in an *in vitro* process, we could start with a subprocess for the formation of mesenchymal cell aggregates that simulates naturally occurring cell condensation and chondrocyte differentiation[26] and then proceed to a second subprocess in which larger constructs are assembled by embedding the aggregates in a hydrogel system.[47] For the design of such a process, infor-

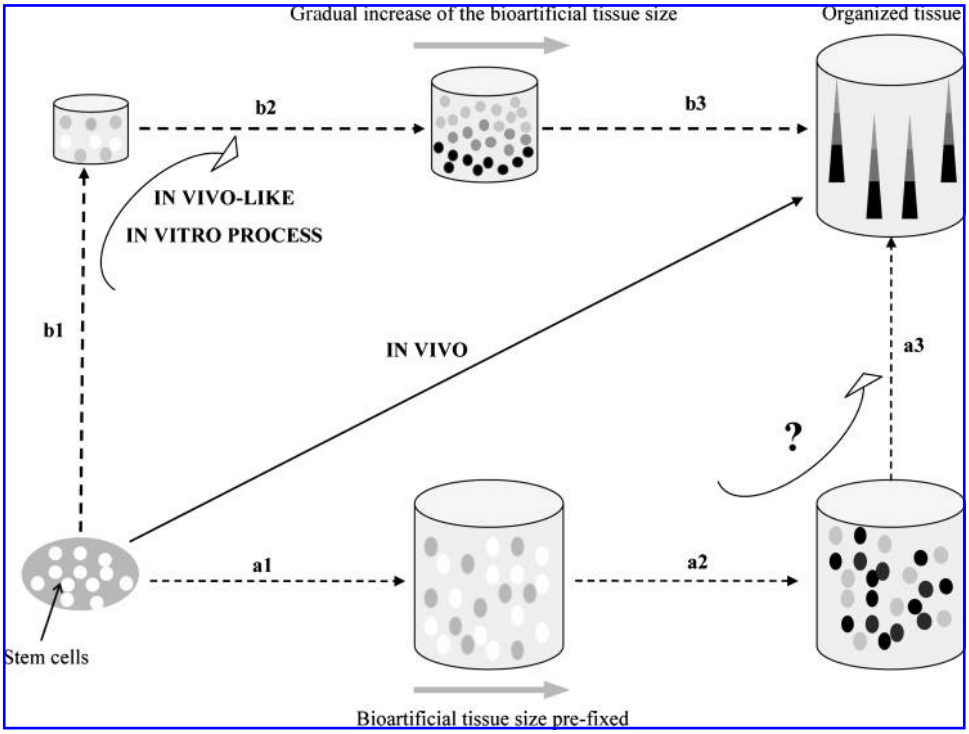

FIG. 3. A multistage *in vitro* process in which the tissue size increases gradually following the advancement of cell differentiation preserves the cell communication that takes place through secreted signals, leading to an organized bioartificial tissue (route b1–b2–b3). Prefixing, however, the bioartificial tissue size to the scaffold size from the beginning of the process will probably lead to a random distribution of differentiated cells because their distances do not evolve gradually to preserve their communication.

mation relating to the way intermediate constructs of the process interact biologically when in contact will facilitate control of the biological properties of the constructs. This is crucial to both structural and functional integration. It is known that cell aggregates are not simple mechanical structures whose only relevant properties are mass and shape when they are assembled to form larger constructs. They are alive, and they communicate with other aggregates. Therefore, the biological properties of multiaggregates formed from their assembly cannot be predicted without referring to phenomena studied in developmental biology. For example, aggregates of differentiating chondrocytes influence each other when they come together.[48] Ossification centers interact and fuse together, forming a single center.[49] Even larger organized structures such as growth plates interact. When surgically excised parts of the growth plate were reinserted ectopically, close to the rest of the growth plate, they influenced the differentiation stage of the chondrocytes of this part.[50]

This concept should be incorporated into process design. Kameda *et al.* showed that isolated micromass cultures exhibit a spatial heterogeneity in the expression of some genes.[48] This heterogeneity changes when different micromasses are in proximity. The expression of Ihh was restricted to the periphery of micromass cultures when the micromass was in isolation. In contrast, placing micromass cultures close to each other drastically reduced the expression of Ihh in the internal peripheries of the micromasses. This shows that when micromasses were placed together forming a larger construct, they interact biologically to minimize heterogeneity and promote biological integration. It also suggests that aggregates formed in a bioreactor system in the first subprocess, which simulates chondrocyte condensation, can be assembled in a second subprocess to increase the size of the tissue and effect their biological integration (Fig. 4a). However, if the assembly of small aggregates happens at late differentiation stages with an ongoing mineralization process inside the aggregates, biological integration is improbable. Instead, a number of randomly dispersed, permanently fixed mineralized nodules inside a larger aggregate (Fig. 4b) is the likely result.

Gradual increase of bioartificial tissue size has been introduced in tissue engineering methods in the context of cell-to-cell communication rather than to imitate natural developmental processes explicitly. These methods provide initial examples of technical approaches that could be used to implement gradual increase of tissue size in biomimetic *in vitro* processes. One of these methods is termed cell sheet engineering, which is the assembly of two-dimensional constructs in three dimensions, developed by Okano and colleagues. This method uses cell culture surfaces coated with a temperature-responsive polymer. When cells reach confluence, they can be detached as a cell sheet by reducing the temperature. This causes the culture surface to become hydrophilic and therefore noncell adhesive. Layering the cell sheets, three-dimensional tissues can be reconstructed. This method has been used to fabricate three-dimensional bioartificial liver constructs that preserve the cell-to-cell contacts

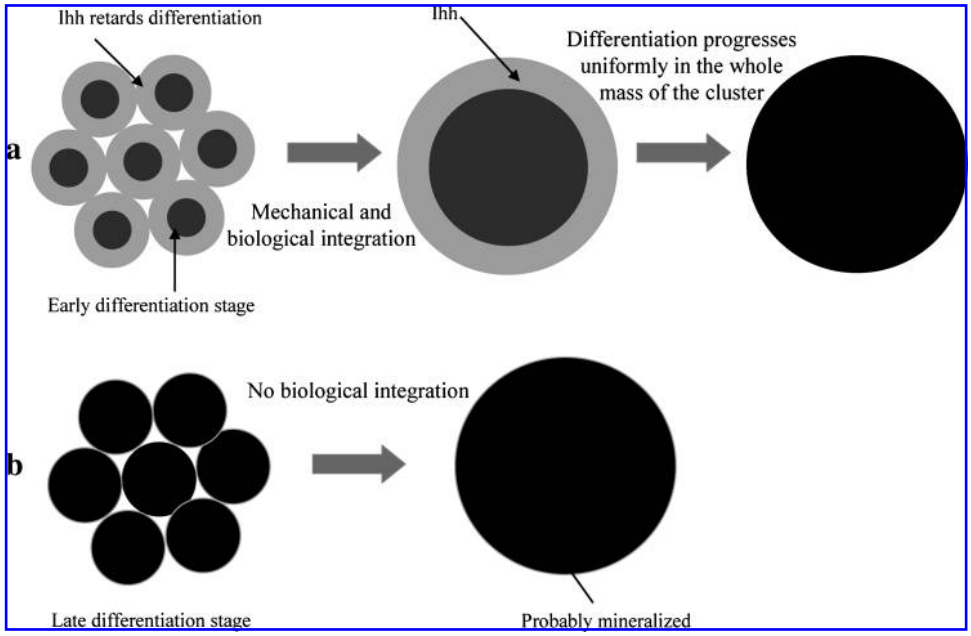

FIG. 4. (**a**) The assembly of early differentiation–stage mesenchymal stem cells to aggregate clusters can lead to their biological integration so that further differentiation can take place uniformly in the whole mass of the cluster. This corresponds to a gradual increase of the tissue size as long as differentiation proceeds. (**b**) However, late differentiation–stage aggregates may not be able to be biologically integrated, especially if mineralization occurs, leading to a cluster with randomly distributed differentiated areas.

and thus promote persistent survivability.[51] It has been also used with layered cardiomyocyte sheets for reconstruction of myocardial tissue. As the authors claimed, their method allowed the formation of gap junctions between cells in the different layers, which is indispensable for the properly organized intercellular electrical communication that leads to synchronized pulsation.[52] This approach, which is similar to what we propose, led to a functional bioartificial construct that exhibited global properties at the tissue level, that is, synchronized pulsation. Another recently developed method of cell sheet engineering, magnetic force–based tissue engineering, enhances cell-to-cell adhesion in the vertical direction. This method decreases cell adhesion on the surface of plates by using ultralow-attachment plates. At the same time, it increases the adhesion between cells bearing magnetic nanoparticles by application of a magnetic field.[53] This method can also provide tubular structures when the cells are attracted around a cylindrical magnet.[54] The controlled formation of folded cell sheets offers the possibility of recapitulating *in vitro* the basic mechanism of folding and refolding taking place in the early stages of *in vivo* development, for example, the formation of germ layers during gastrulation, germ layer transformation (e.g., tube formation in endoderm and formation of the neural folds and neural tube), and interactions between the germ layers (e.g., organ-specific bud formation in the endodermal tube).

Multicellular developmental modules as reliable building blocks of tissues

The use of intermediate tissue forms in multistage *in vitro* processes. Gradual progression of the tissue variables underscores the importance of engineering intermediate tissue forms. As has been suggested by Ingber *et al.*,[13] tissue engineering should shift its exclusive focus from mature tissue forms to preexisting tissue structures that are remodeled during development; *in vitro* processes should be designed accordingly. Intermediate tissue forms that can establish optimal conditions for the continuation of development, such as cartilage for endochondral ossification, can be used as components in processes for generation of other tissues. Another example is the induction of hepatic differentiation and early stages of hepatic morphogenesis in an *in vitro* system that involved the generation of hepatoblasts, as well as endothelial cells, as intermediates that provided the signals for hepatic development.[55] At present, these signals are known only partially,[56] but eventually they will be defined, thereby allowing external factors to be added under precise control. The endodermal cells used by D'Amour *et al.* for the generation of β-cells[19] are another example.

Similarly, osteoclasts, which until recently were not of interest in tissue engineering because they are not structural components of a tissue but rather part of the bone remodeling process, have been used as such an intermediate. Nakagawa *et al.* examined *in vitro* osteoclastogenesis in a bioartifical bone construct to design it in a way that can assure bone remodeling after implantation.[57] As the authors mentioned, the process of bone remodeling must be taken into consideration in the design of bioartificial bone to assure that it will take place *in vivo* after implantation. This requires proper design of the scaffold to allow osteoclast progenitor cells to differentiate, attach, and migrate. Besides bone formation from the differentiation of mesenchymal stem cells isolated from bone marrow, two other aspects were ad-

dressed: isolation of hematopoietic cells that contain osteoclast precursors from bone marrow and addition of these cells to the osteoblast-bearing scaffold. Such a process could also be used for the later stages of *in vitro* endochondral ossification, which besides the formation of developing cartilage and coculture along an interface with osteoblast precursors for proper bone formation, requires the presence of chondroclasts that arise from hematopoietic cells[58] for the resorption of the calcified cartilage that will be replaced by bone.[31] The advantage of such a process is that it can control the relative thickness of cartilage and bone in a bioartificial osteo-chondral tissue through the control of cartilage resorption by adjusting the concentration of chondroclasts and osteoblasts appropriately.

Though not related to developmental processes, difficult problems in bioartificial tissues, such as the vascularization, have recently been addressed combining different cell types in multistage processes. These efforts show that the technical background required for the development of vascularized tissues during *in vitro* development has been prepared. Kelm *et al.*[59] produced primary human myofibroblasts in spheroids and coated them in a second stage with human umbilical vein endothelial cells. In a third stage they encapsulated the coated human myofibroblast spheroids in an agarose mold. The authors observed that the microtissues were assembled into a coherent macrotissue inside the agarose developing a dense network of endothelial cells throughout the whole macrotissue mass. The implantation of the prevascularized macrotissue into chicken embryos showed the development of a vascular system across the macrotissue–embryo interface, while non vascularized macrotissue implants were rejected. Similarly McGuigan and Sefton have coated collagen cylinders containing HepG2 cells with human umbilical vein endothelial cells and assembled the cylinders in a larger tube which developed interconnected channels through which blood or medium could be perfused.[60]

Tsuda *et al.*, using the method of cell sheet engineering fabricated prevascularized bioartificial tissues using multilayered cell construct with layers of micropatterned endothelial cells between layers of fibroblasts.[61] Kaihara *et al.* used micromachining technologies on silicon and pyrex surfaces to generate vascular systems with branched architecture of vascular and capillary networks.[62] The authors cultured hepatocytes and endothelial cells as single-cell monolayers on these two-dimensional molds and they folded them into compact three-dimensional vascularized bioartificial tissue.

In the above methods, we see not only the graded increase of tissue size through sequential steps of the *in vitro* process, but also the ability to construct complex vascularized tissues using different cocultured cell types. With appropriate signals, such methods could also be adapted by coculturing—developing tissues with stem cells to induce them to differentiate gradually to endothelial cells around small tissue constructs that later assembled in to larger ones.

Modules as structurally robust building blocks of complex tissue. Some of the intermediate tissue forms, though transient, have robust characteristics, including anatomic pattern, that allow retention of their integrity *in vitro* without an explicit design of special externally imposed conditions because their integrity depends on intrinsic factors. Abad *et al.*, for example, showed that the polarity of the growth plate with the resting chondrocytes on one side and the hypertrophic cells on the opposite, does not depend on surrounding tissues and remains the same after the growth plate is excised, inverted, and re-implanted[63] (Fig. 5), probably due to intrinsic factors, that is, the Ihh/PTHrP–negative feedback loop.

As mentioned previously, embryologists had since long observed that some parts of developing organisms, such as limb buds or tooth germs, are robust embryonic regions which could be displaced or induced ectopically. Therefore, they exhibit an internal coherence and relative independence

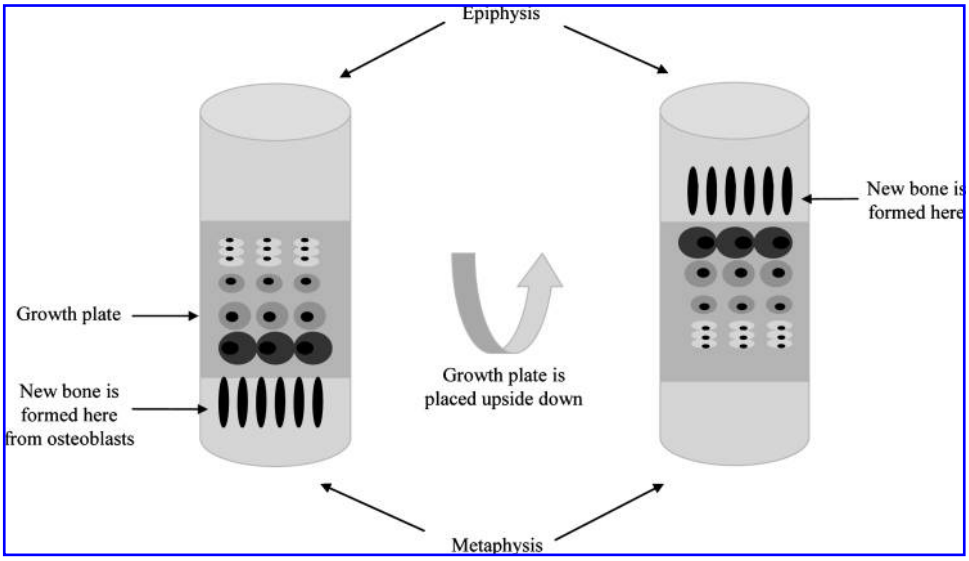

FIG. 5. The growth plate is developmental module, that is, a robust structure whose integrity depends on intrinsic factors. The polarity of the growth plate with the resting chondrocytes on one side and the hypertrophic cells on the opposite does not depend on surrounding tissues and remains the same after the growth plate is excised, inverted, and re-implanted (from the study of Abad *et al.*[63]).

from other parts of the organism. These types of units of embryonic development are called "modules."[64] Such an internal coherence, for example, is exhibited by mesenchymal stem cells during the process of condensation, the first stage of endochondral ossification that induces chondrogenic differentiation.[65] When condensation takes place, the external interactions of the condensed cells with the neighboring tissues have been minimized already through regression of the vasculature, which is a prerequisite for condensation.[66] It is because of its modularity that condensation in the form of micromass cultures became a popular *in vitro* model system for studies of chondrocyte differentiation.[67] Similarly, the endoderm, after pancreatic induction from the mesoderm, could be considered a developmental module because it completes pancreatic tissue specification autonomously, since after this stage the presence of the mesoderm is no longer required.[68] This means that several modular forms appear during development, ranging from cellular ones like cartilage condensation to spatially extended multicellular ones with a heterogeneous cell population like the growth plate. The robustness and autonomy of such modular processes argues in favor of manufacturing schemes that recapitulate developmental stages, to exploit the benefits of regulative, self-controlled behavior in developing processes that are well controlled and thus likely to result in high product consistency.

Modularity is a well-known concept in engineering that has been used for efficient product development. When the interfaces between the product components are uncoupled, which means that the component structure and function do not depend on other components, for example, growth plate organization, the architecture is modular, while if coupled it is integral.[69] In developmental processes, the integration of cells into modules and the relative isolation of the module from other modules of surrounding tissues render the interfaces between them uncoupled. Indeed, to make bioartificial osteochondral tissue, one can design a growth plate without having to design the interface between hypertrophic chondrocytes and osteoblasts. This interface, which is characterized by the presence of the hypertrophic chondrocytes at the end of growth plate, is based on internal growth plate interactions, that is, the negative feedback loop of Ihh/PTHrP that controls chondrocyte differentiation rate. Evolution has provided optimal conditions for bone formation, which takes place during endochondral ossification at the interface between cartilage and bone, facilitating osteoblast recruitment and differentiation. If, however, the osteochondral tissue is made in a non-developmental process, for example, when cartilage and bone components are made separately in different scaffolds, the interface has to be designed artificially; its characteristics will necessarily be limited by the material properties of the scaffold. Any modification of the two scaffolds will require re-design of the interface. Needless to say, no biological interaction can take place through such an interface the way it does *in vivo*, where the osteoblasts penetrate the cartilage mass gradually,[31] allowing the integration of the two tissues to be self-established instead of explicitly designed artificially.

Modular architecture provides tremendous advantages in flexibility and cost effectiveness when exploring a range of candidate products with different properties to help optimize product design. A modular architecture allows the creation of a variety of product properties at the final assembly of the separately fabricated component-modules.[69] In contrast, an integral process, typical in tissue engineering until now, requires an equivalent variety of the entire process, and is much more time consuming and costly. It is therefore incompatible with the business model that was adopted by venture capitalists of short-term instead of long-term investment money, as mentioned by Lysaght in Hunziker *et al.*[23] Similarly in development, modularity allows exploration of diverse sets of product characteristics by combinatorial rearrangement of connections between modules that evolve separately.[70]

The importance of cocultures to construct higher order modules

Under the framework of developmental engineering, a tissue engineer can use methods and tools that, as we have discussed, are sufficiently advanced to accommodate discrete developmental stages. Cocultures of modular tissue forms emerge as the most important operational mode. As the recent literature of tissue engineering suggests, cocultures of intermediate tissue forms can help to assure correct and complete signal activation of the developmental pathway needed for a particular process, since in most cases these signals are not known in sufficient detail to be replaced externally. This is particularly important for the first stage of the process, because due to the path-dependence of development, it sets the optimal conditions for the subsequent stages. If the first stage is implemented *in vitro* with the maximum possible fidelity, activation of the complete set of necessary differentiation pathways is much more likely, as is faithful continuation of the developmental process.

Tissue engineering has already developed coculture methods, for example, multilayered hydrogel systems that allow cell signaling along interfaces between cells in different layers and the separation of these layers without disturbing the cells, as mentioned by Elisseeff in Mikos *et al.*[71] Such a system could place different developmental modules in series or in parallel, or a combination of both, bringing in contact distinct codeveloping and interdependent intermediate tissue forms as needed. For example, coculture of mesoderm with endoderm allows for the induction of pancreatic development through instructive signals from mesodermal to endodermal cells.[72] Until now signals from the mesoderm were considered permissive rather than instructive, that is, the endodermal cells of the gut were already prepatterned. A requirement for this type of prepatterning would impose a formidable or perhaps insurmountable barrier to design of faithful biomimetic *in vitro* processes because at present it is not known how to achieve prepatterning differentially and organ specifically. In such a case we might rather follow the current common practice of trying various combinations of differentiation factors. However, Kumar *et al.* recently provided evidence that the signals of the mesoderm for the initiation of the development of the pancreas are instructive and able to induce the expression of the pancreatic genes even in endodermal positions that normally give rise to other organs.[72] The difference between permissive and instructive signals is critical in process design because instructive signals offer direct control over the process from the initial stage of the differentiation onward. In other words, making use of instructive signals, if they exist,

Table 1. The Interrelation of the Concepts of Developmental Biology, Engineering, and Systems Biology

	Developmental biology	*Engineering*	*Impact in process*
1	Robustness	Stability Reproducibility	Manufacture Regulatory procedures
2	Sequential stages	Observability Controllability	Direct assessment of intermediates Directed interventions
3	Path-dependence	Semiautonomy	Self-designed optimal conditions
4	Gradual/concerted advancement of tissue variables	Interdependence of tissue variables	Self-designed cell organization
5	Modularity	Uncoupled interfaces	Flexibility and cost effectiveness in product development

would assure correct control over the entire process. After induction is complete, the mesoderm layer must be removed because the bone morphogenetic proteins, produced by these cells, though needed in the beginning, later lead to differentiation toward liver.[68] The coculture could continue in another subprocess by replacing the mesodermal cell layer with another intermediate, a layer of endothelial cells that would provide a different set of distinct signals to specify a pancreatic fate.[73] Similarly, the cell sheet engineering or magnetic force–based tissue engineering mentioned previously has been used for cocultures[74,75] and their applicability could be extended to codeveloping tissues.

In another example, Viravaidya *et al.* developed a system of bioreactors modules that can accommodate several tissue cells, such as lung and liver, grown separately in each bioreactor.[76] The system has been used for studies of toxicity including absorption, distribution, metabolism, elimination, and potential toxicity of chemicals that pass through the interconnected bioreactors where the cells are performing different functions. This allows the toxic effect to be studied at the level of body physiology instead only at the tissue level.

The examples above show that use of subprocesses, making use of cocultures to restore necessary cell-to-cell interactions, where appropriate, is a feasible approach to process design.

Conclusions: Developmental Engineering

To describe the fusion of concepts from developmental biology and engineering presented above, we introduce the term "developmental engineering" to denote a methodology for *in vitro* process design from sequential subprocesses that correspond to *in vivo* developmental stages that follow a gradual and concerted progression of tissue growth and cell differentiation leading to the organization of cells into intermediate tissue forms with modular behavior.

We have analyzed the benefits of recapitulating *in vivo* developmental stages with *in vitro* processes from the process design point of view:

1. Such processes are inherently stable compared to concerted, one-step empirical schemes.
2. Critical information from developmental biology related to the sequence of developmental stages and the conditions required for each to take place is already available to inform *in vitro* process design.
3. These stages may be recapitulated separately and sequentially *in vitro* so that the process can be observed and controlled by making interventions appropriate for each particular stage based on information provided by developmental biology.
4. The primary focus should be the first stage of the process, because it establishes optimal conditions for all subsequent stages and therefore facilitates the design and implementation of such conditions to be imposed externally for each stage of the process.
5. Special consideration should be given to the concerted progression of tissue size and cell differentiation in the series of developmental stages performed *in vitro* so that the cell communication that is needed for correct spatial organization of the cells into tissue structures will be maintained.
6. The structurally organized intermediate tissue forms—modules—have internal control of their structure (robustness) and therefore can be retained and handled in more complex processes that assemble them as building blocks into several different final tissue forms without the need to re-design the whole process.

We have also seen examples of how a tissue engineer could modify the methods used currently to recapitulate phenomena of *in vivo* tissue development. This means that the technical tools that tissue engineering has already developed are sufficient to design biomimetic *in vitro* processes, if used appropriately inside the conceptual framework we have presented. The analysis of the empirical concepts of developmental biology and their significance in process design result in a common vocabulary, presented in the columns 1, 2, and 3 of Table 1, between the two fields, so that a tissue engineer will be able to focus the discussion with a developmental biologist on issues directly related to process design.

Acknowledgments

Partial support from the European project LSHM-CT-2007-037862 is gratefully acknowledged. We would like to thank Eleni Nicodemou-Lena, Jan Schrooten, Jeroen Eyckmans for numerous critical discussions on the biological aspects of the article and Andreina Elena Lanzara for the preparation of the figures and meaningful discussions for the final design of the figures, and Steve Bauer, Caitilin Hamill, Deborah Hursh, and Terrig Thomas for critical review of the manuscripts.

Disclosure Statement

No competing financial interests exist.

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Received: October 17, 2008
Accepted: June 8, 2009
Online Publication Date: July 16, 2009