Cardiovascular *in situ* tissue engineering

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Summary

Structural cardiovascular disease is responsible for a significant disease load worldwide. Although currently used replacement procedures are efficacious and change the deadly evolution of many structural cardiovascular defects, the clinically used replacement materials are sub-optimal as they are prone to progressive calcific degeneration or thromboembolic complications. Therefore, the concept of cardiovascular tissue engineering has been initiated, aiming at the fabrication of autologous cell-based constructs with growth, regeneration and remodelling potential. While most attempts have focused on the *in vitro* fabrication of native-analogous cardiovascular constructs, more recent approaches have also focused on the fabrication of more "immature" autologous cell-seeded constructs without *in vitro* tissue processing – mainly aiming at full tissue development *in vivo*. This approach, which is also referred to as the "*in situ* tissue engineering" approach, has shown promising initial success in several pre-clinical as well as initial clinical investigations. Here, we review the concept of *in situ* cardiovascular tissue engineering and systematically compare the "*in situ*" technology to the "classical" *in vitro* tissue engineering approach.

Key words: cardiovascular in situ tissue engineering; cardiovascular in vitro tissue engineering; heart valve replacement; stem cells; bone marrow

Introduction

Structural cardiovascular disease is responsible for a significant global disease load involving congenital and acquired defects. In particular, valvular heart disease represents a frequent structural problem, which leads to substantial morbidity and mortality of patients around the world [1, 2]. If the repair of diseased cardio-vascular structures is not feasible, replacement of the diseased structures is necessary resulting in more than 290,000 heart valve replacements performed annually worldwide [3], which is more than 2000 per year in Switzerland alone [4]. Importantly, these numbers are constantly increasing due to the demographic changes of Western societies. Although the currently used heart valve substitutes are efficacious and lifesaving for affected patients, they are associated with several significant limitations [1, 2]. While mechanical valve prostheses are associated with thromboembolic complications and haemolysis, bioprosthetic valves are prone to progressive calcific degeneration and thus have to be replaced 10–15 years after implantation [1–3, 5]. Beyond that, both types of substitutes have no capacity to adapt to the constantly growing and developing organism of paediatric patients, which results in the need for repeated re-operations of these young patients with congenital heart disease and are thus associated with a substantial load to morbidity and mortality [1–3, 6]. Importantly, these material-associated complications are not only limited to heart valve prostheses, but are typical of almost any of the currently used materials for the treatment of structural cardiovascular disease.

Therefore, the scientific field of cardiovascular tissue engineering has been introduced in order to overcome these considerable shortcomings via the "symbiosis" of technologies derived from engineering sciences with technologies originating in biotechnology sciences. The technology of tissue engineering aims for the fabrication of autologous cell-based, fully matured, native-analogous structures that can ultimately replace diseased structural defects [6, 7]. These autologous constructs may then hold the potential for remodelling and regeneration in order to overcome the progressive degeneration or malfunction of current prosthetic materials [1, 2, 6–8]. Ideally, these constructs should also be covered by an anti-thrombogenic surface to prevent clotting activation. However, most importantly, the "tissue engineered" constructs should

Funding / potential competing interests:
The studies have been supported by the Swiss National Science Foundation and the FP-7 Program of the European Commission.
No conflict of interest relevant to this article was reported.

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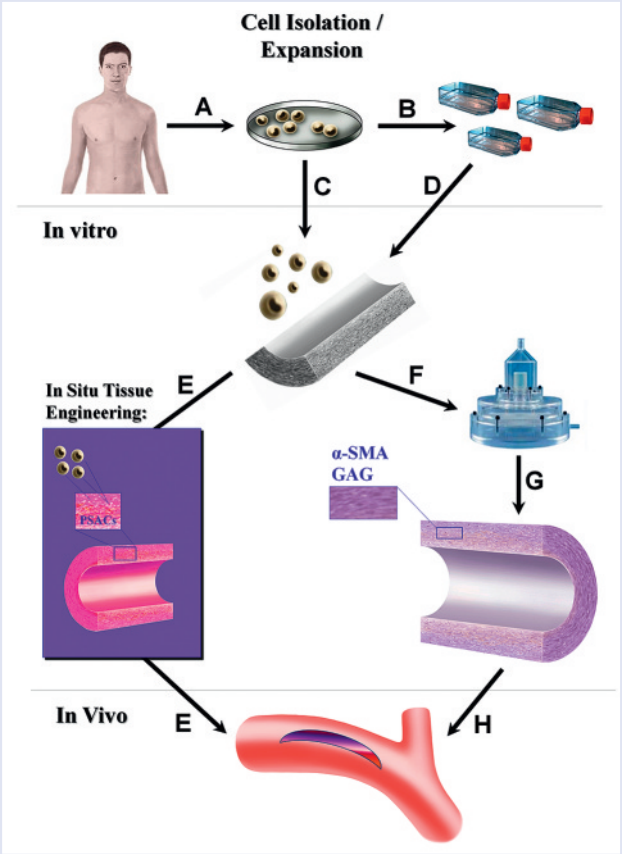
eventually also harbour the potential to adapt to the somatic growth of paediatric recipients in order to prevent life-threatening re-operations as well as to improve the morbidity and mortality of these young patients [1–3, 6–8].

Approaches to cardiovascular tissue engineering

The scientific direction of cardiovascular tissue engineering was initiated more than 15 years ago, when Shinoka and colleagues implanted a bio-engineered heart valve leaflet in a lamb model [9]. Since then this scientific approach has surmounted several experimental hurdles and has currently reached the clinical arena with the first-in-man pilot trials [10–13]. Based on these first steps, the paradigm was mainly to mimic the native structures as much as possible in order to implant a "fully matured", native-like substitute, which was considered to be the ideal replacement structure [7, 14]. This concept is also referred to as *"cardiovascular in vitro tissue engineering"* [1, 2, 6–8, 14] and comprises of the isolation of autologous cells, the *ex vivo* expansion of cells, the seeding of the expanded cells onto a fully biodegradable polymer scaffold, the *in vitro* maturation of the seeded constructs in a bioreactor system as well as their subsequent *in vivo* implantation (fig. 1). However, given the logistic complexity of this time- and cost-intensive approach, this original paradigm has somewhat shifted over the recent years [1, 2, 13], a phenomenon not limited to the field of cardiovascular tissue regeneration. Scientists have tried to circumvent the complex *ex vivo* expansion and conditioning phases to create constructs that can be implanted into the patient much sooner with a minimum of *in vitro* steps which require extensive regulatory approval [13]. Accompanied by the evolving understanding of remodelling and healing of implanted constructs *in vivo*, the paradigm of cardiovascular *"in situ tissue engineering"* was developed. This approach aimed at constructs that are based on autologous cells as well as constructs that hold growth-adaptive capacities; however, full tissue maturation was not pursued any more *in vitro* [15–18]. In contrary to the *in vitro* approach [14, 19–24], the *in situ* approach relies more on the bodies' own regenerative capacities and aims at remodelling and full tissue maturation *in vivo* (or "*in situ*") after the implantation of a bioengineered substitute material based on fully biodegradable scaffold matrices. While the role of seeded cells in the *in vitro* approach was that of tissue formation and extracellular matrix (ECM) maturation, in the *in situ* approach the autologous cells (and/or factors) attached to the scaffold matrix serve as mediators of a remodelling and healing response present after delivery of the substitute material [13, 16]. Interestingly, initial investigations found that pre-seeded cells – such as stem cells – hold the potential to further differentiate and form vascular (or valvular) tissue *in situ* [15]. However, more recent investigations revoked this concept and convincingly showed that seeded cells rather exert a "mediator" function [16, 18]. That is because these initially seeded cells secrete factors, such as "chemokines" (chemoattractive cytokines) and "growth factors", which mediate an inflammatory-driven remodelling response with attraction of immune- and vascular interstitial cells, which guide the formation of neo-tissue in the bioengineered construct *in situ* [13, 16, 18].

Figure 1
Approaches to cardiovascular tissue engineering. Autologous cells are isolated from the patient (A) and either expanded *in vitro* (B) or directly (C) seeded onto the construct (C–D). As part of the "*in situ* tissue engineering approach" the seeded construct can then be directly implanted into the patient (E; PSACs = Patient-derived Seeded Autologous Cells) or placed into a bioreactor system (F), conditioned under native-like flow and pressure conditions with formation of extracellular matrix (G, α-SMA = α-smooth muscle actin; GAG = glycosaminoglycans) and then implanted into the patient (H; *"in vitro* tissue engineering approach").



Cardiovascular *in vitro* tissue engineering: The maturation of native-like structures *in vitro*.

The concept of cardiovascular *in vitro* tissue engineering represents the initial approach to the regeneration and/or replacement of diseased cardiovascular structures. It aims at the *in vitro* (*ex vivo*) fabrication of tissues with an architecture and function as close to the native counterpart as the currently available technolo-

gies permit [1, 2, 6–8]. It is mainly stimulated by the more general concept of mimicking nature in order to replace, and therefore cure, diseased cardiovascular structures using native-analogous, but healthy, structures. Driven by this more general idea, the procedure of cardiovascular *in vitro* tissue engineering has been developed involving the following major steps: (1.) Initially, two specific autologous cell types, that is myofibroblastic as well as endothelial cells, have to be isolated from the patient using sorting and/or enzymatic instillation techniques. (2.) After *in vitro* expansion of these cells up to large quantities, myofibroblastic cells are seeded onto a fully biodegradable scaffold matrix in the shape of the desired construct (e.g., a heart valve). (3.) Following static pre-incubation, these seeded constructs are then placed into bioreactor systems, which mimic the pulsatile flow environment present in the human vascular system. This "biomimetic" conditioning induces the pre-seeded patient cells to secrete extracellular matrix, which forms the basis for developing the interstitial vascular architecture *in vitro*. (4.) These constructs are finally "sealed" using autologous endothelial cells, which renders bioengineered structures with a native-like, layered tissue composition. Importantly, these surface-mediated endothelial cells should prevent clotting activation after implantation of the constructs into the patient.

Although this technology has been extensively investigated *in vitro* [19–21] and successfully transferred to several different animal models [14, 22], it represents not only a highly complex and time consuming technology, but it also raises several regulatory as well as patient-safety issues (e.g., genetic alterations during extensive *in vitro* cell expansion), which need to be addressed prior to human clinical use.

Figure 2
PGA-P4HB composite scaffold matrix prior to cell seeding (* asterisks indicate individual leaflet structures). (Reprinted from [24]: Weber B, Emmert MY, Behr L, Schoenauer R, Brokopp C, Drögemüller C, et al. Prenatally engineered autologous amniotic fluid stem cell-based heart valves in the fetal circulation. Biomaterials. 2012;33(16):4031–43. Copyright © 2012 Elsevier, Oxford, UK, with kind permission.)

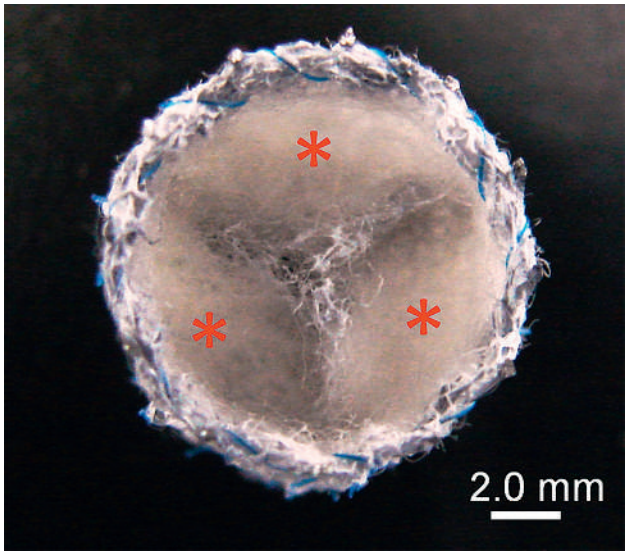


Figure 3
Morphology and marker profile of bone marrow mononuclear cell (BMNC)-derived adherent mesenchymal stem cells (MSCs). A fraction of BMNC-derived MSCs show expression of different markers that are characteristic for a specific subset of stem cells found in the bone marrow aspirate including positive expression of CD90, CD44, CD166 and CD146 (A–D) as well as negative expression of CD45 and CD34 (E–F). (Reprinted from: Weber B, Schermann J, Emmert MY, Gruenenfelder J, Verbeek R, Bracher M, et al. Injectable living marrow stromal cell-based autologous tissue engineered heart valves: first experiences with a one-step intervention in primates. Eur Heart J. 2011;32(22):2830–40 [18]. Copyright © 2012 Elsevier, reprinted with kind permission.)

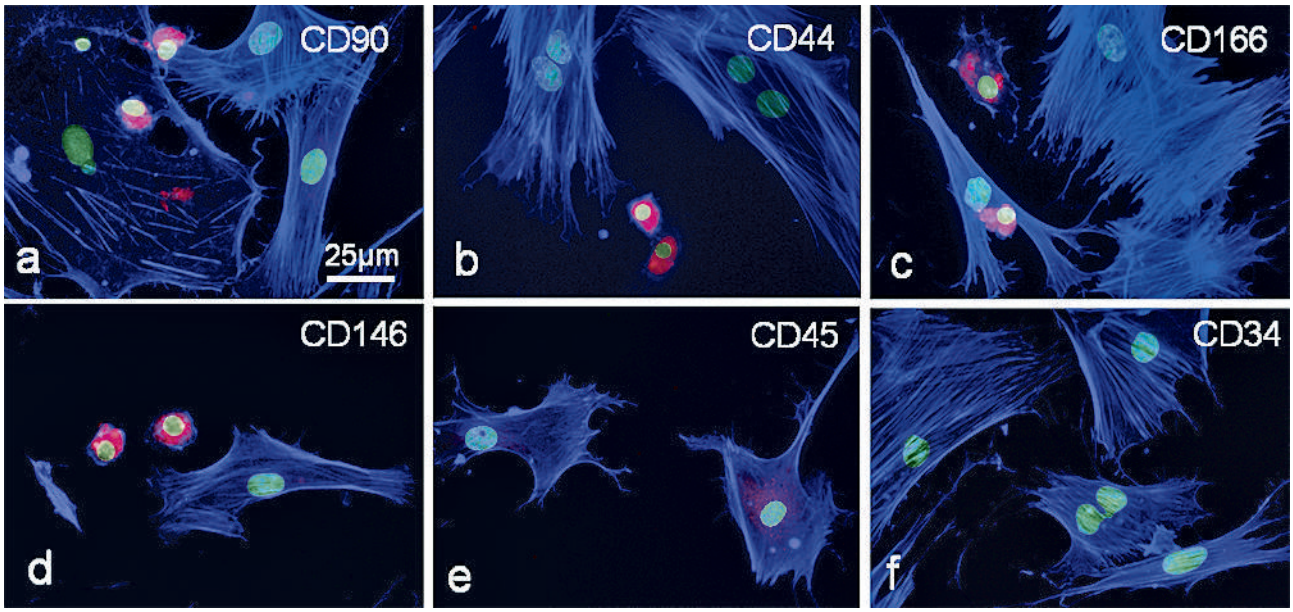
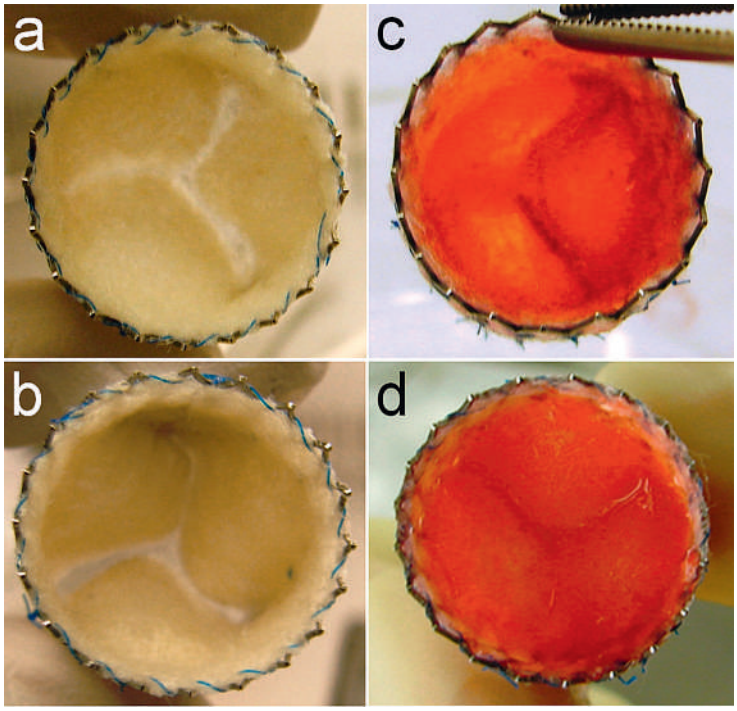


Figure 4
In situ heart valve tissue engineering based on BMNCs. Valve shaped PGA-P4HB scaffolds (A–B) are seeded with autologous BMNCs (C–D) prior to *in vivo* implantation. (Reprinted from: Weber B, Schermann J, Emmert MY, Gruenenfelder J, Verbeek R, Bracher M, et al. Injectable living marrow stromal cell-based autologous tissue engineered heart valves: first experiences with a one-step intervention in primates. Eur Heart J. 2011;32(22):2830–40 [18]. Copyright © 2012 Elsevier, reprinted with kind permission.)



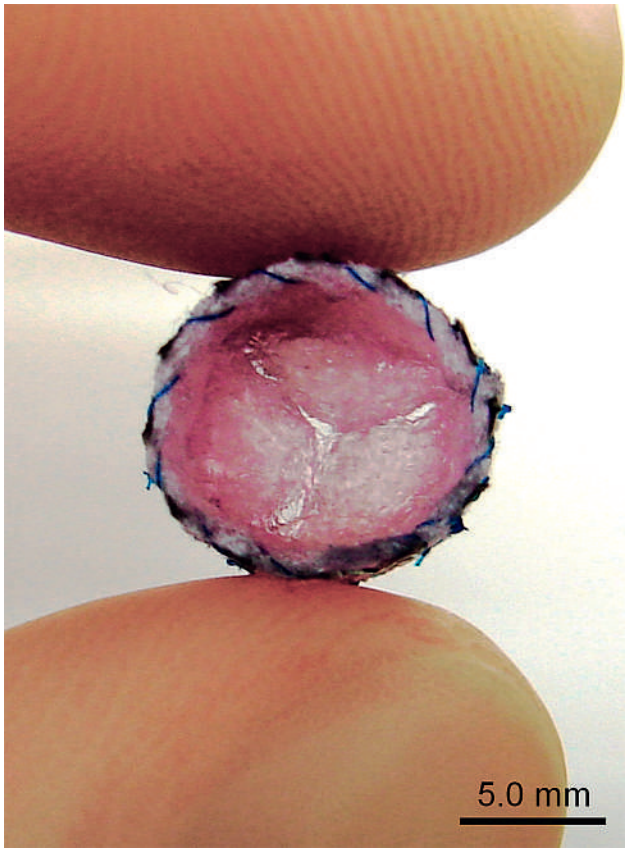
Cardiovascular *in situ* tissue engineering: Supporting the bodies' own regenerative capacities.

Stimulated by these regulatory obstacles and the tremendous need for better clinical options, in particular in paediatric patients, an alternative approach has been developed. This approach, also referred to as the *"cardiovascular in situ tissue engineering"* approach, aims to stimulate and support the bodies' own regenerative capacities in order to create the vascular tissues *in situ* (*in vivo*), rather than fabricating native-like tissues *ex vivo* [1, 2, 13, 15–18]. In principle, this approach is also composed of several steps *ex vivo*: (1.) The isolation of autologous patient cells without extensive *in vitro* cell expansion, (2.) the fabrication of a scaffold in the shape of the desired cardiovascular structure (e.g., a heart valve), and (3.) the seeding of these cells onto a biodegradable scaffold matrix in the shape of the desired construct. It was again the group of Shinoka et al., who first investigated this approach *in vivo* by seeding bone marrow mononuclear cells (BMNCs) onto biodegradable PGA-PCLLA (polyglycolic acid poly-L-lactide-ε-caprolactone) scaffolds and implanting them without any prior *in vitro* incubation [13, 15–17, 24]. Initially it was believed that after implantation a particular fraction of the scaffold-seeded BMNCs – the mesenchymal stem cells (MSCs) – may proliferate and thereby directly contribute to and/or orchestrate the formation of valvular/vascular neo-tissue *in situ* [15]. Interestingly however, more recent investigations involving different animal models and cardiovascular structures [16, 18] clearly demonstrated that the pre-seeded cells do not directly contribute to the vascular interstitium in the long run. Nevertheless, the same investigations clearly revealed that the pre-seeded cells still play a fundamental role for the remodelling and patency of the tissue engineered constructs *in vivo*. It was shown that the seeded cells secrete a series of chemokines, including MCP-1 (monocyte chemotactic protein-1) or IL-6 (interleukin 6), which themselves induce the attraction of monocytes to the site of implantation. These monocytes – also activated by the chemokines and the foreign body locus – secrete several factors, such as VEGF (vascular endothelial growth factor), which supports the development of neovascular tissues within the bioengineered construct [16]. However, the actual underlying mechanisms as well as the origin of the vascular interstitial and endothelial cells remain to be conjectural and have to be addressed in future studies. Besides BMNCs, other cell types have also been investigated as part of the *in situ* tissue engineering approach involving mesenchymal [23] as well as amniotic fluid-derived cells [24]. Although these first mechanistic studies have shed more light onto the mechanisms of cell-mediated cell attraction in tissue engineered constructs and elucidated principal mechanisms involved in cardiovascular tissue engineered construct remodelling in general, several aspects need to be addressed by future mechanistic investigations. For example: What is the origin of the cells repopulating the tissue engineered constructs? What factors are most important to induce the vascular remodelling cascade? What cell sources can be used other than BMNCs?

Experimental *in vivo* experiences: *From bench to bedside.*

Although the small animal models are highly valuable for uncovering potential mechanisms involved in the vascular remodelling of tissue engineered constructs *in situ*, the pre-clinical value of the data generated in rodent models is still limited. Therefore, several groups also implanted BMNC-based, *in situ* engineered, large diameter vascular grafts as pulmonary artery replacements into large animal models [25, 27, 28]. Importantly, in order to study the growth-adaptive potential of the *in situ* engineered BMNC-based vascular grafts, the constructs were implanted into a growing lamb model [25], which was already investigated for *in vitro* engineered vascular grafts in the pulmonary artery position by Hoerstrup et al. [26]. By using magnetic resonance angiography six months after implantation, Brennan et al. found that the mean volume of the vas-

Figure 5
In situ heart valve tissue engineering based on AFCs. Valve shaped PGA-P4HB scaffold seeded with ovine amniotic fluid-derived cells (AFCs) prior to foetal *in vivo* implantation. (Reprinted from [24]: Weber B, Emmert MY, Behr L, Schoenauer R, Brokopp C, Drögemüller C, et al. Prenatally engineered autologous amniotic fluid stem cell-based heart valves in the fetal circulation. Biomaterials. 2012;33(16):4031–43. Copyright © 2012 Elsevier, Oxford, UK, reprinted with kind permission.)



cular grafts was significantly increased when compared to initial volumetric values [25]. Together with the finding of high expression values of venous differentiation markers, these results clearly suggest a growth-adaptive behaviour of *in situ* engineered vascular grafts based on BMNCs. Although these kinds of growth-adaptive, tissue engineered vascular grafts would offer novel treatment options for many paediatric patients, the clinical introduction of tissue engineered heart valves would also certainly bring a fundamental improvement for an even broader patient population [11]. However, as heart valves represent native structures with a much more sophisticated micro-architecture and biomechanical function when compared to vascular grafts, the *in vitro* and *in situ* generation of native-like valves represents a major challenge. By using the BMNC-based tissue engineering approach our group was the first to fabricate *in situ* engineered heart valves that were then implanted into a clinically relevant large animal model [18, 26, 27] raising hope that a clinical solution is within our grasp.

Initial clinical experiences: The first steps to routine use?

Based on the plethora of pre-clinical studies [9, 14–16, 18, 22, 24–28], first groups entered the clinical phase by using BMNC-based *in situ* engineered vascular grafts for total cavopulmonary connection (Fontan) procedures [10–12, 17]. In the first 25 patients in Japan no graft-related mortality was observed. However, in several patients a partial stenosis of the tissue engineered graft lumen was found, which required interventional dilation [10]. Based on these first encouraging clinical experiences, Breuer and colleagues recently entered the clinical phase in the United States using the same *in situ* engineered vascular grafts [11, 12].

The future of cardiovascular *in situ* tissue engineering: *A technology on the cusp of routine clinical use?*

When the concept of cardiovascular tissue engineering was initiated more than a decade ago [7], the primary goal was the *in vitro* fabrication of replacement structures similar to the native counterpart that would allow for one-to-one replacement structures with identical micro- and macro-architecture. Native heart valve leaflets were defined as the gold standard – and this standard had to be copied [1, 2, 14]. Several groups worldwide were primarily aiming at the fabrication of cardiovascular structures mimicking native tissues in histo-morphologic and macroscopic appearance as well as biomechanical behaviour [29].

However, in recent years an alternative procedure of cardiovascular tissue engineering has evolved relying more on the body's own regenerative capacities and supporting the organism to remodel implanted constructs *in situ*. While the *in vitro* approach required time- and cost-intensive *ex vivo* cell expansion and bioreactor phases, the *in situ* approach offers ready-to-go implantable constructs within a single surgical intervention. In addition, it is also an approach that holds a major non-medical advantage over the *in vitro* concept: The regulatory efforts for the *in situ* tissue engineering procedure, which does not involve any (significant) *ex vivo* cell expansion and processing, does certainly not require the same extensive certification processes associated with *ex vivo* cell culture and expansion procedures, which are foreseen by national and international regulatory authorities. These (regulatory) differences offer a possible explanation for the faster clinical introduction and the recent "story of success" of the *in situ* approach. However, in the future the *in vitro* approach will also ultimately make its way into the clinical arena and this is the time when the clinical results will tell which is the more promising concept for the long term treatment of structural cardiovascular disease.

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