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Environmental regulation of valvulogenesis: implications for tissue engineering

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Summary

Ongoing research efforts aim at improving the creation of tissue-engineered heart valves for in vivo systemic application. Hence, in vitro studies concentrate on optimising culture protocols incorporating biological as well as biophysical stimuli for tissue development. Important lessons can be drawn from valvulogenesis to mimic natural valve development in vitro. Here, we review the up-to-date status of valvulogenesis, focussing on the biomolecular and biophysical regulation of semilunar valve development. In addition, we discuss potential benefits of incorporating concepts derived from valvulogenesis, as well as alternative approaches, in tissue-engineering protocols, to improve in vitro valve development. The combined efforts from clinicians, cell biologists and engineers are required to implement and evaluate these approaches to achieve optimised protocols for heart-valve tissue engineering.

1. Introduction

To date, prostheses used in valve-replacement therapies suffer from limitations, mostly related to the lack of living tissue [1]. Tissue engineering aims to reduce these limitations by creating living heart valve replacements that are able to grow and adapt to changes in the physiological environment (Fig. 1). In its most frequent strategy, tissue engineering involves the isolation of cells, in vitro cell expansion, cell seeding on carriers (scaffolds) and mechanical conditioning in bioreactors, prior to implantation (Fig. 2(A)). Although initial feasibility studies showed successful application of tissue engineered heart valve constructs in the pulmonary position in sheep [2], the clinically more relevant application in the aortic position has not yet been achieved. This is mainly due to difficulties in the production of strong, flexible and durable tissue that can withstand systemic pressures for prolonged periods of time. These characteristics are strongly related to the microscopic and macroscopic valvular architecture [1], motivating the numerous efforts to induce this architecture in vitro.

The aortic valve consists of the aortic root comprising three sinuses and three flexible semilunar cusps attached to a ring of fibrous tissue, called the annulus. The cusps are the main load-bearing parts of the valves and they consist of three layers: the collagen-rich fibrosa on the aortic side, the

Table 1. Summary of recommendations and suggestions for future research on heart valve tissue engineering, presented in this paper.

Abbreviations and acronyms: α-SMA, α-Smooth muscle actin; AV valves, Atrio-ventricular valves; BMPs, Bone morphogenetic proteins; ECM, Extracellular matrix; EGF, Epidermal growth factor; EMT, Endocardial-to-mesenchymal transition; EPCs, Endothelial progenitor cells; EPDCs, Epicardial-derived cells; FGF, Fibroblast growth factor; GAGs, Glycosaminoglycans; GTP, Good tissue practice; HA, Hyaluronan; HB-EGF, Heparin-binding epidermal-like growth factor; HSCs, Hematopoietic stem cells; miRNAs, MicroRNA, or micro ribonucleic acids; OFT valves, Outflow tract valves; P4HB, Poly-4-hydroxybutyrate; PGA, Poly-glycolic acid; TGF-β, Transforming growth factor-β; VEGF, Vascular endothelial growth factor-A; VICs, Valvular interstitial cells.

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Use cell sources that can resemble valvular interstitial cells and search for alternative cell sources, like EPDCs or blood-derived fibrocytes
Investigate if matrix proteins can be used to improve the cellular niche on artificial scaffolds, like peristin
Explore new combinations of growth factors to enhance tissue maturation and reduce compaction. Examples are new combinations with BMPs or TGF-β
aim for tissue-engineering protocols based on serum-free culture media, or autologous serum alternatives
Mimic physiological mechanical stimuli for tissue conditioning and allow opening and closing of cusps
Apply techniques derived from other tissue engineering studies, like combined culture of different cells in specialised media or use of microRNAs
intermediate spongiosa, mainly containing glycosaminoglycans (GAGs) and the elastin-rich ventricularis on the ventricular side. In mature valves, two main cell types have been identified: valvular interstitial cells (VICs) are dispersed in all three tissue layers, and endothelial cells cover the cusps (Fig. 3). VICs are thought to be responsible for maintenance and remodelling of the tissue, in particular, of the extracellular matrix (ECM) and thus for the durability and adaptive capacity of the cusps.

The architecture of the aortic root and cusps is organised such that it reduces the mechanical stresses on the valve. The root withstands continuous deformations, whereas the typical fibre architecture of the cusps transmits applied forces from the cusps to the annulus and the aortic wall [3]. Scientific progress has not yet resulted in tissue-engineered prostheses that mimic the adult tri-layered natural aortic valve architecture in vitro, but it is likely that this architecture is achieved in vivo, as was demonstrated for engineered valves placed in the pulmonary position [2].

As suggested by Butcher and co-authors in 2007 and 2008, the embryonic development of heart valves, or valvulogenesis, can be used as a template by tissue engineers to optimise reconstruction of native aortic valves [4,5]. This has resulted in suggestions for new tissue-engineering strategies, such as the use of growth factors and specific mechanical...
stabilization protocols. However, there are additional, perhaps more challenging, lessons available from valvulogenesis that can be incorporated. In the present review, we evaluate up-to-date knowledge of human aortic valve development, integrating the roles of the biomolecular and biomechanical environments during pre- and postnatal morphogenesis of the aortic valve. Next, we provide suggestions for aortic valve tissue-engineering protocols derived from our analysis, with specific emphasis on cell sources, growth factor cocktails and serum alternatives, matrix proteins and mechanical stimulation. Lastly, we discuss alternative strategies that have not yet been studied in relation to valvulogenesis.

2. Natural valve development

2.1. Initiation of embryonic valve formation

Heart valve development starts with formation of the cardiac jelly at embryonic day 8.5 (E8.5) in mice, and in the middle of the third week after conception in humans. The cardiac jelly is formed by myocardial secretion of GAGs, such as hyaluronan (HA) [6]. This initial synthesis of ECM is thought to be stimulated by bone morphogenetic protein-4 (BMP4) in the outflow tract (OFT) at E8.5 (Fig. 4(A)) [4]. This is followed by cardiac cushion formation at E9 in mice and day 20 in humans, through a process known as endocardial-to-mesenchymal transition (EMT, Fig. 4(B)) [4,7]. During this process, endocardial cells become activated, lose their cell-adhesion molecules, migrate into the cardiac jelly and transform into mesenchymal cells [4,8]. Both cardiac jelly formation and induction of EMT rely on the presence of matrix proteins that stimulate the invasion of mesenchymal cells into matrix and initialise transformation [9,10].

The human embryonic heart starts to contract at 65 beats per minute (bpm) at the beginning of the fourth week after gestation and creates unidirectional flow few days later [11,12]. Thus, EMT starts under (mild) cyclic shear stresses, while transvalvular pressure is believed to be zero [12]. Although cardiac contraction and EMT start at the same time, the relative and combined contribution of haemodynamics and growth factors to valvulogenesis at this stage remain to be elucidated. Shear forces are likely to contribute to cardiac cushion formation and EMT, but they are not indispensable in the presence of specific growth factors [13,14] and might hence be relevant for fine-tuning morphogenesis.

2.2. Regulation of EMT

The most frequently described molecular processes involved in the initiation of EMT are down-regulation of vascular endothelial growth factor (VEGF) [7,15] and up-regulation of BMP2 [4,16,17]. Interestingly, the role of BMP2 in EMT was primarily identified in atrio-ventricular (AV) valves, but not in OFT valves. At this location, BMP4 has been suggested as an alternative [18,19], but the exact mechanism of EMT initiation in OFT valves is still not clear. The onset of EMT in OFT valves is followed by (de)activation of complex signalling networks by growth factors that have been described in more detail by others and which are beyond the scope of this article [4,19,20]. To mention, some of the relevant growth factors involved are transforming growth factor (TGF)-ß2 and ß3, fibroblast growth factor (FGF)-4 [21–23], BMP4 [19], epidermal growth factor (EGF) [19] and heparin-binding epidermal-like growth factor (HB-EGF) [24]. These factors can influence proliferation, migration into the matrix or differentiation of endocardial cells into mesenchyme (Fig. 4(B)).

In chick AV valves, transition of cardiac jelly into cardiac cushions coincides with tissue stiffening and increased tissue thickness due to higher cell mass and collagen deposition [14]. This leads to preliminary valvular functioning of the cushions in the regulation of blood flow. Mathematical models of embryonic haemodynamics suggest that subsequent "leafing" of the cushion in direction of the flow results from differences in flow patterns (laminar or vortex) up- and downstream of the cushions (Fig. 4(C)) [25]. The primitive cusps remain short and thick up to the seventh week of human development, while the heart rate increases to 180 bpm and arterial blood pressure stays below 1 kPa (10 mm Hg) [11,12].

Together, these findings indicate that biomolecular factors, such as growth factors and transcription factors, as well as haemodynamic forces, contribute to the regulation of EMT and early morphogenesis of valvular leaflets.

2.3. The elongation phase

Valvular maturation starts in humans at embryonic day 52 (E14.5 in mice) and occurs in several stages: mesenchymal proliferation first causes a phase of cusp elongation, which is followed by differentiation of the cells and remodelling of the ECM [26]. During the elongation phase, FGF4 stimulates proliferation of distal mesenchymal cells [4,23], while TGF-ß2, HB-EGF and EGF are thought to act as inhibitors of this process to prevent hyperplasia (Fig. 4(D)) [4,19,24,27].

During the differentiation phase, proliferation stops and signalling cascades involved in EMT are partially reversed
Further, BMP2 is expressed in the OFT cushions at E12.5, potentially inducing valve maturation through the expression of the matricellular component periostin (Fig. 4(D)) [28]. Periostin has been located in both OFT and AV valve cusps where it plays an important role in valve maturation through stimulation of mesenchyme invasion and production and compaction of matrix [21,28,29]. The differentiation phase continues into the second trimester of human development and coincides with the expression of growth factors, such as FGF4 and BMP2, as potential regulators of mesenchymal proliferation and matrix remodelling.

2.4. Final stage of valvular maturation: pre- and postnatal remodelling

During the last phase of valvular development, additional matrix components are formed and ultimately arranged into the tri-layered architecture observed in mature valves. These changes are depicted in Fig. 4(E)–(G) and include:

1. A decrease in cell proliferation from 30% in the endocardial cushions, to 5% prenatally and 1% in the postnatal valve [30].
2. Up-regulation of \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA) by interstitial cells in mouse AV valves between E15.5 and E18.5, associated with activated myofibroblasts and contractile properties [31,32]. In mature, postnatal valves, \( \alpha \)-SMA-expression is lost, indicating differentiation towards the final, quiescent fibroblast phenotype [33].
3. In humans, the elastin content increases from 1% during the first part of the second trimester (14–19 weeks after gestation) to 4% at the third trimester (20–39 weeks after gestation) and 25% postnatally [31].
4. Where collagen exists only in small, disorganised fibres in earlier stages of valvulogenesis, collagen content reaches a maximum during the maturation period. After this, only the thickness and orientation of collagen fibres change: the frequency of thin collagen fibres decreases from 72% in the second trimester to 24% in the third,
while the frequency of thick fibres increases from 5% to 30%. During postnatal remodelling, mature collagen fibres compose 47% of the total surface area [31]. This maturation could be driven by changed haemodynamic loads, such as high shear stresses and increased transvalvular pressures during this period.

(5) The heart rate remains high at 155–160 bpm at 20 weeks after gestation and only gradually decreases to 140 bpm prior to labour [12], followed by a more gradual decrease to 70 bpm at mature age.

(6) Aortic blood pressure increases from 4 kPa (28 mm Hg), to 6 kPa (45 mm Hg) at 40 weeks of gestation [34]. Postnatally, the shunts between the pulmonary and systemic circulation close and the aortic pressure increases to 9/5 kPa (70/40 mm Hg) after birth, slowly rising to 16/12 kPa (120/90 mm Hg) in adults [31].

(7) Shear stresses in adults are estimated to be 8.0 Pa on the ventricular side of the cusps in adults, and between −0.8 and 1.0 Pa in the sinuses on the aortic side [35]. These different shear stresses are thought to induce differences in matrix depositions on different sides of the cusp [20].

The mechanisms underlying the transduction of mechanical stimuli to cellular behaviour are still only partly known but may involve the inhibition of BMP2 signalling [36]. In addition, TGF-β3 is thought to increase collagen production and both BMP4 and TGF-β3 have been suggested to play a role in semilunar valve maturation [37,38]. Although mechanical stimuli may become more important for regulation of valvulogenesis in maturing valves, it would again be interesting to determine the most favourable combination of growth factors and mechanical stimuli, to optimise valvular tissue development and maturation in tissue-engineering strategies.

3. Implications of valvulogenesis for tissue engineering

In the following sections, different steps of currently used in vitro heart-valve tissue engineering protocols will be analysed with respect to potential improvements derived from valvulogenesis.

3.1. Available cell sources to mimic valvulogenesis

Valvular cells are predominantly endocardial-derived mesenchymal cells and endothelial cells [39]. Vascular myofibroblasts have been suggested as cells of mesenchymal origin for use in heart-valve tissue engineering [31,40,41]. They can be easily harvested from vascular grafts and allow concomitant isolation of vascular endothelial cells as a source for valvular endothelial cells [40,42,43]. However, relatively invasive procedures are required to obtain venous grafts and the grafts may not be available from all patients.

Alternatively, adipose- or bone marrow-derived mesenchymal cells are used for heart-valve tissue engineering [44–47]. These cells can be differentiated towards endothelial cells and thereby allow formation of a valve construct with both mesenchymal cells and endothelial cells from a single biopsy [48]. Limitations of these cells might be the invasive harvest procedure and their susceptibility for osteogenic differentiation, which has been shown repetitively for mesenchymal stem cells as well as for haematopoietic stem cells (HSC) [49]. It has even been suggested that the natural ongoing replacement of VICs with HSC in vivo can lead to valvular pathologies in time [49]. Thus, future studies should determine whether valvular constructs produced with mesenchymal progenitors are prone to calcification in vivo.

Recently, studies have focussed on the use of endothelial progenitor cells (EPCs) circulating in the bloodstream. An advantage of these cells is the relatively non-invasive way in which they can be obtained. Moreover, EPCs can differentiate into mature endothelial cells and are able to undergo transdifferentiation into mesenchymal cells [50], which mimics EMT processes as witnessed during valvulogenesis. EPC-like cells have already been used for reseeding of decellularised allograft pulmonary valves [51] and synthetic poly-glycolic acid (PGA) scaffolds [52], while future applications aim at in vivo recellularisation of implanted scaffolds [53].

Although EPC characterisation is far from complete, earlier in vitro work indicated that the EPC population comprises at least two different cell types referred to as ‘early’ and ‘late’ EPCs [54]. These different EPC populations produce different pro-inflammatory cytokines that could limit their use for heart-valve tissue engineering. Early EPCs produce tissue factor, for instance, which could increase the risk of thrombosis on the graft surface, whereas late EPCs produce monocyte chemoattract protein-1, a pro-inflammatory cytokine that may stimulate invasion of inflammatory cells [55]. It should be further investigated whether the production of these cytokines limits the application of EPCs for tissue engineering and if pharmaceutical treatment is required to reduce their release [55]. Thus, EPCs are clinically accessible in a relatively non-invasive manner, but their true potential for clinical applications remains to be determined.

Another interesting cell source are the cells derived from the proepicardium, known as epicardial-derived cells (EPDCs), which give rise to cardiac fibroblasts and have a speculated role in valve development [56]. EPDCs and cardiac fibroblasts are able to produce periostin and collagen type I, relevant for heart-valve tissue formation [56]. Future studies should elucidate if EPDCs or cardiac fibroblasts can be easily isolated from adult patients and if they can be useful for heart-valve tissue engineering purposes.

3.2. Use of matrix proteins in heart-valve tissue engineering

Despite the relevance of matrix proteins in the regulation of valvular interstitial cell behaviour, they are generally not used in tissue-engineering protocols. The application of decellularised allografts to preserve the highly specialised valvular matrix and architecture may favour this natural cellular environment, or niche, but low availability of donor valves limits the use of this technique [41,51,57,58]. Scaffolds for tissue engineering are predominantly made out of synthetic polymers such as PGA or natural polymers such as fibrin [50,52,59]. An extracellular matrix niche that
resembles the matrix environment during valvulogenesis may be obtained by coating of synthetic scaffolds with defined matrix proteins, to manipulate cell behaviour and ultimately, tissue properties.

An interesting candidate protein is periostin, which might be involved in maturation of the valve and modulation of cellular phenotype [21,28,29,38]. The lack of periostin has even been related to calcific aortic valve disease, stressing its relevance for normal valve development [60]. Incorporation of periostin in collagen gels showed beneficial effects on invasion of cells into the gels and enhanced collagen remodelling, as indicated by condensation and compaction [21]. Care should be taken, however, to control matrix compaction due to remodelling, since it may lead to retraction of valvular cusps and impaired function of engineered valves. Keeping the delicate balance between healthy and excessive compaction will be an important problem to overcome in the coming years [61].

3.3. Tailored culture media for specific stages of heart-valve tissue engineering

The previous sections have shown that multiple growth factors are expressed in spatiotemporal patterns during valvulogenesis. In the last few years, the first articles on heart-valve tissue engineering have been published using different growth factors to improve tissue properties [45,62,63]. However, research has focussed primarily on the growth factors most commonly used in cell culture, such as FGF2, EGF and TGF-β. Although the use of FGF2 and EGF can be useful for tissue-engineering strategies in general, their family members FGF4 and HB-EGF are specifically beneficial for aortic valve development. Moreover, it has been shown that BMPs are indispensable for valvulogenesis, but they are not reported for heart-valve tissue engineering purposes. This might be explained by their role in osteogenic differentiation [64] and the development of diseased and stenotic valves in vivo [65–67]. However, HB-EGF signalling was suggested to interfere with BMP-dependent signalling pathways [68,69] and to reduce chondrogenic differentiation [27]. Thus, using BMPs in combination with other growth factors, as occurs during valvulogenesis, could result in protection from osteogenesis during tissue culture. Likewise, FGF2 can disrupt TGF-β-mediated myofibroblast activation, thereby preventing unbalanced extracellular matrix production [70]. Exploring the addition of less conservative growth factor cocktails to culture media should therefore be considered when optimising tissue-engineering strategies.

Such cocktails can be sequentially added in vitro based on their temporal presence and regulatory roles in valvulogenesis. The first step in general tissue-engineering paradigms is ex vivo expansion of endothelial and/or mesenchymal cells, followed by cell seeding and tissue culture. Proliferation of endothelial-like cells can be induced in vitro using VEGF-containing media [7,71]. Next, these cells can be seeded as an endothelial surface layer on tissue-engineered constructs or allografts. As a source of mesenchymal cells, EPCs can be transdifferentiated by TGF-β-mediated induction of EMT, as seen in natural valvular development [22,72]. If mesenchymal target cells are directly isolated, like venous myofibroblasts or bone marrow-derived cells, proliferation can perhaps be stimulated by the addition of FGF4 to the culture media [23,73]. When sufficient numbers of cells have been acquired, cells can be seeded on scaffolds and growth factors can be added to induce tissue formation. Growth factors that can enhance tissue formation, according to valvulogenesis, are BMPs and TGF-β [26,28,38,64]. To prevent stiffening of the tissue constructs by fibrosis or osteogenesis, we hypothesise to add HB-EGF and FGF2, respectively. Further experiments are required to determine at which time points growth factors should be added, at which concentrations, and whether they should be added in bursts or via gradual release. Eventually, this should lead to tailored culture media for different stages in tissue engineering to mimic natural valvular development more closely.

3.4. Serum-free or autologous culture conditions?

The Food and Drug Administration (FDA) has formulated Good Tissue Practice-guidelines (GTP) for the use of cell-based products in clinical application to prevent the introduction of animal components and contamination in patients [74]. Possible complications might be rejection of the implanted construct through an immune response against animal proteins, as has been shown for cells expanded in bovine serum [75,76]. Although this may not seem relevant for in vitro bench studies, these guidelines may affect tissue properties, for example, when using serum alternatives to prevent cross-species contamination due to non-autologous serum [43]. As an alternative, serum-free culture media are becoming commercially available. Use of specialised serum-free culture media, with growth factor additives optimised for each different step of heart-valve tissue engineering, could reduce expansion time, induce tissue formation and allow clinical application. It should be considered, however, that cellular behaviour in these highly defined culture media most likely does not reflect cellular behaviour after implantation. Exposure to blood subjects the cells to completely different concentrations of humoral factors such as growth factors, cytokines and hormones, and leads to different cellular responses. A promising alternative was studied by our group through the replacement of bovine serum with autologous platelet-lysate [43] or serum as a source for nutrients and growth factors. Supplementing autologous serum alternatives with growth factors could stimulate proliferation and tissue formation of cells, while mimicking in vivo circumstances and valvulogenesis more closely. Future studies need to address whether fully autologous culture of heart valves, hence using the patient’s plasma or serum and cells, can be achieved and allows clinical application of heart-valve tissue engineering.

3.5. Mechanical conditioning

As for most load-bearing tissues, the concept of in vitro mechanical conditioning, for example, in bioreactors, to improve functional tissue architecture and properties is also well acknowledged for heart-valve tissue engineering and has been confirmed in various in vitro studies [5,77–79]. The most important mechanical stimuli during heart-valve development are shear stress and (transvalvular) pressure. Involvement of shear stresses has been suggested in
processes directing EMT, valvular morphogenesis and stratification [20,25]. Further, analysis of collagen content and collagen ultrastructure has shown that the increase in transvalvular pressure and/or shear stresses during valvulogenesis leads to thickening of the lamina fibrosa and to strain-related changes in orientation of fibres [80]. Similar strain-related changes can be observed when comparing adult pulmonary and aortic valve cusps. The collagen-fibre architecture of aortic cusps is modestly, but significantly, different to accommodate for the higher transvalvular pressure loads during diastole [81]. For aortic heart-valve tissue engineering, it is therefore beneficial to mimic native-like fibre architecture and mechanical functioning by applying (increased) transvalvular pressures during culture.

Since collagen fibres are the most important load-bearing structures in heart valves, collagen content and fibre thickness have been used as a marker for mechanical properties [1,82]. However, maturation of collagen fibres through cross-link formation is also important. Balguid and co-authors have demonstrated that the application of cyclic strains enhanced collagen cross-link formation in engineered tissues, as compared to unloaded controls. Cross-link density was further found to be highly correlated to tissue strength in vitro, as well as in native aortic valve leaflets [82]. Cross-links are essential for tensile strength and mechanical stability of the collagen microfibrils and protect the microfibrils against enzymatic degradation [83]. Mechanical stimulation to improve cross-link density can therefore be considered an important additional regulator of tissue maturation and mechanical properties in engineered valves, next to collagen content and fibre organisation [84].

Lastly, valvulogenesis suggests that physical stimuli are important for the formation of the tri-layered architecture of aortic valve cusps. Mechanisms behind stratification likely involve different flow conditions on each side of the cusp, resulting in differentially expressed genetic profiles by endothelial and matrix-producing cells [4,20,85]. Ultimately, these differences might influence expression of proteins involved in matrix deposition or degradation, leading to specific niches favourable for development of specific matrix proteins [20,85]. At this moment, there are only few studies available that investigate the synergistic or inhibitory effects of mechanical stimulation on growth factor and protease expression [77,78]. In the future, however, such studies might shed more light on the formation of specialised collagen and elastin layers and indicate whether growth factors and mechanical stimulation should be applied together or separately.

The above studies show that mechanical conditioning of engineered valves in bioreactors is indispensable prior to implantation and that cyclic, strain-based conditioning is beneficial for the production of strong aortic valves. These protocols, however, are only partially related to the haemodynamic processes during valvulogenesis. Potential mechanical regulation of, for example, EMT and tissue stratification, as well as interaction with biomolecular regulation of these processes, is hardly studied. This should be the subject of future studies and include the use of bioreactors that allow for controlled application of different flow and pressure profiles to opening and closing valves.

4. Discussion and future perspectives

The aim of this overview is to provide an up-to-date analysis of natural aortic valve development, the regulatory roles of biomolecular and biomechanical stimuli during different stages of valvulogenesis and their relevance for optimising heart-valve tissue engineering strategies. Several concepts from valvulogenesis have already been applied in tissue engineering, but new and, perhaps, more challenging strategies can be suggested from our analysis. If one desires to mimic valvulogenesis as closely as possible, we suggest the combined or subsequent application of:

1. Progenitor cell sources that may reconstitute both valvular endothelial and matrix-producing interstitial cells. However, alternative sources such as cells directly derived from the heart, such as cardiac fibroblasts or EPDCs, should also be considered, because they produce matrix proteins relevant for heart valve and are used to a mechanically dynamic environment.

2. Addition of different growth factors relevant for valvulogenesis to culture media, such as BMPs and FGF4: this can potentially reduce expansion time and increase tissue formation in vitro. BMPs, for instance, are indispensable for heart-valve formation, but are not used for valve tissue engineering. Specific combinations of growth factors in cocktails might solve undesired side effects of the use of these BMPs.

3. The use of specific matrix proteins such as periostin in scaffolds: natural valve development shows that matrix proteins can influence cellular behaviour and using them could lead to tissue formation that resembles heart valves more closely.

4. Mechanical stimulation to enhance collagen formation, organisation and cross-link formation to improve tissue strength during valvular maturation. In addition, valvulogenesis suggests that shear stress and transvalvular pressure contribute to other stages of valvular development and should therefore be considered for optimising tissue-engineering strategies.

5. Alternatives for the use of animal-derived products in culture media required to expand cells in vitro. Highly specific culture media can be developed to mimic different stages of valvulogenesis. Alternatively, the use of media with autologous serum or plasma combined with growth factor cocktails, or as an alternative source for growth factors, can be investigated.

Our suggestions are incorporated in Fig. 2(B). Complete mimicking of the complex and highly organised valve and copying all natural factors involved, such as cells, growth factor combinations and mechanical conditioning, is a highly challenging and unrealistic approach and may likely be unnecessary. To determine the most relevant processes and regulators, we suggest to compare stages in tissue-engineering strategies with developmental stages in valvulogenesis and to evaluate the effects of identified regulators of valvulogenesis on tissue-engineering outcomes. Obviously, this approach can and should be combined with new emerging strategies that are not well studied or understood in natural valve development.
An example of these new strategies is the recent successful clinical application of a tissue-engineered airway [86]. In this study, a layered construct was created using two different autologous cell types and specialised culture media for each individual cell type. This concept can also be considered for heart-valve tissue engineering. For instance, the attachment of TGF-β to scaffolds within the construct to induce EMT can be combined with endothelial growth promoted via VEGF on the outside.

Second, several groups have reported the presence of fibroblast progenitor cells, called fibrocytes, in the blood [87]. These cells have been suggested to be involved in the repair of damaged tissues, including heart valves, and are therefore a potentially attractive source of mesenchymal cells for heart-valve tissue engineering [88]. However, future studies should point out if these cells can be isolated, expanded and used for heart-valve tissue engineering [88].

Another example are microRNAs (miRNAs), which have been shown to block synthesis of specific target proteins in vivo [89]. MiRNAs are suggested to be important regulators of tissue-engineered aortic valves within reach [90–92].

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