5 DISCUSSION

The application of patches or grafts in cardiovascular surgery and particularly in pediatric cardiac surgery is a widely accepted surgical technique for repair or reconstruction of cardiovascular structures [6, 8]. Currently, the patch materials used clinically are limited to prosthetic materials, autologous pericardium and allogenic or xenogenic (glutaraldehyde-fixed) pericardium [8]. All materials used for patch repair or reconstruction have certain limitations such as inability to grow, repair, and remodel, which leads to aneurysm formation in patch aortoplasty, inelasticity of the prosthetic materials and an increased risk of hemolysis induced by contact of the blood with the prosthetic materials [94, 95]. Although the mechanisms of aneurysm formation after patch repair are not completely known, the inelasticity of the glutaraldehyde-fixed pericardium and of the prosthetic material may be a key factor. Aneurysm formation and the inability of patches to grow or to remodel are important sources of morbidity and mortality after repair or reconstruction of cardiovascular structures, the latter imposing substantial problems specifically to the pediatric cardiac surgery patient population [42, 43]. Pediatric patients often have to undergo multiple reconstructive surgical procedures, so that the use of autologous pericardium for repair or reconstruction of congenital defects is also limited [30].

Calcification is the most frequent cause of clinical failure of bioprosthetic tissues [40, 41]. The allogenic pericardiac tissues are subject to increase calcification after implantation in children, and scarcity remains a significant problem for pediatric patients [36-38]. Xenograft pericardium tissue patches are superior in blood and tissue compatibility, but are inferior in *in vivo* durability, mainly due to calcification.

Prosthetic replacements lack growth potential and can become obstructed by tissue ingrowth or calcification, leading to the need for multiple replacements [44, 45]. Furthermore, all synthetic material is thrombogenic and, after implantation, the risk of thrombembolic and infectious complications potentially increases [46].

The optimal cardiovascular patch materials should be characterized by long durability, absence of thrombogenicity, resistance to infections, lack of antigenicity, potential for growth and ability to prevent patch dilatation and thinning. To meet these requirements, our laboratory applies the principles of tissue engineering in an attempt to develop viable

autologous replacements for deficient cardiovascular structures. The possibility of using living constructs for tissue replacement offers numerous potential advantages over currently used implants, including integration into the surrounding tissue, non-thrombogenicity and the ability to grow, repair and remodel which should finally lead to better long-term results [49].

One approach in tissue engineering is to seed autologous cells onto biodegradable polymers and to establish optimal *in vitro* conditions to guide tissue development and to create cell-polymer constructs with a high degree of maturity prior to implantation. Without *in vitro* seeding and tissue cultivation, host fibroblasts will colonize the biodegradable graft. After bioabsorption of the scaffold, only a scarred patch will replace the revascularization structures. Fibrous tissue lacks elasticity and can thin and dilate because of high circulatory pressure. In order to fabricate such constructs *in vitro* it has been widely confirmed that a dynamic tissue environment significantly enhances tissue maturation and mechanical properties [70, 96, 97]. Pulsatile flow or fluid dynamics have a well-known impact on cell morphology [98], proliferation [99], and composition of extracellular matrix that might lead to surgically feasible tissue engineered constructs suitable for implantation [100-102]. Therefore, we have developed a new pulsatile flow system that provides biochemical and biomechanical signals to regulate autologous patch tissue development *in vitro*. Using this newly developed bioreactor system we have fabricated a viable patch tissue construct with ovine cells. Although our early *in vitro* results appear promising, we do not yet know whether it is possible to fabricate implantable human vascular tissue in our newly developed *in vitro* system. The tissue engineered patches have to consist of functional human tissue at the time point of implantation. Therefore, this study focused on using human vascular cells to fabricate patch tissue.

In this study, we demonstrated the feasibility of seeding human SMCs onto a biodegradable scaffold to construct a TE patch for potential human implantation. We selected cultured vascular smooth muscle cells (SMCs) obtained from human pediatric ascending aorta to seed the P4HB polymers because SMCs can be harvested from a number of sources in the donor. In addition, the SMCs are readily obtainable from patients and easily cultured. Studies have shown that SMCs also stimulate angiogenesis in the graft [103], which is necessary for its long-term survival. During the graft survival period, the patch's nutrient supply and waste removal may initially depend on diffusion between the blood and the TE patch; later the neovascular system of the patch constructs takes over. Finally, SMCs proliferate and

hypertrophy in response to stress and can form an extensive elastic extracellular matrix, that could maintain the elasticity of the blood vessel wall [103, 104].Therefore, theoretically the muscle cell-seeded patch should be able to prevent patch dilatation and thinning under high pressure circulatory conditions. In the experiment, to obtain a high purity of SMCs, the tunica adventitia of the aorta was peeled off to minimize fibroblast contamination and the cell culture medium utilized was DMEM, which is unsuitable for the growth of endothelial cells.

The ideal biodegradable material should permit the diffusion of nutrients and metabolic waste necessary for cell growth; enable cell adhesion, migration, proliferation, and differentiation; facilitate extracellular matrix formation; and permit endothelialization of the endocardial surface. The material should also be biocompatible and bioabsorbable at a rate compatible with the repair process so that the patch ultimately becomes a tissue supplied by blood vessels. Polymer scaffolds used in this experiment were P4HB. We previously described the use of P4HB for cardiovascular tissue engineering [64]. P4HB is a rapidly absorbable biopolymer biologically derived from bacteria, that is strong and pliable. Because of the thermoplastic properties of P4HB, it can be molded into almost any shape and thus be applied widely in cardiaovascular tissue engineering. In addition, the P4HB based tissues showed supraphysiologic mechanical strength, which is an importantant advantage for potential surgical applications. In our experiment, the gross appearance of the TE patch showed that after conditioning in the pulsatile bioreactor, all TE constructs maintained intact structure without any rupture. We anticipate that *in vivo* study will demonstrate that the P4HB based TE patch is sufficiently strong to resist damage from the contracting myocardium and high pressure of circulation system. The experiment also demonstrated that our newly developed bioreactor system did not impair the integrity of the TE constructs and may be suitable for long term dynamic conditioning.

More efficient *in vitro* cell seeding and cell-scaffold cultivation may be important in building a successfully tissue engineered graft for *in vivo* use. Cell seeding may be performed under static or dynamic conditions. Although optimal cell seeding conditions are unknown, higher seeding efficiency and more uniform cell distribution on the scaffold are important issues. Static cell seeding mothods have been successful when seeding polymer films and thin scaffold [105]. In our study, we chose a static cell seeding model because P4HB scaffolds used in the experiment were thin and flat film with the thickness of 0.4-0.5 mm and cell suspension could be dripped evenly onto the two-dimensional scaffold; thus, uniform cell

distribution could be achieved under static seeding condition. In order to raise the cell seeding efficiency, the flat surface of the polymer scaffold was rubbed with a metal brush and the scaffolds were prewetted with FBS. Meanwhile, we designed two metal rings made of stainless steel to enhance the effect of cell seeding.

Our results demonstrate positive evidence that the seeded SMCs adhered to, migrated into, proliferated in, and differentiated in the porous P4HB scaffold to form viable, oriented and confluently layered tissue without any signs of contamination in our bioreactor system, as shown in Figure 14. The cells appeared viable on the polymeric patch scaffold. In addition, after conditioning in our bioreactor, each polymeric scaffold was partially absorbed and replaced with cells and extracellular matrix. In contrast to the static controls, the conditioned patch constructs were completely covered with organized tissue in a layered fashion and did not form unorganized tissue particles. Additionally, more cells penetrated and grew into the deeper layer of the conditioned scaffolds. We thus fabricated a living, tissue engineered construct *in vitro* for potential use in cardiovascular surgery. The histology of the patch constructs which were not exposed to pulsatile flow (static controls) showed a loose, less organized tissue formation.

The SEM examination of the TE patch demonstrated that the SMCs in conditioned patches attached well to the surface of the polymeric scaffold, exhibited a characteristic elongated bipolar spindle shape and were oriented parallel to flow direction, whereas those in static controls were polygonal in shape and randomly oriented. This emphasizes the fact that the biomimetic *in vitro* environment influences the degree of tissue organization and the cell orientation.

Studies have shown that mechanical stress is an important parameter in bioreactor design because mechanical stress in fluid dynamics has a well-known impact on human vascular cell morphology, proliferation, orientation, organization and the composition of extracellular matrix [106]. The orientation of cells and the collagen bundles along the shear stress field was demonstrated in a pulsatile flow chamber when culturing heart valve and small vessels [97, 107]. Moreover, the orientation of SMCs and collagen fibers is more prominent under dynamic stress loading conditions than those under static stress loading conditions [108]. In our pulsatile bioreactor design, we also considered the factor of mechanical stress. The medium inlet is pointed directly at the patch, whereas the direction of the medium outlet is

away from patch. In this way, the flow direction is parellel to the patch surface and a shear stress is established. Meanwhile, in our experimental setting, dynamic tension was induced on the patch constructs by their being stretched periodically and arching into the cell medium chamber due to pumping of the respirator. At the same time, the cellular surface was continuously exposed to varying rates of pulsatile flow. Thus we combined flow and dynamic biomechanical stress in order to provide physical signals comparable to those to which a patch is exposed under *in vivo* conditions.

Our new dynamic bioreactor allowed for adjustable pulsatile flow and varying levels of pressure. For different tissue engineered constructs, the use of pulsatile flow is critical, because the peaks of the pulsatile flow pattern lead to high distension forces at the fixation area and might destroy the new tissue. In this experiment, the bioreactor was adjusted to a low flow of 250 ml/min and a systolic pressure of 8 mmHg. Under these conditions, the polymer construct remained intact without any sign of rupture. Over the experiment period, the bioreactor system was easily fitted into a standard cell incubator, representing a highly isolated dynamic cell culture setting with maximum sterility, optimal gas supply and stable temperature conditions especially suited for long-term experiments. At same time, the bioreactor system was compact without any sign of leakage. This is very important for the avoidance of contamination of the incubator.

Additionally, our results further demonstrate formation of extracelluar matrix proteins (collagen, elastin) under pulsatile flow conditions as measured by Biocolor assays and stained positive for smooth muscle cells and fibronectin. Staining for *a*-Smooth muscle actin and fibronectin revealed positive signals throughout the TE patches. ASMA positive cells were detectable throughout the constructs, demonstrating SMC populations. Fibronectin was detectable in the patch constructs suggesting that cellular interactions with ECM were established. Quantitative collagen analysis revealed the values of bioreactor conditioned TE patch to be significantly higher compared with static controls. These findings indicate that the new bioreactor system has a beneficial impact not only on cell function but also on the composition of extracellular matrix, and additionally facilitates the development of new tissue and enhances tissue maturation. Interestingly, there was no significant difference between the conditioned patch and the static patches as to the quantitative elastin analysis. This suggestes that the mechanical stress, which was mimicked by the bioreactor system, may not affect elastin synthesis as it does collagen synthesis. There was also no positive staining for CD31 or

CD34, indicating the absence of endothelial cells in TE patch and no cell contamination. This is the result expected because no endothelial cells were seeded on the patch.

Our early *in vitro* results appear promising and we have created a viable human tissue construct on a porous, elastic, and biodegradable polymer *in vitro*. However, the results are still preliminary and numerous issues remain to be addressed before the clinical application of tissue engineered patches will be possible. Future advances will likely be made in polymer scaffold design, cell sources, in optimal human vascular cell seeding methods, and in culture conditions. Further experiments are needed to determine the optimal conditions for human cardiovascular tissue formation *in vitro*.