Evolving Technology

In vitro engineering of heart muscle: Artificial myocardial tissue

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Introduction: Myocardial infarction followed by heart failure represents one of the major causes of morbidity and mortality, particularly in industrialized countries. Engineering and subsequent transplantation of contractile artificial myocardial tissue and, consequently, the replacement of ischemic and infarcted areas of the heart provides a potential therapeutic alternative to whole organ transplantation.

Methods: Artificial myocardial tissue samples were engineered by seeding neonatal rat cardiomyocytes with a commercially available 3-dimensional collagen matrix. The cellular engraftment within the artificial myocardial tissues was examined microscopically. Force development was analyzed in spontaneously beating artificial myocardial tissues, after stretching, and after pharmacologic stimulation. Moreover, electrocardiograms were recorded.

Results: Artificial myocardial tissues showed continuous, rhythmic, and synchronized contractions for up to 13 weeks. Embedded cardiomyocytes were distributed equally within the 3-dimensional matrix. Application of Ca^{2+} and epinephrine, as well as electrical stimulation or stretching, resulted in enhanced force development. Electrocardiographic recording was possible on spontaneously beating artificial myocardial tissue samples and revealed physiologic patterns.

Conclusions: Using a clinically well-established collagen matrix, contractile myocardial tissue can be engineered in vitro successfully. Mechanical and biologic properties of artificial myocardial tissue resemble native cardiac tissue. Use of artificial myocardial tissues might be a promising approach to reconstitute degenerated or failing cardiac tissue in many disease states and therefore provide a reasonable alternative to whole organ transplantation.

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Cardiac transplantation represents a life-saving and life-extending treatment modality for end-stage heart failure. Although advances in surgical techniques, immunosuppression, and postoperative care have improved survival and quality of life, the shortage of donor organs has induced research efforts to develop alternative approaches. One strategy is the in vitro engineering of myocardial tissue.[1-4]

Several cardiomyocyte 3-dimensional in vitro culture systems have been devel-

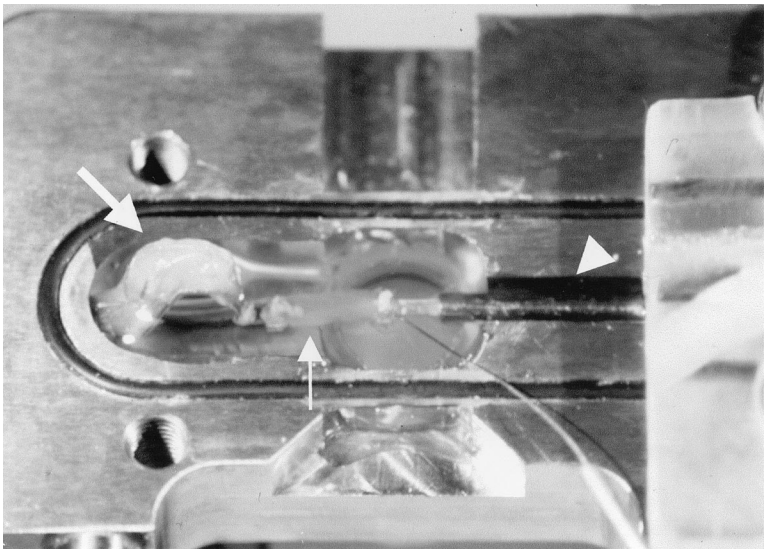

Figure 1. Force measurement assembly. An AMT strip *(thin arrow)* is fixed between the force sensor *(left side, arrow)* and a mobile holding arm *(right side, arrowhead).*

oped with synthetic polymers or biologic components as the underlying matrix. Li and associates[3] have shown that cardiac cells can attach to scaffolds to form contractile cell-polymer constructs. Li and others constructed a viable cardiac graft that contracted spontaneously in culture conditions. In most cases cells were derived from fetal rat ventricular muscle and seeded onto biodegradable material. Bursac and associates[4] have examined the effects of specific variations in a cell-polymer bioreactor model system, performed electrophysiologic studies, and compared constructs with native cardiac tissue. Eschenhagen and coworkers[5-7] subjected 3-dimensional cultured cardiomyocytes to chronic stretching to study important features of cardiac diseases, such as myocardial hypertrophy, which correlates with poor prognosis in heart failure. This group also performed pharmacologic studies on engineered heart tissue.

With regard to the potential clinical application, however, all current models have significant drawbacks or limitations, including size, cellular distribution, viability, and mechanical stability. Therefore we developed a novel type of artificial myocardial tissue (AMT). AMT is based on a clinically approved collagen matrix. Cell isolation and casting of AMTs have been optimized. The development of AMTs might be an important step toward a successful, cell-based restoration of diseased areas of the heart with poor function.

Materials and Methods

Isolation of Cardiomyocytes

Neonatal Wistar rats (days 1-3) were decapitated according to the National Institutes of Health and United States Drug Administration guidelines for the care and use of laboratory animals. Cardiomyocytes were isolated essentially as described by Eschenhagen and coworkers.[5] Cardiomyocyte yield and cellular vitality was assessed microscopically.

Preparation of AMT

Pieces of a preformed collagen scaffold (20 mm $\times$ 15 mm $\times$ 2.5 mm, Tissue Fleece, bovine collagen type I, Baxter, Hyland Immuno GmbH) were placed in cell-culture dishes and into rectangular wells of the same size that had been cut out of layers (approximately 5-mm thick) of silicone rubber (Dow Corning).

One milliliter of the cell suspension containing 2×10^6 cells was added to the collagen fleece. The mixture was allowed to gel at 37°C for 4 hours. In a next step 4 mL of culture medium was added to each well. AMTs were cultured in Minimal Essential Medium (Life Technologies) plus 10% fetal calf serum (PAA) and 110 μmol/L 5-bromo-2′-deoxyuridine (Sigma Chemicals). Microscopic examination of cellular distribution and viability, monitoring of contractility, and exchange of culture medium was performed daily.

Histology

AMTs were removed from their silicone wells and fixed in 3% formaldehyde (Sigma Chemicals) at a pH of 7.4. After dehydration, AMTs were embedded in paraffin blocks. Longitudinal sections and cross-sections (10 μm) were cut and stained with hematoxylin and eosin.

Force Measurements and Electrical Stimulation

AMTs were cut into strips of 10 mm $\times$ 1.5 mm and were mounted on a chamber, as described by Kraft and coworkers (Figure 1).[8] By using Histoacryl (Braun), AMT strips (n = 10) were glued onto metal holding arms on both sides of the chamber, which was filled with culture medium. The temperature inside the chamber was kept constant at 37°C by using 2 water-cooled Peltier elements.

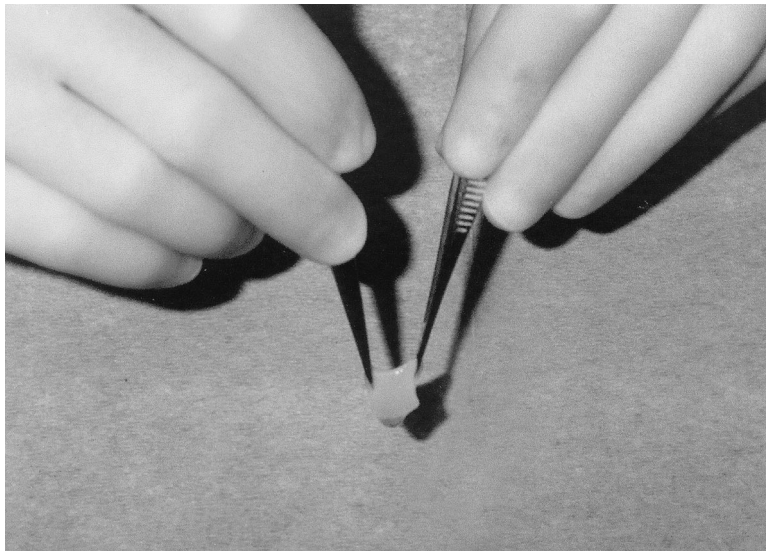

Figure 2. AMT stability.

Physiologic oxygen and carbon dioxide levels in the medium were maintained by means of an external supply. One of the strip holders was connected to a force transducer (SensoNor). The opposite holder was flexible to enable stretching of the AMT strips. AMT-based force generation was recorded digitally and later used for offline analysis. After baseline measurements, stimuli, including Ca (a single dose of 0.25 mmol/mL) and epinephrine (100 μg), were administered to the culture medium. Force generation was measured. Progressive stretching of each strip at various lengths was performed in 10-second intervals.

Electrocardiograms to monitor spontaneous electrical activity of AMTs were performed by using a Grass SD-9 system. Two separate electrodes were placed inside the AMT and connected to a standard electrocardiographic monitor. A constant electrode distance of 5 mm was used in all experiments. Bipolar signals were recorded.

Results

Shape, Texture, and Stability

Before 3-dimensional seeding, trypan blue staining of AMTs revealed cellular vitality of 80% $\pm$ 18%. Gelation of the collagen-cell mixture in culture lasted until day 2. During this process, the cell-seeded and hydrated matrix shrunk, and the AMTs lost contact with the surrounding silicone walls and the bottom of the culture dishes. At that point, they started floating in the culture medium. The definite size of the AMTs was approximately 15 mm $\times$ 10 mm $\times$ 2.5 mm. In this shape they remained stable throughout the entire culture period of 14 $\pm$ 2 weeks without cellular detachment. After gelation, the AMT texture was flexible and solid, allowing removal of AMTs from the petri dish without loss of integrity (Figure 2). AMTs displayed significant elasticity and allowed for stretching of up to 150% of the original length.

Histology

Hematoxylin and eosin–stained specimens showed a homogeneous cell population throughout all layers (center to the edges), as shown in Figure 3. In addition to single cells, cell clusters of different sizes were also found within the AMTs.

Contractility

Contractions became apparent 36 hours after casting and reached maximal frequency and strength on day 4 in 87% of AMTs. Contractions were synchronous along the total AMT length. As observed macroscopically and microscopically, contractions within AMTs were transmitted in wave form. In culture 40% of AMTs displayed continuous contractility for 12 weeks. The frequency of contractions was measured daily and ranged between 40 and 220 beats/min, with an average frequency of 125 $\pm$ 35 beats/min.

Force Measurements

All AMT strips contracted spontaneously and continuously. Force recordings were performed from 10 different AMTs before and after stretching. Force development in AMTs reached 8.6 $\pm$ 3.6 μN. Stretching resulted in a significant increase in force ($P < .0001$). Maximal force was achieved at 2.5 mm of stretching (25% of initial length of strip), leading to an increase in force of 119% (18.8 $\pm$ 3.7 μN) compared with that seen in unstretched AMTs (Figure 4). Stretching by more than 3 mm was not possible without detachment from the holding arm. Stretching, as well as application of Ca^{2+} or epinephrine, did not affect the rate of spontaneous contraction. Increase of Ca^{2+} concentrations in the culture medium from 1.8 to 2.8 mmol/L resulted in a 103% increase in maximal force (14.1 $\pm$ 3.9 μN, Figure 5).

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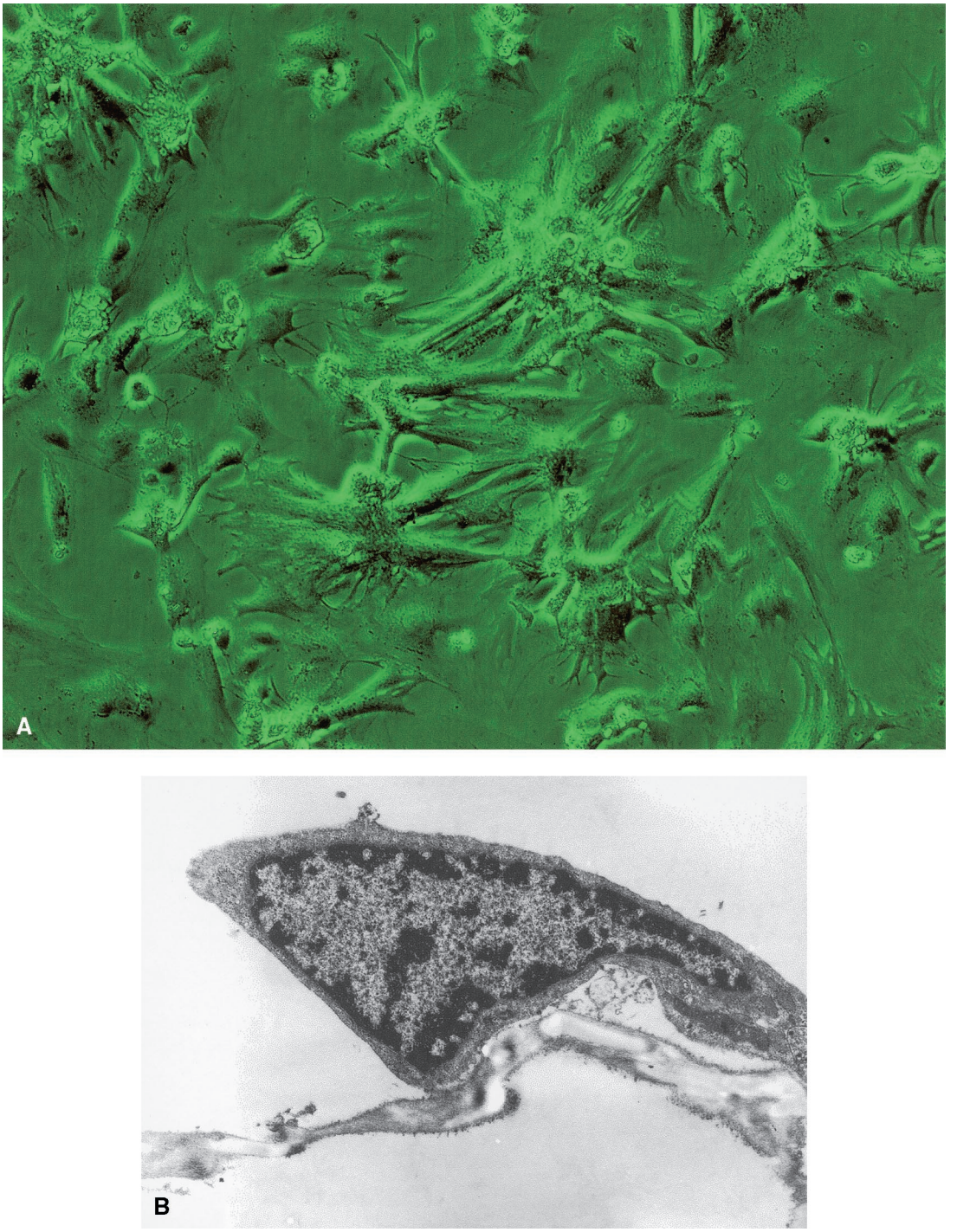

Figure 3. A, Cellular seeding of an AMT: fluorescent stain of AMT after 12 weeks of culture, which demonstrates intercellular intercalations. B, Electron microscopic study reveals a tight junction between cell and collagen fibril.

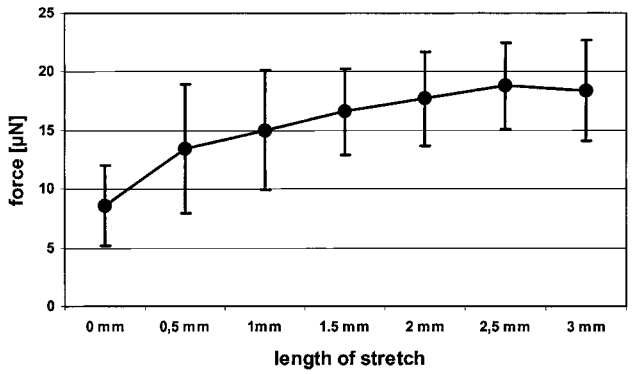

Figure 4. Stretching of AMTs resulted in increased force development, dependent on the length of stretch. We measured force immediately after stretching AMT strips for 10 seconds (n = 10).

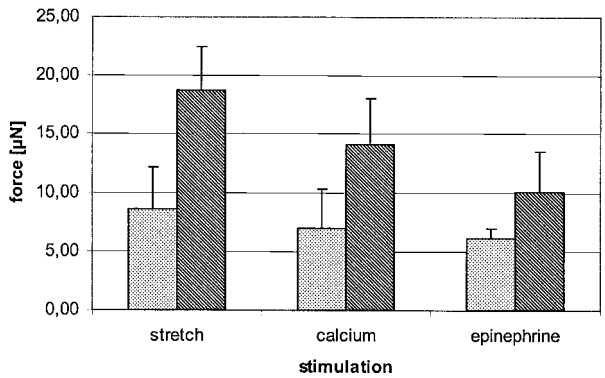

Figure 5. Effect of stretch and pharmacologic agents on force development (*left column,* baseline; *right column,* stimulated). Depicted is the force development in unstimulated AMTs after 10 seconds of stretching (at 2.5 mm), after addition of a further 1 mmol/L Ca^{2+} in culture medium, and after administration of 0.1 mg of epinephrine (n = 10 each).

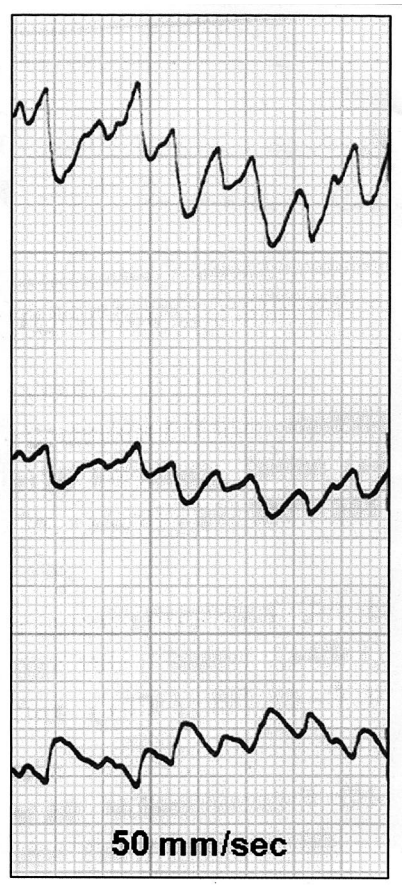

Figure 6. A representative electrocardiographic recording of AMT is shown. A double-peak signal represents a single complex of spontaneous electrical activity without stimulation.

The mean baseline measurement revealed a maximal force of 6.9 ± 3.4 μN. Epinephrine increased the maximal force from 6.1 ± 0.9 to 10.1 ± 3.4 μN (65.6%).

Electrographic Recordings

Electrocardiograms showed amplitudes that ranged between 0.5 and 4.6 mV (mean, 2.1 ± 1.3 mV). The obtained curves resembled bundle-branch blocks or ectopic ventricular activity (Figure 6).

Discussion

Ischemic heart disease and myocardial infarction are major causes for end-stage heart failure. Currently, heart transplantation is the sole therapeutic option for many of these patients. Because of the progressive lack of donor organs, many listed patients die while waiting for a suitable organ. High-risk coronary surgery, left ventricular restoration with or without mitral valve surgery, implantation of artificial assist devices, and biventricular pacemaker implantation are widely used as treatment concepts in ischemic heart failure. During the last few years, other strategies have evolved, aiming at restoring diseased areas of the heart. These approaches include cellular transplantation, as well as in vitro engineering of bioartificial myocardial tissue.

Thus far, it is unknown whether cell transplantation strategies are able to successfully restore severely injured myocardium. Damaged tissue within infarction areas rapidly undergoes irreversible necrosis, leading to structural alterations, such as scar tissue development. Recent results of animal studies suggest improvements of heart function after injection of skeletal myoblasts[1] or bone marrow stromal cells.[2] However, it is controversial whether these effects are due to the ability of these cell types to create sufficient amounts of new myocardium-like tissue within the infarction area and to participate in synchronized heart contrac-

tion. It was criticized that the reported effects are simply caused by remodeling of connective tissue and extracellular matrix.

An alternative approach might be the replacement of diseased myocardium with heart tissue engineered in vitro. However, all approaches to in vitro–engineered myocardium-like tissue reported previously[3,7] require further improvements to enable clinical use. Potential drawbacks of these engineered 3-dimensional cultures are as follows: (1) obtained 3-dimensional constructs are too small to allow surgical implantation into infarction areas; (2) cellular distribution and viability are not homogeneous; (3) myocyte function is limited by various factors, such as impaired nutrient availability or defective culture geometry; (4) 3-dimensional constructs lack adequate plasticity and mechanical stability for implantation purposes; (5) used materials, especially matrix proteins, are not exactly defined or clinically approved; and (6) production costs are high because of expensive additives and collagen compounds, such as Matrigel.

We have produced a myocardium-like 3-dimensional tissue, which can be manufactured in various shapes to fit into infarction scars. Our 3-dimensional culture system is based on a clinically approved bovine collagen–based matrix, initially designed as a hemostypticum for topical surgical application. By pouring cells into a single-component scaffold, the seeding procedure can be simplified and shortened significantly. During tissue regeneration and remodeling, it is known that autologous cells migrate into the scaffold. Finally, the natural collagen network disintegrates after implantation and is replaced with an autologous matrix. Accordingly, migration of myocardial cells into "tissue fleece" occurred and resulted in artificial myocardium-like tissue with improved mechanical stability compared with that of the initial matrix material. Structural integrity of AMTs was maintained during the entire culture period, resembling mechanical properties of native cardiac muscle.

In addition to their function as an extracellular scaffold, matrix proteins supply important migration and proliferation signals through specific interactions with cellular receptors. These matrix-cell receptor interactions are essential to regulate cellular organization within areas of remodeling tissue. Unfortunately, cell-matrix interactions on the molecular level are widely unknown. Although detailed molecular mechanisms remain to be analyzed, our results suggest that collagen fleece provides all signals essential to support the spreading, attachment, and synchronous contractions of neonatal cardiomyocytes in the 3-dimensional scaffold. The observed histologic pattern resembles a compact multilayer structure.

High densities of contracting cardiomyocytes were detected not only in the peripheral areas of AMTs but also in the center. Low central-cell density, as observed by others working on myocardial tissue engineering, has been explained by oxygen and nutrient deficiency[4,6] in the thicker central areas of the unvascularized 3-dimensional cultures. Although central areas of AMTs in our studies were about 2.5-mm thick, we did not observe lower cell densities compared with those at the edges of the scaffold. Whether this is due to a better nutrient supply of collagen fleece compared with that seen with other scaffold mixtures remains unknown and is subject of ongoing studies at our institution. For this purpose, we have constructed a novel bioreactor system with pulsatile flow designed to stimulate angiogenesis in 3-dimensional engineered tissues.

One major characteristic of cardiac muscle is the ability of spontaneous and rhythmic contractions. Macroscopically visible contractions result from synchronized excitations of single cardiomyocytes connected through gap junctions. Spontaneous and synchronous contractions occurred approximately 36 hours after casting in our AMTs. Maximal contractions were observed after day 4 and were maintained for up to 12 weeks of culture. This, to our knowledge, represents the longest duration of in vitro contractile activity in the literature. Future experiments will focus on the longevity of AMTs after in vivo implantation. Comparable with a heart, contractions spread along the total length of our engineered myocardium-like tissue in a wave-like manner. Force recordings of native and stretched AMTs revealed stretch-dependent regulation of contractile force, which is in accordance to the Frank-Starling mechanism. Furthermore, regulation of contractile force by extrinsic factors could be measured. Increase of Ca^{2+} and addition of epinephrine to the culture medium in a physiologic range lead to enhanced force development. In contrast to the report of Bursac and associates,[4] beating activity in AMTs was not transient but continuous, even without stimulation or pacing. The obtained amplitudes were higher than those previously reported by Bursac and associates, and this might indicate a more physiologic structure and function of our engineered heart tissue.

In conclusion, we have engineered a novel and promising type of myocardium-like tissue that resembles native cardiac muscle in many aspects. In addition, AMTs might serve as a basis for the development of tissue, which is capable of replacing human myocardium in many disease states of the failing heart. Future progress in stem-cell technology, as well as discovery of factors responsible for proliferation of adult cardiomyocytes, combined with suitable techniques of gene transfer might allow for the production of autologous artificial myocardium-like tissue that is capable of correcting myocardial injury and restoring impaired heart function. Finally, vascularization of in vitro–engineered tissues might result in the generation of a complete bioartificial heart.

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