# Tissue Engineering of Viable Pulmonary Arteries for Surgical Correction of Congenital Heart Defects

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*Background.* **Tissue-engineered pulmonary arteries could overcome the drawbacks of homografts or prosthetic conduits used in the repair of many congenital cardiac defects. However, the ideal scaffold material for tissue-engineered conduits is still subject of intensive debate. In this study, we evaluated an acellularized allogeneic matrix scaffold for pulmonary artery tissue engineering with and without in-vitro reseeding with autologous endothelial cells in the pulmonary circulation in a growing sheep model.**

*Methods.* **Ovine pulmonary arteries (n = 10) were acellularized by trypsin/ethylenediamine tetraacetic acid incubation. Autologous endothelial cells were harvested from carotid arteries, and the pulmonary conduits were seeded with endothelial cells. We implanted in-vitro, autologous, reendothelialized (group A, n = 5) and acellularized pulmonary conduits (group B, n = 5) in the pulmonary circulation. The animals were sacrificed 6 months after the operation. Explanted valves were examined histologically and by immunohistochemistry.**

*Results.* **The conduit diameter increased in both groups (group A, 44% ± 11%; group B, 87% ± 18%; $p < 0.05$). In group A, however, a proportional increase in diameter was present, whereas in group B, a disproportionate increase resulting in aneurysm formation was observed. Histologically, the conduit wall integrity was destroyed in group B and preserved in group A. In group B, the extracellularmatrix degenerated with a reduced amount of collagens and proteoglycanes. Furthermore, no elastic fibers were detectable. In contrast, the extracellularmatrix in group A was close to native ovine tissue.**

*Conclusions.* **Tissue-engineered pulmonary conduits (autologous endothelial cells and allogeneic matrix scaffolds) functioned well in the pulmonary circulation. They demonstrated an increase in diameter and an extracellular matrix comparable to that of native ovine tissue.**

**(Ann Thorac Surg 2006;81:1466–71)**


A large proportion of children with congenital heart defects may require implantation of conduits to reconstruct pulmonary arteries or the right ventricular outflow tract. Currently, homograft or prosthetic conduits are the conduits of first choice. However, drawbacks of these conduits are a lack of growth potential, a lack to remodel, the tendency to degenerate, and susceptibility to infection. Thus, these children may face several reoperations for pulmonary artery conduit replacement.

Creation of bioartifical conduits based on the principal concept of tissue engineering with the use of preformed scaffold material with in-vitro seeding of cellular tissue components could overcome the inherent problem of homograft or prosthetic conduits. Previous studies focusing on biodegradable polymer scaffolds for tissue engineering of heart valve and pulmonary artery resulted in formations with abnormal extracellular matrix (ECM) (1-5). In-vivo and in-vitro studies with acellularized allogeneic matrix proofed the concept of biological matrices for the generation of viable cardiac tissue [6–8]. However, only limited data are provided on acellularized biological scaffolds for tissue-engineered pulmonary arteries. The purpose of this study was to evaluate the concept of tissue-engineered pulmonary arteries based on an allogeneic scaffold in a large growing animal model with an implantation period as long as 6 months.

## Material and Methods

### *Acellularization of Pulmonary Artery Bifurcations*

Ovine pulmonary artery bifurcations (n = 10) were obtained from immature sheep (16 ± 2 kg) under sterile conditions. Conduits were stored at 4°C. Within 30 minutes the conduits were acellularized in a bioreactor. The bioreactor was filled with 0.05% trypsin (Biochrom KG, Berlin, Germany) and 0.02% ethylenediamine tetraacetic acid (EDTA [Biochrom]) for 48 hours, followed by phosphate-buffered saline (PBS) flushing for 48 hours to remove cell debris. All steps were conducted in an atmosphere of 5% $CO_2$ and 95% air at 37°C with the bioreactor rotating at a speed of 7 rpm. Samples of the conduit were taken before and after treatment to verify complete acellularization.

Accepted for publication Aug 29, 2005.

Presented at the Fortieth Annual Meeting of The Society of Thoracic Surgeons, San Antonio, TX, Jan 26–28, 2004.

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Published by Elsevier Inc

0003-4975/06/$32.00
doi:10.1016/j.athoracsur.2005.08.083

### *Cell Isolation and Culture*

The techniques of cell isolation and cell culturing have been described in detail elsewhere [9]. Autologous cells were retrieved from short segments of the right carotid artery, which were harvested from five lambs (14 ± 2 kg), which were planned for subsequent implantation.

### *Cell Seeding*

Acellularized conduits (group A, n = 5) were coated with endothelial cells, resulting in a uniform cellular restitution of the pulmonary conduit surface. Three separate cycles of endothelial cell seedings were performed. In each cycle $1 \times 10^6$ endothelial cells were seeded onto the pulmonary conduit scaffold fixed into a bioreactor and cultured in static nutrient medium (DMEM, Gibco BRL Life Technologies, Gaithersburg, MD) for 4 hours (37°C, 5% $CO_2$), followed by rotation of the bioreactor (12 hours, 0.1 rpm). Samples of the conduit were taken to prove seeding of endothelial cells.

### *Surgical Techniques*

The in-vitro reseeded pulmonary conduits were implanted into the same 5 sheep, from which the carotid arteries were harvested initially (group A). The acellularized conduits were implanted in the remaining 5 sheep (group B). The mean weight of immature sheep amounted to 16.3 ± 2 kg. Anaesthesia was induced with 30 mg/kg of ketamine and maintained with an intravenous bolus injection of 2 mg/kg propofol. The heart was exposed by a left anterolateral thoracotomy entering the chest through the fourth intercostal space. Systemic anticoagulation was induced with heparin (400 IU/kg). After cannulation of the femoral arteries and right atrium, a normothermic cardiopulmonary bypass was established. On bypass, 0.01 mg/kg fentanyl and 0.02 mg/kg pancuronium were administered. With the heart beating, the main pulmonary artery was transected, and the pulmonary trunk removed. The conduits were orthotopically implanted by using running 5-0 monofilament sutures (Prolene; Ethicon, Somerville, New Jersey). Heparin was reversed with 300 IU of protamine per kilogram after weaning from bypass. The chest was closed in layers using resorbable sutures. An intercostal nerve block with 0.25% bupivacaine was administered. No further anticoagulation was applied. All animals received cefazolin, 1,000 mg daily, for the first postoperative week. For pain control, intramuscular buprenorphin injections were performed during the first 3 days and later as required. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 85-23, revised 1985). The animals were sacrified after 6 months.

### *Postoperative Evaluation*

The animals were examined by echocardiography immediately after the operation, and before termination. After termination, specimens of conduit wall were prepared for histology and immunohistochemistry.

ECHOCARDIOGRAPHY. Epicardial echocardiography examinations were performed immediately after the operation, and before termination (Sonos 5500 Cardiac Imager, 7.5-MHz phased-arrayed transducer; Hewlett-Packard; Agilent Technologies, Palo Alto, CA) to evaluate conduit diameter. Changes in diameter were expressed as percentage. The native pulmonary trunk, to which the allogeneic pulmonary artery conduit was attached, served as the reference diameter for evaluation of postoperative allogeneic pulmonary conduit diameter. Proportional increase in diameter: diameter of the implanted allogeneic pulmonary conduit at the time of termination equals the diameter of the native pulmonary trunk at the time of termination. Disproportionate increase in diameter: diameter of the implanted allogeneic pulmonary conduit at the time of termination exceeded the diameter of the native pulmonary trunk by more than 20% at the time of termination.

VIABILITY TEST. To verify the viability of endothelial cells after in-vitro repopulation, circular segments were excised before implantation and examined by a live-dead assay. A commercially available cell viability assay based on simultaneous determination of live and dead cells was used (Live/Dead Viability/Cytotoxity Kit for Animal Cells; Molecular Probes; Invitrogen GmbH, Karlsruhe, Germany).

HISTOLOGY. For determination of cellular and extracellular components of the pulmonary conduits, histologic staining and histochemical assays were performed. Histologic characterization of conduit matrix components was conducted by means of standard hematoxylin-eosin, Movat pentachrome, and van Giesson staining. Histologic comparison between explanted pulmonary artery tissue and native ovine pulmonary artery tissue was performed. Conduit wall integrity was defined as a histologic composition of the ECM in terms of cellularity, and semiquantitative reduced amounts of stainable collagen, glycosaminoglycans, proteoglccans, and stainable elastin fibers comparable with native ovine pulmonary artery tissue.

IMMUNOHISTOLOGY. Snap-frozen conduit wall specimens were used for immunohistologic analysis. Immunohistochemical staining for endothelial cell characterization was performed by applying the avidin-biotin-peroxidase technique. Endothelial cells were characterized by the presence of factor VIII-related antigens (primary antibody, v. Willebrandt factor; clone 8/86, DAKO Diagnostika, Hamburg, Germany). A goat anti-mouse antibody (DAKO) served as a secondary antibody. Negative controls were simultaneously performed. Normal mouse or rabbit serum served as a primary antibody and a specific mouse or rabbit antibody served as the secondary antibody.

## Results

All animals survived the operative procedure and recovered uneventfully.

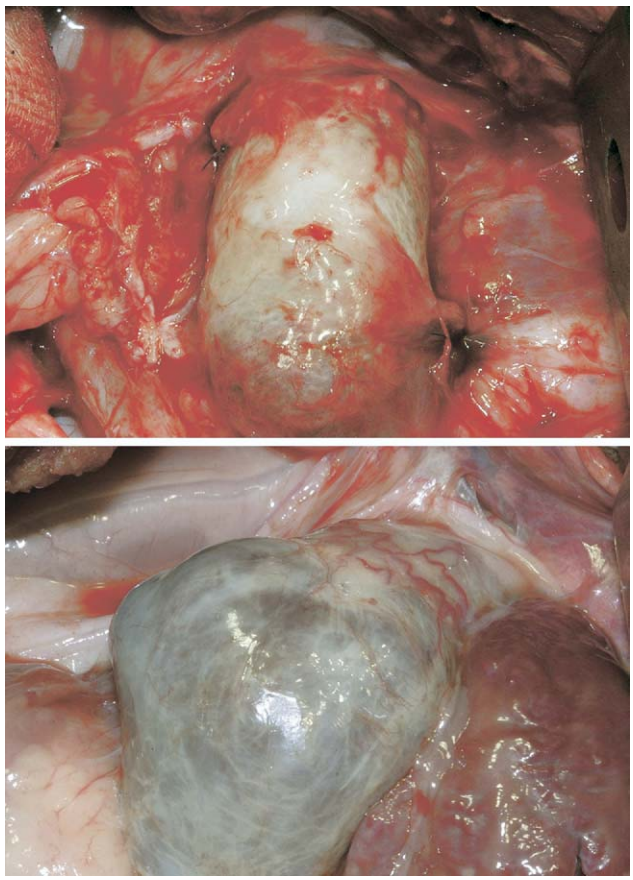

*Fig 1. Allogeneic pulmonary conduits after 6 months in-situ. (A) In-vitro reendothelialized conduits; the explanted conduits showed a diameter and normal appearance of the pulmonary arterial wall. (B) Acellularized implanted conduits; gross dilatation of the pulmonary conduit with a thin aneurysmatic arterial wall.*

### *Echocardiographic Findings*

After 6 months, the conduits showed no evidence of thrombus formation or calcification in either group. All conduits showed an increase in diameter compared with the time of implantation. In group A, however, a proportional increase in diameter was present, whereas in group B, a disproportionate increase was observed (group A; 44% ± 11%, group B; 87% ± 18%; $p < 0.05$; Fig 1).

### *Histologic Examination*

The viability test of specimen taken immediately before implantation of the in-vitro repopulated conduits revealed viable endothelial cells. Hematoxylin-eosin staining of the conduits after explantation showed a complete endothelial lining in all conduits after 6 months. An increase in cellular repopulation of the valve interstitium was observed in group A, indicating an increased cell turnover. The characterization and semiquantification of conduit matrix components showed normal amounts of stainable collagen, elastin, glycosaminoglycans, and proteoglycans content in group A. Although the in-vitro repopulation resulted in a tissue formation resembling native ovine tissue, the increased cellularity of the ECM could either indicate that the repopulation process is a still ongoing process or the beginning of a fibrosis. In contrast, in group B, a decreased cellular repopulation of the interstitium was observed. The Movat pentachrome stain showed a severe degenerated ECM with reduced amouts of stainable collagen, glycosaminoglycans, and proteoglycans, and no stainable elastin fibers (Fig 2).

### *Immunohistochemical Staining*

Factor VIII stained cells were found in all conduits on the internal surface of the pulmonary conduits (Fig 3).

## Comment

In this study, we have shown that the implantation of in-vitro reendothelialized allogeneic pulmonary conduits

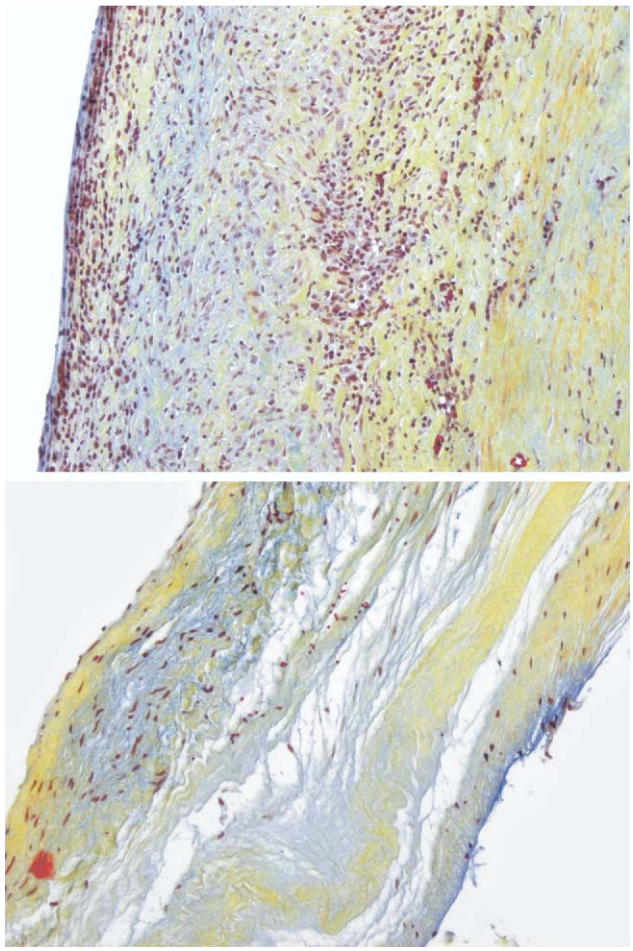

*Fig 2. Movat pentachrome stain of the extracellular matrix of allogeneic pulmonary conduits after explantation. (A) In-vitro reendothelialized implanted conduit after 6-month implantation period, with normal content of collagen (yellow), elastic fibers (red), and proteoglycanes (bright blue) resembling native ovine pulmonary artery tissue but with increased cellularity. (B) Pathologic composition of the extracellular matrix acellularized implanted conduits after 6 months with reduced collagen (yellow) and proteoglycane (bright blue) content and no stainable elastin fibers. Furthermore, a decreased interstitial cellularity is shown.*

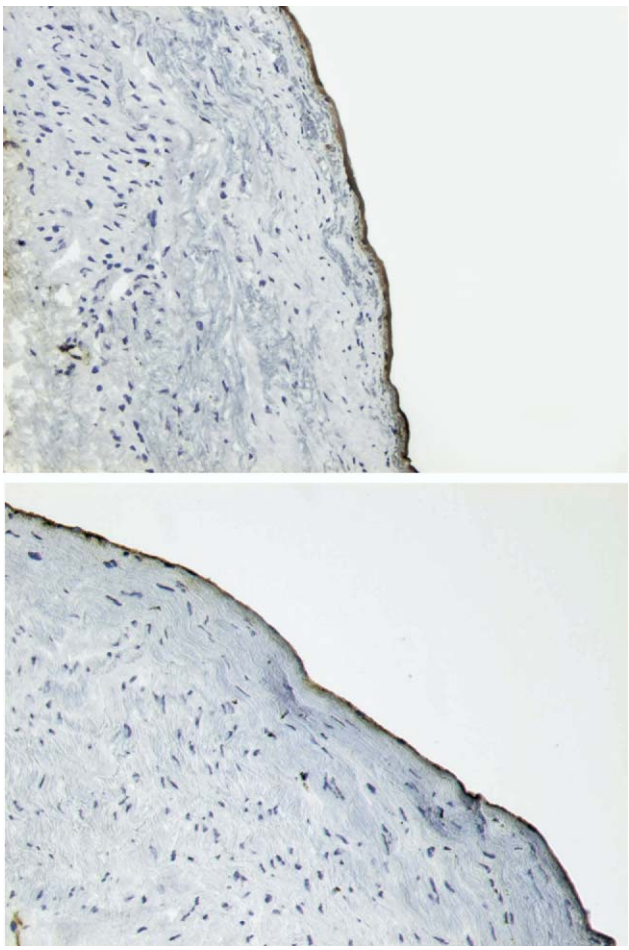

*Fig 3. Hematoxylin-eosin and van Willebrandt factor staining of explanted allogeneic pulmonary conduits after 6 months. (A) In-vitro reendothelialized pulmonary conduit; normal endothelial cell monolayer. (B) Acellularized allogeneic conduits 6 months after implantation, showing normal endothelial cell monolayer.*

leads to viable and well-functioning pulmonary artery conduits in an immature growing sheep model. Follow-up examinations for as long as 6 months after implantation resulted in an ECM composition resembling native ovine pulmonary artery tissue. Furthermore, we were able to detect a proportional increase of the conduit diameter.

The durability of conduits for use in children with congenital heart disease is limited because neither homografts nor synthetic grafts have growth potential. That may lead to a progressive increase in pressure gradients over time, necessitating reoperation for replacement of stenotic conduits [10, 11].

Several groups have been focusing on engineering conduits, which should be vital, nonthrombogenic providing growth potential [5, 12–15]. Only Shinoka and coworkers [5] created a viable conduit following the principles of tissue engineering using a three-dimensional synthetic polyglycol acid fibers matrix that was repopulated in vitro with autologous endothelial cells, fibroblasts, and smooth muscle cells. Although the conduits functioned well in the pulmonary circulation for as long as 169 days, the analysis of conduits showed an abnormal low collagen content and an increase in the calcium content within the ECM [5]. A tissue-specific ECM is a prerequisite for normal cell metabolism, cellular function, interaction, and adhesion [16–18]. The absence of an extracellular molecular network and of ECM proteins, responsible for cellular attachment, in biodegradable polymer scaffolds, might result in a disturbed cell-to-ECM interaction. This could explain the abnormal collagen, elastin, and glycosaminoglycan concentration in the ECM of tissue-engineered cardiac tissue based on biodegradable polymer scaffolds [1–5]. The consequences of an abnormally composed ECM are manifold. The different matrix ECM components are mutually dependant; furthermore, these components may influence the ability of regeneration and the function of the matrix. Hence, it can be speculated that an abnormal ECM composition may lead to premature degenertation of tissue-engineered conduits. In addition, a potential problem of polymer scaffold is the lack of extracellular matrix proteins, which play a pivotal role in physiological cell-to-cell and cell-to-ECM interaction.

Acellularized allogeneic matrix scaffolds have already shown superior adhesive properties of endothelial cells, and in-vivo studies proofed the concept of biological matrices for generation of viable cardiac tissue [6–9]. The degeneration of the acellularized implanted conduits in this study clearly demonstrate that in-vitro repopulation with endothelial is mandatory to create viable and functioning conduit. It can be speculated that the endothelial cells lining the luminal surface of the conduit create a protecting barrier for the extracellular matrix; this could guarantee a restricted migration of fibroblasts and myocytes. Thus, a controlled remodeling and regeneration of the allogeneic matrix and replacement of the allogeneneic matrix proteins with autologous matrix proteins may result.

Previously, we have shown that the source of biological acellularized tissue (xenogeneic versus allogeneic) has an impact on the quality of tissue-engineered cardiac tissue [19]. In the previous study, however, we applied a static acellularization process in contrast to the dynamic acellularization process used in this study. Hence, there is evidence that the acellularization process influence the quality of tissue-engineered cardiac tissue, based on biological three-dimensional scaffolds. The degenerated tissues of acellularized implanted pulmonary conduits resulted in aneurysm formation, in contrast to the in-vitro reendothelialized conduits, and a proportional increase in diameter could be observed. This finding is in concert with the findings of Simon and coworkers [20]. In a clinical study, implantation of acellularized xenogenic Synergraft conduits resulted in a strong inflammatory response and no repopulation of the grafts with human cells, resulting in conduit failure in 3 patients with one lethal conduit rupture at the seventh postoperative day due to structural disintegration of the graft [20]. Although implantation of in-vitro reendothelialized conduits resulted in a tissue formation resembling native ovine tissue, an increased cellularity was observed, which could indicate an increased cell turnover within the ECM, finally resulting in fibrosis.

We would like to emphasize that these data do not indicate that clinical use of acellularized in-vitro repopulated allogenic pulmonary arteries is justified at this time. In this context, it is important to recall the data published by Simon and coworkers [20], who reported on early failure of xenogeneic acellularized conduits in pediatric patients, with sudden death for 3 of 4 patients; in all deaths, the Synergraft was severely degenerated—indicating that premature implantation of tissue-engineered grafts may have catastrophic results.

In conclusion, the results of this study demonstrate that a more detailed knowledge of tissue development is mandatory to generate viable and functioning pulmonary artery conduits withstanding physiologic stress in the pulmonary circulation in the long run. Although in-vitro reendothelialized conduits revealed viable and functioning conduits tissue for as long as 6 months, it can not be excluded that these conduits will degenerate. Further studies with different scaffold materials, cell types, in-vitro reseeding protocols, and longer follow-up periods are mandatory until tissue-engineered conduits can be implanted clinically.

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## DISCUSSION

**DR ANDREW C. FIORE** (St. Louis, MO): When you seeded these allografts, did you use any kind of cell labeling techniques to identify the fact that those seeded cells were intact in the allograft conduit?

**DR LEYH**: Thank you very much for the kind question. Well, we actually did not label these cells. But what we did is, actually, in another experience, we explanted these conduits after 1 hour, 12 hours and 24 hours, and we are still able to see endothelial cells in the in vitro repopulated conduit. In contrast to the acellularized conduits, we were not able to detect any cells after 24 hours. But anyway, we have no data whether these cells, we actually found after explantation, are still the cells we seeded onto before implantation.

**DR HENRY L. WALTERS III** (Detroit, MI): You pointed out one of the disadvantages of homografts. This is their tendency to degenerate. Another disadvantage, of course, is their relative lack of availability, especially in certain sizes. I assume that the clinical application of your scheme in humans would again involve harvesting cadaveric allografts; therefore, availability would still be a problem. Is this the direction that we should pursue in tissue engineering as opposed to trying to develop scaffolding materials such as PGA, chitosan, and so forth, which is something we're working on in our laboratory?

Also, have you tried to harvest allograft pulmonary arteries with valves and submit them to the same kind of treatment?

**DR LEYH**: Well, with respect to your first question, obviously, this is the kind of initial study where we proved the concept of tissue engineering with regard to scaffold material. So I think endothelial cells that are obtained from the carotid artery is not a proper way for clinical use. Our sources might be more

applicable, like progenitor cells or any other cells; but there is still a lot of work to do in this field.

Coming to the second question, yes, we used the same concept for pulmonary arteries or for tissue engineering of pulmonary conduits with a valve-sparing conduit. But when we used this concept, we used a synergetic scaffold material; and we almost found similar results.

**DR FIORE** (St. Louis, MO): I just want to ask you one other question. From your experience, what do you think the most optimal source of autologus endothelial cells should be?

**DR LEYH**: Honestly, I can't answer your question. But I believe that endothelial cells are not the optimal source for tissue engineering. Other cells might be much better.

**DR SCOTT M. BRADLEY** (Charleston, SC): You showed that both the in-vitro and the in-vivo approach led to an endothelial cell layer when you removed the conduits. Could you give us some thoughts on why, if both the conduits acquire an endothelial layer, the in-vitro conduits had such a markedly improved extracellular matrix. Is it a time effect, or is it something else?

**DR LEYH**: There might be sort of a time effect associated with the results presented. I believe that, in my opinion, the in-vitro seeding with endothelial cells may have sort of a protective mechanism on the extracellular matrix, since the material sort of preserved interaction between the matrix and the endothelial cells.

And that might result, or it's just a hypothesis, this can result in a sort of controlled migration of cells within the matrix, so that controlled tissue regeneration will take place on these conduits. And in contrast, if there are no cells, then maybe it's sort of uncontrolled migration of cells into a matrix, and this might result in degeneration of the matrix. But it's just a hypothesis.