CULTURE OF CARDIOGENIC STEM CELLS ON PCL-SCAFFOLDS: TOWARDS THE CREATION OF BEATING TISSUE CONSTRUCTS

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ABSTRACT
Post-myocardial infarction pathologies are additionally compromised by the poor self-repair capacity of the myocardium and the limited possibilities for clinical interventions for regeneration. Cell therapy and in particular tissue engineering evolved as attractive therapeutic options to counteract the high mortality after myocardial infarction and aim to regenerate the myocardium by the delivery of functional cells or tissue constructs. The implantation of stem cell-derived cardiomyocytes, embedded in a biologically degradable matrix, constitutes an intriguing approach, and scaffolds for the culture of cardiomyocytes are increasingly studied. To this end, we developed an electrospun, plasma coated scaffold for the culture of embryonic stem cell (ESC)-derived cardiomyocytes. ESCs were differentiated into cardiomyocytes by the "hanging drop" method and subsequently cultured on a fibrous poly(ε-caprolactone) scaffold. We could demonstrate that the cardiomyogenic phenotype is maintained on the fibrous matrices. Spontaneously beating areas were highly prevalent and α-actinin expression indicated the differentiation into cardiomyocytes. Our results validate the feasibility of cardiomyogenic culture on the designed matrix and warrant further research on the ex vivo generation of a cardiac construct.

KEY WORDS
Tissue Engineering, Embryonic Stem Cells, Cardiomyocytes, Electrospinning

1 Introduction
Among the many causes for cardiac diseases, ischemic heart disease is strongly prevalent and the cause for myocardial infarction (MI). Occlusions in the coronary arteries, which are caused by cholesterol deposits within the vessels, limit oxygen and nutrient supply of the affected tissue, resulting in ischemia. Total arterial closure leads to MI, causing massive cell death and dramatic functional consequences for the heart including scar formation, conduction and contraction deficits and reduction in cardiac force. Ventricular dilation and increased susceptibility for arrhythmias are the consequence and ultimately lead to heart failure (HF). Currently, there is no cure for patients suffering from chronic MI, and the only salvage therapy consists of surgical procedures comprising the implantation of left ventricular assist devices and finally cardiac transplantation. These treatments are, however, only palliative and fail to regenerate injured myocardium.

The adult mammalian heart is a highly specialized organ, which consists of a large variety of terminally differentiated cells. This high degree of differentiation is necessary to reach the sophisticated level of morphological and functional specialization, but it represents a problem in disease, when cells start to fail in their function. In contrast to skeletal muscle, the heart contains only a small fraction of stem cells with cardiogenic potential, so called cardiac progenitor cells [1, 2, 3]. The density of these cells is, however, too low to ensure endogenous tissue repair in disease or after damage such as MI. Recent advances in stem cell research bring new hope for the diseased heart. One major goal in cardiac stem cell research and tissue engineering focuses on the identification of the ideal cell type that can functionally replace damaged cardiomyocytes after injury in order to prevent the deleterious sequence of events leading to cardiac remodelling and HF. Due to their pluripotency, stem cells have gained a lot of attention for cardiac repair, and the potential to direct their differentiation towards cardiomyocytes makes them good candidates for cardiac tissue engineering. Early clinical trials focused on the use of bone marrow-derived mesenchymal stem cells, and despite persisting controversies in the mechanism of action, injection of undifferentiated stem cells into the heart resulted in beneficial effects on cardiac performance after myocardial injury [4, 5]. However, no cardiogenic differentiation or functional integration has been clearly reported, suggesting that the positive influence was induced by the release of paracrine...
factors favorable to cell survival, angiogenesis and stem cell recruitment [6, 7].

Another possible source of cells for cardiac tissue engineering is provided by embryonic stem cells (ESCs). ESCs are pluripotent cells gained from the inner cell mass of the blastocyst stage of an embryo. The great advantage of these cells is that they can be cultured and kept in a proliferative state while staying pluripotent. Differentiation into the cardiogenic lineage is successfully induced by specific changes in the culture conditions, resulting in contractile embryoid bodies, which are composed of a high percentage of cells with cardiomyogenic potential. These stem cell-derived cardiomyocytes can be isolated and used for in vitro and in vivo studies and for the development of new treatment strategies for myocardial repair. The implantation of ESCs into MI rodent models has been associated with an improvement in heart function and a reduction in ventricular remodeling [8, 9, 10]. However, the in vivo differentiation of ESCs into cardiomyocytes at the site of implantation is still controversial [11]. In addition, the risk of teratoma formation is dependent on the selection and the degree of purity of ESC-derived cardiac progenitors [12]. The development of alternative strategies to trigger survival and maturation of ESC-derived cardiomyocytes is of paramount clinical relevance. A new approach for cardiac repair comprises the ex vivo production of three dimensional (3D) tissue constructs with the aim to transplant them epicardially to the damaged myocardium. Recent evidence indicates beneficial effects of this matrix-based cell delivery with respect to cell survival and retention at the site of interest. Early studies used skeletal myoblasts [13, 14] and mesenchymal stem cells [15, 16, 17] as cell models for cardiac 3D constructs. The resulting 3D cell patches were then transferred onto the infarcted myocardium. Despite demonstrated functional benefits, none of these cells have shown any significant cardiac differentiation or functional integration after implantation [18]. For this purpose, stem cells and, ideally, stem cell-derived cardiomyocytes cultured on artificial 3D scaffolds present some advantages. In the best case, the cell patches will not only confer mechanical stability to the weakened heart, but also provide functional integration into the existing myocardium ensuring electro-mechanical coupling between new cells and host tissue and active participation in myocardial contraction. Therefore, tissue engineering represents a promising way to deliver mature cardiac tissue to the site of the infarct, facilitate graft survival and may allow for the replacement of infarcted and fibrotic tissue in the diseased heart. Apart from the correct choice of cell source, another critical parameter is the optimal selection of scaffold material. From a large range of different synthetic and/or naturally occurring polymers, scaffolds were processed and evaluated in vitro with respect to cell-scaffold interactions. Architectural and topographical features as well as chemical and physical surface properties of the scaffold play an essential role for the morphological behavior of the respective cell types growing on such patches [19, 20, 21].

In this study, we investigated the growth properties and scaffold interactions of ESCs and ESC-derived cardiomyocytes on plasma coated poly(ε-caprolactone) (PCL) scaffolds. PCL is a biodegradable polyester with important human applications, such as sutures or as drug delivery device [22]. Currently, PCL is being investigated for its use as implantable biomaterial and scaffold for tissue engineering in various sectors of medicine. In our previous studies, we have shown that PCL matrices are compatible with culture of several cell types (MSCs, skeletal myoblasts and endothelial cells [17, 23]). Here, different scaffold dimensions and arrangements were tested and the morphological interactions of the two developmentally different cell types (ESCs and ESC-derived cardiomyocytes) with the fiber constructs were evaluated. In addition, functional differentiation into the cardiogenic lineage was assessed by cardiac α-actinin staining. For the first time, we cultured and characterised ESC-derived cardiomyocytes on plasma coated PCL substrates and therewith provide the basis for further research on stem cell based cardiac tissue engineering.

### 2 Material and Methods

#### 2.1 Substrate Production and Characterization

Fibrous, plasma coated cell culture substrates were produced as previously established and reported by our group [23]. In short, 3 g of poly(ε-caprolactone) (Sigma-Aldrich, Buchs, Switzerland) were dissolved in 20 mL of a solvent mixture of chloroform:methanol (9:1) or Acetic Acid:pyridine (100:1) (Sigma-Aldrich, Buchs, Switzerland) to produce micron- or nano-scaled fibers, respectively. The solution was processed by electrospinning at an electrical field strength of 1 kVcm⁻¹, a distance of 15 cm between spinneret and collector and a flow rate of 10 μLmin⁻¹ or 30 μLmin⁻¹, respectively. Fibers were collected on a rotating drum (ca. 1000 rpm), resulting in matrices of parallel-oriented fibers. Surface functionalization was accomplished in a RF plasma coating process applying previously established parameters [24]. Briefly, a gas mixture of CO₂, C₂H₄ and Ar (all gases of >99.9% purity and provided by Carbagas, Gümlingen, Switzerland) was introduced to the reactor chamber. Plasma polymerization was carried out at a pressure of 0.1 mbar, a power input of 34 W and for a duration of 15 minutes. An oxygen functional hydrocarbon layer of 12 nm thickness was generated. Functional groups were confirmed by X-ray photoelectron spectroscopy (XPS). Table 1 summarizes the fiber characteristics used in this study.

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<th>fiber diameter (μm)</th>
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<td>n-AL</td>
<td>0.25 ± 0.09</td>
<td>no coating</td>
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<tr>
<td>m-AL</td>
<td>2.7 ± 0.5</td>
<td>no coating</td>
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<tr>
<td>n-AL-PC</td>
<td>0.25 ± 0.09</td>
<td>plasma coated</td>
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<td>m-AL-PC</td>
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Fiber diameter and substrate morphology were assessed based on scanning electron microscopy (SEM) images. Substrates were gold sputtered (Polaron Equipment, SEM coating Unit E5100, Kontron AG, Switzerland; 5 mA, 1 mbar, 7 min), images were acquired at an accelerating voltage of 2 kV and 10 μA current flow on a Hitachi S-4800 instrument (Hitachi High-Technologies, US & Canada, Illinois, USA).

For cell culture, matrices were blanked by biopsy punches (Miltex, Rietheim-Weilheim, Germany) and fixed with minutaie insect pins (EntoSPHINX, Pardubice, Czech Republic) on silicone-coated culture dishes (Sylgard-184 two component silicone, Dow Corning Corporation, Midland, USA). Matrices were sterilized under UV-light overnight.

2.2 Embryonic Stem Cell Culture

Mouse embryonic stem cells (ESC, cell line CGR8) were kindly provided by Dr. Marisa Jaconi (University of Geneva, Switzerland) and expanded under classical culture conditions [25, 26]. Briefly, ESCs were seeded on gelatine-coated dishes and cultured in Glasgow minimum essential medium (G.MEM BHK-21) supplemented with 10% fetal bovine serum, MEM sodium pyruvate, MEM non-essential amino acids, penicillin/streptomycin and 2-mercaptoethanol (all from Gibco, Invitrogen, Carlsbad, USA). Differentiation was suppressed by Leukemia Inhibitor Factor (LIF, EMD Millipore Corporation, Billerica, USA) at a concentration of 1000 UmL⁻¹. ESCs were trypsinized and subcultured every 48 hours.

Cell seeding on the matrices was accomplished by gently depositing a cell suspension of 0.25·10⁶ ESCs in 50 μL medium. Cells were let to adhere for 3 hours prior to medium addition. Medium was changed every second day. Medium was changed every second day. Differentiation was propagated over a time period of 4 days and quantified with a commercially available cell proliferation assay (CyQuant, cell proliferation assay, Invitrogen, Carlsbad, USA). According to the manufacturer, ESCs were lysed, sonicated, vortexed and spectrofluorometrically assessed (Infinite 200, Tecan, Trading AG, Männedorf, Switzerland) at 480 nm excitation and 520 nm emission wavelength.

To examine cell morphology, ESCs were fixed in 4% formaldehyde (Institute of Pathology, Inselspital Bern, Switzerland), dehydrated in an ascending ethanol series and dried in hexamethyldisiloxane (HMDSO, Sigma-Aldrich, Buchs, Switzerland). Cell morphology and cell-matrix interactions were studied based on electron microscopy images, recorded after gold sputtering.

Cardiac phenotype was assessed by immunohistological staining for cardiac α-actinin. Briefly, cells were fixed in 4% formaldehyde and permeabilized in 0.2% triton X-100 (Sigma-Aldrich, Buchs, Switzerland). Unspecific sites were blocked in 3% bovine serum albumin (BSA, Sigma-Aldrich, Buchs, Switzerland). Cells were incubated with the primary antibody (mouse anti α-actinin, Sigma-Aldrich, Buchs, Switzerland), followed by a fluorescently labelled secondary antibody (Alexa 488 conjugated anti mouse, Molecular Probes, Invitrogen, Carlsbad, USA). Fluorescent images were taken on a confocal microscope (Olympus Fluoview FV1000, Olympus Switzerland Ltd.) or on a fluorescent microscope (Nikon, Eclipse 2000, Tokyo, Japan).

Statistical analysis was accomplished with Origin 8.1. Results are presented as mean ± SD. Individual sample groups were compared by a one way ANOVA, followed by a pair-wise comparison with a Bonferroni corrected unpaired t-test. Results were accepted as statistically different for p<0.05.

3 Results and Discussion

3.1 Substrate Characterization

Fibrous matrices were produced by electrospinning and, depending on the solvent, displayed diameters of 0.25 ± 0.09 μm (nano-scaled) or 2.7 ± 0.5 μm (micron-scaled) (Figure 1 and Table 1). XPS analysis confirmed the plasma deposition of an oxygen functional hydrocarbon film (data not shown). The plasma coating rendered the fibers highly hydrophilic and provided carboxyl, carbonyl and hydroxyl groups for enhanced cell adhesion [23].

Matrix evaluation was accomplished in three steps. First, undifferentiated embryonic stem cells (ESC) were seeded on the fibrous scaffolds and assessed for viability and proliferation. In a second step, ESC were developed into embryonic bodies and subsequently seeded on the matrices and assessed for cardiac differentiation. In a third step, ESC derived single cardiomyocytes were seeded on the
matrices and evaluated for cardiac phenotype maintenance and spontaneous beating.

Fiber size as well as surface chemistry were previously demonstrated to significantly affect ESC adhesion, proliferation and differentiation [20, 21]. In addition, we have previously shown that plasma coated fibers, compared to uncoated fibers, enhanced cell adhesion and viability of mouse skeletal C2C12 cells along with a higher cell number [23]. Consequently, we investigated ESC viability, morphology and proliferation on four different scaffolds, displaying varying fiber diameters and distinct surface coatings, denoted n-AL or m-AL and n-AL-PC or m-AL-PC, respectively (see Table 1).

3.2 ESC Viability, Proliferation and Morphology

Cytocompatibility and suitability of the produced 3D scaffolds for ESC culture were investigated in high density cultures. Twenty four hours post seeding, an MTT test revealed cell viability on all four matrices (Figure 2). On nano-scaled fibers, cell viability was reduced compared to micron-scaled fibers, indicated by a sparse blue coloration. The patterned color allocation demonstrated heterogeneous cell distribution or viability on the scaffold. On micron-scaled fibers, however, an intense homogeneous blue coloration was found. Importantly, cell viability and color intensity was independent of the surface coating on micron-scaled fibers. Consequently, further experiments were accomplished with micron scaled fibers, both uncoated and plasma coated.

Cell number on m-AL and m-AL-PC matrices was assessed based on DNA quantification (CyQuant) over a time frame of 4 days and compared to culture conditions on TCPS dishes. The relative number of cells normalized to day 0 are reported in Figure 3. During the first 3 days of cell culture, cell number continuously increased on m-AL-PC substrates, followed by a drop on day 4. Cell numbers were comparable to TCPS culture. On m-AL substrates on the other hand, cell number was significantly decreased on day 3, compared to TCPS culture. Similar cell numbers on m-AL-PC substrates to standard TCPS cultures further indicate the cytocompatibility of the designed matrix. The fibrous architecture and the chemical surface profile did not hamper cell proliferation.

The altered chemical surface composition on plasma coated, compared to uncoated substrates, induced a distinct ESC morphology. On the functionalized matrix, cells displayed a fibroblast-like morphology with bipolar or multipolar elongations along with lamellipodia and filopodia that adhered to the matrix (Figure 4a). ESCs were stretched out and bridged over the gaps between individual fibers (Figure 4b, c). On uncoated matrices, however, a spherical morphology was found, combined with the absence of membrane extensions. Cells only rarely bridged between fibers and rather adhered to single fibers only (Figure 4d, e, f).

ESCs displayed different morphologies on the substrates, indicating a clear preference for plasma coated substrates.

A preferred adhesion to plasma coated substrates and an optimal cell proliferation (Figure 3), resulted in the se-
3.3 Embryoid Body Culture on the Matrices

For cardiogenic differentiation, EBs were prepared using the "hanging drop" method, grown in low adhesion culture dishes and subsequently transferred to fibrous matrices. Between 6 and 17 EBs were harvested and seeded on the matrices. After one day in culture on the scaffolds, EBs did not present any beating areas, whereas 48 hours post seeding, EBs presented 1 to 2 beating areas per EB, increasing to up to 4 beating areas per EB after 72 hours culture. Beating areas grown on the matrices could be detected under light microscopy and were comparable in size and activity to TCPS controls. Importantly, ESC that were not differentiated by the hanging drop method did not attain a cardiogenic phenotype on the substrates and no beating areas were apparent.

In SEM images, EBs were clearly visible as large cell aggregates of spherical, densely clustered cells. Towards the periphery, cells presented a flattened morphology that was in tight contact with the underlying fibers (Figure 5).

Immunohistochemistry revealed cardiac α-actinin(+) cells, with a characteristic striation pattern (Figure 6) and confirmed cardiomyogenic differentiation of ESCs. Cardiac α-actinin(+) staining was found in cells at the periphery of EBs, clearly distinguished from surrounding, negatively labelled tissue. Areas with positive staining corresponded to areas with high beating activity.

In EBs, different beating areas with asynchronous rhythmicity can be detected, and the ESC-derived cardiomyocytes within the EBs present an immature phenotype with irregularly organized striation pattern, when compared with the sarcomeric organization of adult cardiomyocytes. EB culture was only maintained for 72 hours, and it remains elusive, to which extent synchronous beating and/or organized striation patterns may develop during longer culture periods.

Additionally, EBs constitute an assembly of very heterogeneous cell types and differentiation states. In order to achieve a monolayer of spatially organized cardiomyocytes, beating areas were excised from EBs on TCPS, dissociated into single cells and individually seeded onto the matrices.

3.4 Cardiomyocyte Phenotype Maintenance on the Matrices

Isolated cardiomyocytes were cultured on fibrous m-AL-PC matrices and were shown to maintain their phenotype over a 3-day culture period. Cardiomyocytes presented spontaneous beatings and were positively labelled for α-actinin.
Figure 6. Fluorescent image of $\alpha$-actinin$^\text{(*)}$ labelled ESC-derived cardiomyocytes. A characteristic striation pattern is apparent in cells at peripheral areas of the EB. Scale bar 10 $\mu$m.

Figure 7 shows a comparison of $\alpha$-actinin$^\text{(*)}$ ESC-derived cardiomyocytes grown in 2D culture conditions on a control TCPS (a, b) and in 3D culture conditions on an m-AL-PC scaffold. Characteristic striation pattern of $\alpha$-actinin expression is clearly present after several days in 3D culture systems. Thus, the fibrous matrix, its architecture and chemical composition, do not interfere with the differentiation status of the cardiomyocyte culture.

Very little is known about the specific cell-material interactions and in particular the triggered differentiation of ESCs on a 3D matrix. Earlier studies on ESC-derived cardiomyocyte culture on synthetic films emphasized on the importance of adequate surface coatings [19]. Laminin or collagen IV coated polyurethane (PU) films, as opposed to uncoated films, induced enhanced adhesion and beating areas. Coatings with extracellular matrix proteins induce, however, certain risks and uncertainties with respect to batch to batch reproducibility, foreign body response to the xenogenic origin or protein contaminations. Introducing functional groups in a plasma process on the other hand generates highly reproducible coatings that are devoid of naturally occurring proteins but similarly enhance cell adhesion. We showed that cell spreading was enhanced on plasma coated substrates, compared to hydrophobic, uncoated PCL fibers.

Anisotropic structure and force generation is of paramount importance to the myocardium to execute its contractile function. This impedes specific challenges with respect to the choice of scaffold architecture. In particular, inducing spatial orientation in order to allow electromechanical coupling between cardiomyocytes should be considered for the in vitro generation of cardiac tissue. To this end, matrices of parallel oriented PU electrospun fibers have been shown to better maintain ESC-derived cardiomyocytes, indicated by an improved sarcomeric organization and an elongated, spatially organized cardiomyocytes morphology along the fibers [27].

In a recent article by Gupta et al. [21], finely orchestrated mechanical and chemical scaffold properties by co-block polymer synthesis resulted in enhanced cardiomyogenic protein expression after seeding of EBs onto the fibrous membranes. On a 4%PEG-86%PCL-10%carboxylated-PCL scaffold co-polymer, enhanced cardiogenic hallmarks were found. Albeit multiple studies focused on the comparison and evaluation of a wide variety of scaffold architectures and compositions, to our knowledge no studies have been accomplished to investigate the cardio-inductive potential of the respective scaffolds. Cardiomyocytes were previously generated by the "hanging drop" method or in bioreactors [20] and subsequently seeded on scaffolds. The formation and culture of EBs induces an additional step during cell culture and with respect to clinical applications, where time is money, the direct seeding of isolated cells and the differentiation into beating cardiomyocytes by inductive properties of the substrate alone would be highly preferred. However, it remains elusive, whether the designed scaffold is cardio-inductive under appropriate culture conditions, and if the traditional induction of cardiomyogenesis via the "hanging drop" method can be bypassed.

4 Conclusion

Structural and functional integrity of individual cells within the engineered tissue is a prerequisite for successful cardiac patch generation and functional integration upon implanta-
tion into the heart. We successfully cultured ESC-derived cardiomyocytes on synthetic matrices of PCL. EB forma-
tion, subsequent isolation and seeding of beating areas were
shown to result in α-actinin(+), contractile cardiomyocytes
on the patches. Our results demonstrate that ESC prolifer-
ation as well as cardiogenic phenotype are not hampered,
yet supported on the electrospun matrices. Future work
needs to consider seeding of ESC-derived cardiomyocytes
at higher density and the formation of a homogeneous mono-
or multilayer of synchronously beating cardiomyocytes. For
in vivo evaluation and particularly translational medical re-
search, ESC-derived cardiomyocytes need to be carefully
characterised and selected prior to implantation.

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