Behavior of CMPCs in unidirectional constrained and stress-free 3D hydrogels

Citation for published version (APA):

Marion, van, M. H., Bax, N. A. M., van Turnhout, M. C., Mauretti, A., van der Schaft, D. W. J., Goumans, M. J. T. H., & Bouten, C. V. C. (2015). Behavior of CMPCs in unidirectional constrained and stress-free 3D hydrogels. *Journal of Molecular and Cellular Cardiology*, 87, 79-91. <https://doi.org/10.1016/j.yjmcc.2015.08.010>

Document license:
TAVERNE

DOI:
[10.1016/j.yjmcc.2015.08.010](https://doi.org/10.1016/j.yjmcc.2015.08.010)

Document status and date:
Published: 01/10/2015

Document Version:
Publisher's PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.tue.nl/taverne

Take down policy

If you believe that this document breaches copyright please contact us at:

openaccess@tue.nl

providing details and we will investigate your claim.

Behavior of CMPCs in unidirectional constrained and stress-free 3D hydrogels

Mieke H. van Marion ^{a,*}, Noortje A.M. Bax ^{a,b}, Mark.C. van Turnhout ^a, Arianna Mauretti ^a, Daisy W.J. van der Schaft ^a, Marie José. T.H. Goumans ^c, Carlijn V.C. Bouten ^{a,b}

^a Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands

^b Institute for Complex Molecular Systems, Eindhoven University of Technology, Eindhoven, The Netherlands

^c Department of Molecular Cell Biology and Center for Biomedical Genetics, Leiden University Medical Center, The Netherlands

ARTICLE INFO

Article history:

Received 25 March 2015

Received in revised form 3 August 2015

Accepted 10 August 2015

Available online 14 August 2015

Keywords:

3D cell culture

Tissue engineering

Cardiomyocyte progenitor cells (CMPCs)

Matrix remodeling

Cardiac regeneration

Mechanical forces

ABSTRACT

Cardiomyocyte progenitor cells (CMPCs) are a candidate cell source for cardiac regenerative therapy. However, like other stem cells, after transplantation in the heart, cell retention and differentiation capacity of the CMPCs are low. Combining cells with biomaterials might overcome this problem. By serving as a (temporal) environment, the biomaterial can retain the cells and provide signals that enhance survival, proliferation and differentiation of the cells. To gain more insight into the effect that the encapsulation of CMPCs in a biomaterial has on their behavior, we cultured CMPCs in unidirectional constrained and stress-free collagen/Matrigel hydrogels. CMPCs cultured in 3D hydrogels stay viable and keep their cardiomyogenic profile independent of the application of strain. Moreover, the increased expression of Nkx2.5, myocardin and cTnT in 3D hydrogels compared to 2D cultures, suggests enhanced cardiomyogenic differentiation capacity of cells in 3D. Furthermore, increased expression of collagen I, collagen III, elastin and fibronectin and of the matrix remodeling enzymes MMP-1, MMP-2, MMP-9, and TIMP-1 and TIMP-2 in the 3D hydrogels is indicative of an enhanced matrix remodeling capacity of CMPCs in a 3D environment, independent of the application of strain. Interestingly, the additional application of static strain to the 3D hydrogels, as imposed by hydrogel constraint, stabilized CMPC viability and proliferation, resulted in enhanced cardiac marker protein expression and appeared crucial for cellular organization and morphology. More specifically, CMPCs cultured in 3D collagen/Matrigel constrained hydrogels became readily mechanosensitive, had a rod-shaped morphology, and responded to the applied strain by orienting in the direction of the constraint. Overall, our data demonstrate the applicability of CMPCs in a 3D environment since encapsulation of CMPCs may stabilize survival and proliferation, can enhance the differentiation and remodeling capacity of the cells, and could induce cellular re-organization, which all may contribute to an improved efficiency of cardiac stem cell therapy.

© 2015 Published by Elsevier Ltd.

1. Introduction

Stem cell therapy is a new approach for heart regeneration, which aims at creating de novo cardiac tissue and preventing fibrosis. Cardiac stem cell therapy efficiency has been studied extensively in both animal studies and clinical trials [1–5]. Overall, a small increase in cardiac function is reported [1–3,6], mainly ascribed to paracrine signaling since cell retention is very low and stem cell differentiation is often not observed [3,6,7]. One possibility to improve the

efficiency and effectiveness of stem cell therapy is to combine cells with biomaterials [8]. The biomaterial can be applied as tissue patch or serve as injectable cell carrier. Injection of biomaterials with cells or biomaterials alone has shown to prevent negative remodeling after MI and passively contributes to the regeneration process by changing the local mechanical properties of the heart tissue [9–14]. By encapsulating stem cells in a biomaterial, cell survival and retention are increased and paracrine signaling is induced [9–13]. Furthermore, biomaterials may serve as a proper micro-environment for stem cells to integrate and differentiate.

Traditional culture methods rely on two-dimensional (2D) adhesive substrates. However, upon encapsulation in a biomaterial, cells experience a three-dimensional (3D) rather than a 2D environment. The interactions of cells with the biomaterial, cellular organization and spatial distribution in a 3D environment resemble the in vivo situation much more [15]. The transition towards a 3D environment

* Corresponding author.

E-mail addresses: m.h.v.marion@tue.nl (M.H. van Marion), n.a.m.bax@tue.nl (N.A.M. Bax), m.c.v.turnhout@tue.nl (M.C. van Turnhout), a.mauretti@tue.nl (A. Mauretti), d.w.j.v.d.schaft@tue.nl (D.W.J. van der Schaft), M.J.T.H.Goumans@lumc.nl (M.J.T.H. Goumans), c.v.c.bouten@tue.nl (C.V.C. Bouten).

can result in different cellular behaviors, such as changes in proliferation and differentiation [16]. For example, Liu et al. [17] demonstrated that embryonic stem cells cultured in 3D show an increased expression of genes that regulate cell growth, proliferation, differentiation and extracellular matrix (ECM) production and remodeling. More specifically, Duan et al. [18] demonstrated that ESCs cultured in hydrogels composed of collagen and decellularized ECM, promoted cardiomyogenic differentiation and maturation. Therefore, 3D culture systems can give important insight on how stem cells respond after transplantation into the heart. Upon transplantation into the heart, stem cells also become exposed to mechanical loads. The contractile and pulsatile forces of the beating heart can be considered as cyclic strains and stresses at the cellular level [19]. Strain is known to affect the proliferation, cellular organization and differentiation of stem cells [20]. For example, Huang et al. [21] showed that cyclic mechanical straining of bone marrow derived mesenchymal stem cells results in increased expression of cardiomyocyte genes and an increase in the gap-junction protein connexin43. Furthermore, Fink et al. [22] and Hansen et al. [23] demonstrated that neonatal rat cardiomyocytes cultured in 3D longitudinally constrained hydrogels show alignment, increased cardiomyocyte maturation, and gap junctional coupling. This indicates that the application of constraint or dynamic strain to stem cells may stimulate differentiation towards a more mature phenotype tissue integration. However, to be able to sense and respond to mechanical forces, cells need to be able to interact with the ECM or biomaterial via specific anchoring proteins [24].

There are multiple stem cell sources for cardiac regenerative therapy. Cardiomyocyte progenitor cells (CMPCs) form an interesting candidate cell source for regenerative cell therapy since these cells reside in the heart itself. CMPCs have the ability to differentiate into mature cardiomyocytes and are able to improve cardiac function after MI via paracrine effects. Additionally, these cells have matrix production and remodeling capacity [25–30]. Although the properties of CMPCs are promising for application in cardiac regenerative therapy, the effects observed *in vivo* are only temporarily beneficial [25,26,29]. Therefore, this present study aims to elucidate whether 3D culture of CMPCs, by encapsulation in a biomaterial, can increase the regenerative capacity of CMPCs. By comparing unidirectional constrained hydrogels with stress-free (unconstrained) 3D culture conditions, the importance of contact with the host tissue can be elucidated. The constrained culture also provides application of static strain which gives insight in the ability of CMPCs to respond to mechanical forces. Therefore, viability and proliferation of CMPCs in a 3D collagen/Matrigel based hydrogel were studied in both constrained and stress-free culture conditions. Additionally, cardiac differentiation capacity and the ability of CMPCs to produce ECM and matrix remodeling proteins in 3D were investigated.

This study shows that CMPCs can be cultured in 3D without losing their early cardiomyogenic profile. Differentiation as well as the ECM remodeling capacity of CMPCs is increased in 3D hydrogels compared to 2D cultures, independent of the application of strain. Moreover, CMPCs cultured in 3D collagen/Matrigel hydrogels respond to static mechanical strain as induced by constraint by cell alignment and constrained culture stabilizes cell proliferation and organization. These results demonstrate the applicability of CMPCs in a 3D environment, and that pretreatment of CMPCs in a 3D culture system may enhance their *in vivo* regenerative capacity for cardiac regenerative therapy compared to 2D cultured CMPCs.

2. Materials and Methods

2.1. Culture of cardiomyocyte progenitor cells

Human fetal CMPCs were isolated from fetal hearts that were collected after elective abortion based on individual informed consent and after approval by the Medical Ethics committee of the Leiden

University Medical Centre. The isolated CMPCs were cultured and subsequently immortalized by lentiviral transduction of hTert and BMI-1 as described previously [29,30]. Immortalized L9TB CMPCs were cultured in growth medium (GM) consisting of M199 (Gibco)/EGM2 (3:1) supplemented with 10% fetal bovine serum (FBS) (Greiner bio-one), 1% non-essential amino acids (Gibco), and 1% penicillin/streptomycin (Lonza) on 0.1% gelatin (Sigma)/PBS (Sigma) coated flasks.

2.2. Culture of 3D cell-hydrogel constructs

For 3D culture, CMPCs were encapsulated in collagen/Matrigel hydrogels, composed of 50% rat tail collagen type I (3.2 mg/ml, BD Biosciences), 8.3% growth factor reduced Matrigel™ (BD Bioscience), 2.7% NaOH (0.5 M, Sigma-Aldrich) and 39% SP⁺⁺ growth medium. To induce static strain, cell-hydrogel constructs were casted in and between two house-shaped Velcro attachment points (5 × 5 mm, 12 mm interspace) in non-adhesive 6-Well tissue culture plates (Fig. 1A) as previously described [31–33]. These constructs are further referred to as unidirectional constrained constructs. To create stress-free constructs, cell-hydrogel mixture was casted as droplets in non-adhesive 6-well plates (Fig. 1B).

Both types of constructs consisted of 400 µl collagen/Matrigel hydrogel solution mixed with 800,000 CMPCs. After polymerization of the constructs for 1 h at 37 °C, growth medium was carefully added to the plates. It was ensured that the constructs did not attach to the culture plates. Constructs were cultured for 9 days and growth medium was replaced twice a week. To induce cardiomyogenic differentiation, half of the constructs were subjected to the differentiation protocol as previously described for 2D monolayer culture [29]. Briefly, after 1 day of culture the constructs were treated with 5 µM 5-azacytidine (Sigma) for 72 h in differentiation medium (IMDM (Iscove's Modified Dulbecco's Medium)/Ham-F12 (1:1) (Gibco), 2% horse serum, 10^{−4} M ascorbic acid (Sigma), 1% non-essential amino acids, 1% insulin–transferrin–selenium (Lonza), and 1% penicillin/streptomycin) to inhibit proliferation of the cells [29]. After 72 h, the medium was changed followed by transforming growth factor (TGF-β1) stimulation on day 7 (1 ng/ml; Sigma) (IFDIFF media) [27,29]. Constructs were sacrificed at day 1 and day 9, and analyzed for viability, proliferation, cardiac differentiation, and mechanical responsiveness. Furthermore, expression of ECM genes and proteins was investigated. Cell culture and differentiation of both types of 3D constructs were performed in three independent experiments.

2.3. Cell viability

Viability of CMPCs in constrained and stress-free constructs was determined using a cell tracker green (CTG) (Molecular Probes) and propidium iodide (PI) staining (Molecular Probes). Constructs were washed twice with PBS and incubated with 10 µM OregonGreen 488 labeled CTG in growth or differentiation medium for 15 min at 37 °C. After vigorous washing with PBS, constructs were incubated with 10 µM PI in growth or differentiation media for 30 min at 37 °C. After incubation, constructs were washed with PBS, submerged in media and visualized using a Zeiss confocal microscope (N = 6 per test group). Images were analyzed for viability using a custom-made MATLAB script (based on [34]). Briefly, based on visual inspection a threshold was defined for each channel (red (PI)/green (CTG)), separately. These channel specific thresholds were used to convert each image into a binary image. In these binary images, the number of connected regions (i.e., groups of connected pixels) per channel was counted using built-in MATLAB functions. A connected region was considered to represent a cell when it contained at least 6 pixels (pixel size 900 nm × 900 nm). Dead cells were counted in the PI channel, and living cells were counted in the CTG channel.

Fig. 1. Constrained and stress-free constructs. Microscopy images of (A) constrained and (B) stress-free constructs after 1 day of culture. One of the house-shaped Velcro attachment point is marked with the white lines (A).

2.4. Quantitative PCR

mRNA was isolated from 1 sample per test group per experiment ($N = 3$) using the Qiagen RNeasy isolation kit (Qiagen) according to the manufacturer's instructions. Gels were grounded in RLT buffer containing 1% β -mercapto-ethanol using an Ultra-Thurrax (IKA T10 basic). RNA was amplified and converted to cDNA using a QuantiTect whole transcriptome amplification kit (Qiagen) as described by the manufacturer. Primers for qPCR were designed with Beacon Designer 7.0 (Premier Biosoft International). Primer sequences and annealing temperatures are presented in Table 1. cDNA samples were subjected to qPCR using iQTM SYBR@ Green Supermix (Bio-Rad) and the Bio-Rad IQTM5 detection system (Version2.0).

2.5. Immunofluorescent staining

Immunofluorescent staining was performed on constrained and stress-free constructs to assess the proliferation, cardiomyogenic differentiation and matrix production of the encapsulated CMPCs.

Table 1
Primer sequences.

Primer	Sequence	Tm
Nkx2.5	fw CCC CTG GAT TTT GCA TTC AC	60
	rv CGT GCG CAA GAA CAA ACG	
GATA4	fw TCC AGC AAC TCC AGC AAC	60
	rv AGA CAT CGC ACT GAC TGA G	
Myocardin	fw AGA TGG ATG AAC TCC TGG AC	60
	rv AGT TGG ACT TCG GGA AGA TC	
Cardiac troponinT (cTnT)	fw CGA CGA GAG GAG GAG AAC	60
	rv CCG CTC TGT CTT CTG GAT GTA AC	
Collagen I	fw AAT CAC CTG CGT ACA GAA CGG	60
	rv TCG TCA CAG ATC ACG TCA TCG	
Collagen III	fw ATC TTG GTC AGT CCT ATG C	60
	rv TGG AAT TTC TGG GTT GGG	
Elastin	fw CTG GAA TTG GAG GCA TCG	60
	rv TCC TGG GAC ACC AAC TAC	
Fibronectin	fw AAG ACC AGC AGA GGC ATA AGG	60
	rv CAC TCA TCT CCA ACG GCA TAA TG	
MMP-1	fw CGC ACA AAT CCC TTC TAC CC	55
	rv CTG TCG GCA AAT TCG TAA GC	
MMP-2	fw ATG ACA GCT GCA CCA CTG AG	60
	rv ATT TGT TGC CCA GGA AAG TG	
MMP-9	fw TGG GGG GCA ACT CGG C	60
	rv GGA ATG ATC TAA GCC CAG	
TIMP-1	fw TGA CAT CCG GTT CGT CTA CA	49
	rv TGC AGT TTT CCA GCA ATG AG	
TIMP-2	fw GGA GGA ATC GGT GAG GTC	60
	rv AAC AGG CAA GAA CAA TGG	

Abbreviations used in this table: fw, forward primer sequence; rv, reverse primer sequence; Tm, temperature.

Constructs were washed three times with PBS and fixated for 1 h in 3.7% formalin (Merck) in PBS. After fixation, constructs were washed with PBS again and incubated in 30% sucrose (Merck) overnight. Constrained gels were cut loose from the Velcro attachment point. Gels were embedded in TissueTek cryopreservation solution, snap/frozen in ice cold isopentane, and stored at -20°C . Slices of $10\text{ }\mu\text{m}$ thickness were cut in longitudinal cross-sections using a cryotome (Microm HM550), which were stored at -80°C until staining. Just before staining, sections were thawed and air-dried at room temperature. For immunofluorescent staining, sections were permeabilized with 0.5% Triton X-100 (Merck) in PBS for 10 min. Nonspecific binding of antibodies was blocked with 1% horse serum in PBS for 20 min. Subsequently, cells were incubated overnight at 4°C with primary antibodies in PBS containing 10% horse serum. All antibodies and their dilutions are listed in Table 1. Cell proliferation was assessed by staining for the Ki-67 cell cycle marker. Primary antibodies against GATA4, Nkx2.5, α -actinin, cardiac troponinI (cTnI) and vimentin were used to determine whether the cells kept their cardiomyogenic expression profile. To determine matrix production by the encapsulated CMPCs, primary antibodies against collagen I, collagen III, fibronectin and elastin were included. To distinguish between the rat collagen I which was used in the collagen/Matrigel hydrogel and the human collagen I produced by the CMPCs, a primary antibody specific for human collagen I was used (Table 2). Cross-reactivity of the human collagen I antibody with rat collagen I has been tested and gave negative results (data not shown).

After primary antibody incubation, cells were washed in 0.1% Tween-20 (Merck) in PBS and incubated with secondary antibodies for 1 h at room temperature (listed in Table 1). TRITC labeled phalloidin (Sigma) (incubation for 1 h at room temperature) was used to stain the actin cytoskeleton. Cells were counter stained with DAPI (Sigma) (1:500) to visualize the cell nuclei. Slides were embedded in Mowiol mounting medium, covered with cover glasses and visualized using a Zeiss Axiovert 200 M (fluorescence) microscope ($N = 6$ per test group).

2.6. Gelatin zymography

Gelatin zymography was performed to semi-quantitatively determine the proteolytic activity of MMP-2 and -9 in media samples from constrained and stress-free constructs. Zymography was performed as described previously [35]. In short, $10\text{ }\mu\text{l}$ media was loaded and separated on a 1.5 mm 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (Bio-Rad) that was polymerized in the presence of 0.6% (w/v) gelatin. After running, the gels were rinsed twice in 2.5% Triton X-100 for 30 min at 37°C to activate the MMPs and incubated overnight in substrate buffer pH 8.5 (50 mM TRIS, 5 mM CaCl_2). Gelatinolytic activity was visualized by staining the gel with Coomassie Blue solution (0.25% (w/v) Coomassie Brilliant

Table 2
Antibodies used for immunofluorescent staining.

Antigen	Source	Cat. no	Clone	Isotype	Label	Species	Dilution
Nkx2.5	SC	sc-14033	H-114	IgG	–	Rabbit	1/200
GATA4	SC	sc-25310	G-4	IgG2a	–	Rabbit	1/200
α -Actinin	SA	A7811	EA-53	IgG1	–	Mouse	1/400
cTnI	SC	sc-15368	H-170	IgG	–	Rabbit	1/250
Ki-67	TS	rb1510P0	–	IgG	–	Rabbit	–
Phalloidin	SA	49409	–	–	Atto 488	Rabbit	1/200
Collagen I	SA	C-2456	COL-1	IgG1	–	Mouse	1/100
Collagen I	AB	ab138492	–	IgG	–	Rabbit	1/200
Collagen III	SA	ab7778	–	IgG	–	Rabbit	1/200
Elastin	AB	ab21610	–	IgG	–	Rabbit	1/200
Fibronectin	SA	F3648	–	IgG	–	Rabbit	1/200
Vimentin	AB	20346	VI-10	IgM	–	Mouse	1/1000
Mouse IgG1	IG	A21121	–	IgG1	Alexa Fluor 488	Goat	1/300
Mouse IgG1	MP	A21127	–	IgG1	Alexa Fluor 555	Goat	1/300
Mouse IgG2a	MP	A21131	–	IgG2a	Alexa Fluor 488	Goat	1/300
Mouse IgM	MP	A21426	–	IgM	Alexa Fluor 555	Goat	1/500
Rabbit IgG	MP	A21428	–	IgG (H + L)	Alexa Fluor 555	Goat	1/200
Rabbit IgG	MP	A31572	–	IgG (H + L)	Alexa Fluor 555	Donkey	1/300

Abbreviations used in this table: AB, Abcam; SA, Sigma Aldrich; SC, Santa Cruz; IG, Invitrogen; MP, Molecular probes, TS, Thermo Scientific.

Blue R-250 (Sigma), 50% (v/v) methanol, 10% (v/v) acetic acid (Sigma)) for 2 h at room temperature, followed by destaining in destainer solution (4% methanol, 10% acetic acid) for 1 h. The amount of active or latent MMP-2 (resp. 66 kDa and 72 kDa) and MMP-9 (resp. 86 kDa and 92 kDa) were visualized using the Proxima C16 Phi + (Isogen). Intensity of the bands ($\text{INT} \times \text{mm}^2$ with global background correction) was measured using Quantity One image analysis software (Biorad) ($n = 2$). Intensity of the bands was normalized to the intensity of the constrained samples at day 1.

2.7. Internal stress formation

Retraction of the constrained constructs was determined as measure for internal stress formation ($N = 6$). After 1 and 9 days of culture, constructs were cut at one side, next to the Velcro attachment point. Pictures were taken before and 5 min after cutting. Tissue retraction was calculated from the length of the sample before and after cutting, and expressed as % of the original length.

2.8. Cell orientation analysis

Cell orientation was determined from confocal z-stack images of the mid-plane of CTG stained constructs ($N = 4-6$, 1 image per sample). Using an in-house algorithm based on the method of Frangi et al. [36], the orientation of each structure to be part of a vessel (fiber-like structure) was calculated [37,38]. In our case, cells that have an elongated shape are considered as vessel. In brief, for each pixel the Hessian and corresponding Eigenvalues and Eigenvectors were determined. From this, the vesselness (the probability that a pixel belongs to a vessel), as determined by Frangi et al. [36], was calculated, followed by an estimation of the structure orientation. The orientation is plotted in a histogram, showing the fiber fraction per angle ($0^\circ-180^\circ$). The algorithm was implemented in Mathematica (Wolfram Research), histogram data was plotted using MATLAB (Mathworks).

2.9. Statistical analysis

All data is presented as mean $\pm$ standard deviation. qPCR data was quantified using the comparative threshold cycle (C_t) method of Livak et al. [39]. qPCR data is presented as fold change ($2^{-\Delta\Delta C_t}$), by comparing signals of the CMPCs embedded in collagen/Matrigel to those of 1 day 2D cultured undifferentiated CMPCs [30], both relative to an internal control, TOP-1. Cell proliferation was quantified by counting (by two independent researchers) the number of Ki-67 positive cells relative to the total number of cells. Statistical analysis was performed by

comparison between groups (constrained vs stress-free) and within groups by using a one-way Kruskal–Wallis with Dunn post-hoc test or a one-way ANOVA with Bonferroni correction for multiple comparisons depending on the Gaussian distribution. Statistical significance was defined when $P < 0.05$. Graphics and statistical analysis were performed with GraphPad Prism software (version 5.04).

3. Results

3.1. Viability and proliferation of CMPCs in a 3D environment

Since this is the first time that CMPCs are cultured in 3D constructs, viability and proliferation were elucidated. Previously, the morphology of CMPCs in 2D was described as small spindle-shaped like cells [27]. CMPCs embedded in constrained 3D constructs elongated in the direction of the constraint, as observed by the CTG staining, while CMPCs embedded in stress-free constructs remained much more rounded, though some cells had thin embranchments (Fig. 2). Semi-quantitative analysis of the amount of viable cells present in the constructs at day 1, revealed high viability of $70.5\% \pm 11.3$ and $78.3\% \pm 12.9$ in both constrained and stress-free constructs, respectively (Fig. 2A,D,I,L).

Both the culture time of 9 days ($78.0\% \pm 15.0\%$, $P < 0.05$) and the initiation of cardiomyogenic differentiation ($77.6\% \pm 8.4\%$) slightly increased viability of CMPCs in the constrained constructs (Fig. 2B–D). In stress-free constructs, after 9 days of culture the viability slightly decreased in both growth medium ($70.0\% \pm 10.4\%$) as well as in differentiation medium ($70.5\% \pm 10.9\%$) (Fig. 2J–L).

Cell proliferation was determined by the analysis of staining with Ki-67. On day 1, $24.6\% \pm 8.7\%$ of the cells in the constrained constructs and $29.9\% \pm 3.5\%$ in the stress-free constructs were proliferating (Fig. 2H,P). In constrained constructs during extended culture time the amount of proliferation cells almost halved to $14.1\% \pm 2.1\%$ ($P < 0.05$) in growth medium and $13.2\% \pm 2.8$ ($P < 0.01$) in differentiation medium (Fig. 2E–H). On the other hand, the number of Ki-67 positive cells in stress-free constructs was almost completely abolished after 9 days under normal culture conditions ($1.6\% \pm 1.4\%$) ($P < 0.001$), while in differentiation medium there was a strong decrease in the number of proliferating cells ($5.2\% \pm 1.7\%$) (Fig. 2M–P).

3.2. Cardiac specific development of CMPCs in 3D

Gene expression of cardiac transcription factors GATA4, Nkx2.5 and myocardin, and the cardiac marker cTnT were determined as confirmation of the commitment to the cardiac lineage (Table 3, Fig. 3 [30]).

Fig. 2. Viability and proliferation in constrained and stress-free constructs. Confocal images show living (green, CTG) and dead cells (red, PI) in constrained (A–C) and stress-free constructs (I–K). Semiquantitative analysis of the amount of viable cells in (D) constrained and (L) stress-free constructs. Immunofluorescent images showing Ki-67 positive cells (green) in constrained (E–G) and stress-free constructs (M–O). (H) and (P) represent the quantification of the amount of Ki-67 positive cells in constrained and stress-free constructs, respectively. (A,E,I,M) 1 day of culture; (B,F,J,N) 9 days of culture in growth medium (GM); (C,G,K,O) 9 days in culture in differentiation medium (DM). Data on number of Ki-67 positive cells and quantification of viability are represented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $N = 6$. Scale bar: 50 μm .

Culturing CMPCs in a 3D environment induced the expression of the cardiac transcription factor Nkx2.5 and cardiac marker myocardin compared to the 2D cultures (Table 3, Fig. 3B,C [30]), which is indicative of an increased cardiomyogenic differentiation potential in a 3D environment. The expression of the cardiac transcription factor GATA4 in a 3D environment was comparable to previously published 2D data (Table 3, Fig. 3A [30]). Extended culture time and subjection to the differentiation protocol in constrained constructs decreased the expression of Nkx2.5, while in stress-free constructs this decrease was not present (Fig. 3B). Moreover, during exposure to differentiation medium the expression of the transcription factor and regulator of the cardiac differentiation pathway, myocardin significantly increased by 2.3 fold ($P < 0.05$) in constrained constructs, while in stress-free constructs the expression of myocardin during differentiation was comparable to the expression at day 1 (Fig. 3C). Furthermore, the expression of

cardiomyocyte marker cTnT was significantly increased by 450 fold ($P < 0.05$) in the stress-free constructs exposed to differentiation medium, while in constrained constructs expression was constant over time and in both types of culture media (Table 3, Fig. 3D). Although the expression of cTnT at d9 in stress-free constructs seems to be highly expressed, the expression at day 1 is very low. Therefore, this change in expression profile can be seen as an on-off response. Construct constraintment did not substantially improve the differentiation capacity of CMPCs compared to a stress-free environment (Fig. 3).

However, at the protein level construct constraintment seemed to result in an enhanced expression as shown by the more pronounced staining and organization of cardiac markers (Fig. 4). Both constrained and stress-free constructs showed protein expression of Nkx2.5 and GATA4 (Fig. 4A,B,F,G). Remarkably, protein expression of GATA4 was

Table 3
Expression cycle threshold (Ct).

Gene	Process of differentiation in 2D			Constrained constructs			Stress-free constructs		
	Day 1	Week 1	Week 2	Day 1	Day 9 (GM)	Day 9 (DM)	Day 1	Day 9 (GM)	Day 9 (DM)
<i>Collagen I</i>	16.11 ± 0.59	14.53 ± 0.41	14.57 ± 0.54	18.39 ± 0.28	18.19 ± 0.58	19.03 ± 1.00	20.61 ± 0.50	19.18 ± 0.21	18.45 ± 0.39
<i>Collagen III</i>	22.83 ± 0.28	17.55 ± 0.42	17.20 ± 0.50	21.14 ± 0.48	17.22 ± 0.53	18.14 ± 0.57	21.60 ± 0.69	17.67 ± 0.80	16.26 ± 0.79
<i>Fibronectin</i>	16.68 ± 0.47	15.77 ± 0.59	15.40 ± 0.54	9.39 ± 0.70	9.90 ± 0.26	8.13 ± 0.81	8.70 ± 0.12	8.96 ± 0.25	8.78 ± 0.26
<i>Elastin</i>	39.70 ± 1.05	28.46 ± 1.35	26.24 ± 1.76	34.67 ± 1.94	35.88 ± 1.29	28.81 ± 0.23	36.93 ± 0.25	36.92 ± 0.62	27.61 ± 1.64
<i>MMP-1</i>	21.28 ± 0.54	26.24 ± 1.36	26.13 ± 0.82	15.88 ± 0.36	22.20 ± 0.65	22.07 ± 0.90	16.00 ± 0.42	21.48 ± 0.42	21.39 ± 1.16
<i>MMP-2</i>	20.51 ± 0.43	17.81 ± 0.69	14.78 ± 0.26	13.37 ± 0.41	11.43 ± 0.27	10.32 ± 0.14	12.42 ± 0.30	11.07 ± 0.50	10.00 ± 0.24
<i>MMP-9</i>	33.22 ± 1.28	31.26 ± 1.41	32.42 ± 0.65	30.71 ± 0.85	31.86 ± 0.45	26.33 ± 3.24	30.19 ± 1.24	26.75 ± 1.62	23.85 ± 2.25
<i>TIMP-1</i>	16.41 ± 0.57	17.06 ± 0.67	16.87 ± 0.32	13.11 ± 0.24	12.35 ± 0.44	15.50 ± 1.25	12.50 ± 0.30	11.08 ± 0.12	14.69 ± 0.48
<i>TIMP-2</i>	18.54 ± 0.48	17.74 ± 0.21	17.90 ± 0.25	17.92 ± 0.64	17.90 ± 0.15	18.05 ± 0.54	17.85 ± 0.27	18.50 ± 0.17	17.93 ± 0.21
<i>GATA 4</i>	21.92 ± 0.87	22.10 ± 0.68	22.23 ± 0.36	19.14 ± 0.75	18.93 ± 0.51	19.66 ± 0.79	18.84 ± 0.53	19.26 ± 0.29	19.67 ± 0.73
<i>Nkx2.5</i>	22.28 ± 0.54	22.93 ± 0.34	23.15 ± 0.31	16.00 ± 0.48	17.18 ± 0.33	18.27 ± 0.68	18.73 ± 0.25	17.18 ± 0.63	17.74 ± 0.39
<i>Myocardin</i>	31.98 ± 1.20	31.73 ± 1.27	31.46 ± 1.42	25.31 ± 0.49	26.56 ± 0.24	25.19 ± 0.64	25.76 ± 0.49	25.62 ± 0.61	24.91 ± 0.26
<i>cTnT</i>	34.77 ± 0.76	28.79 ± 0.57	28.32 ± 0.50	32.88 ± 1.14	31.77 ± 0.30	31.41 ± 1.31	33.71 ± 1.09	31.18 ± 1.66	24.48 ± 0.98
<i>TOP</i>	23.67 ± 0.61	24.11 ± 0.18	24.00 ± 0.16	21.278 ± 0.26	21.76 ± 0.35	22.25 ± 0.67	22.24 ± 0.86	22.00 ± 0.33	21.71 ± 0.25

The cycle threshold (Ct) for each gene varied over the time course and after addition of differentiation media (DM). Not applicable Ct values were set a value of 40. The table represents mean Ct values ± SD. Data of the process of differentiation in 2D were used as published previously [30]. High expression genes gave a low Ct value [8–20], moderate expression was shown by Ct values of [20–26]. Low and hardly expressed genes gave a high Ct value of 26–34 and 34–40, respectively.

abolished in constructs cultured in growth medium (Appendix Fig. 1), while the gene expression level of GATA4 was similar to the levels observed after 1 day of culture or after 9 days of culture in differentiation medium. Interestingly, constrained constructs displayed a more intense expression of the sarcomeric protein α -actinin and the cardiac marker cardiac troponinI (cTnI) (Fig. 4C,D). However, the expression was cytoplasmatic, and no cross-striations, characteristic of mature cardiomyocytes, were present yet. In stress-free constructs protein expression of α -actinin and cTnI was mainly observed in and around the nucleus (Fig. 4H,I). Vimentin, a marker for intermediate filaments, was

present in the cytoplasm or in small dots in both constrained and stress-free constructs, but no fiber-like structures, characteristic of mesenchymal or fibroblast-like cells were observed (Fig. 4E,J).

3.3. Extracellular matrix production and remodeling of CMPCs in 3D

CMPCs in 3D constructs express the ECM proteins collagen I, collagen III and fibronectin and the ECM modulators MMP-1, MMP-2, MMP-9, TIMP-1 and TIMP-2 (Table 3, Fig. 5 [30]). No substantial differences between constrained and stress-free constructs were observed

Fig. 3. Cardiomyogenic differentiation profile of CMPCs cultured in a 3D environment. Expression of cardiac transcription factors GATA4 (A), Nkx2.5 (B) and myocardin (C), and cardiac marker cardiac troponinT (cTnT) (D) in CMPCs cultured in constrained and stress-free constructs compared to 2D cultures [30]. The data are represented as mean fold change ± SD. *P < 0.05 vs day 1 in 2D cultures, **P < 0.01 vs day 1 in 2D cultures, \$P < 0.05 vs day 1 3D constrained construct, #P < 0.05 vs day 1 3D stress-free construct.

Fig. 4. Protein expression of cardiac transcription factors and cardiac markers in constrained and stress-free constructs. Representative immunofluorescent images of (A,F) GATA4 (green), (B,G) Nkx2.5 (red), (C,H) α -actinin (green), (D,I) cardiac troponinI (red) and (E,J) vimentin (red) in constrained and stress-free constructs after 9 days in culture with differentiation medium. Cell nuclei are stained with DAPI (blue). Images of both constructs at day 1 and day 9 cultured with growth medium can be found in Appendix Fig. 1. Scale bar: 50 μ m.

Fig. 5. Expression of matrix proteins in CMPCs cultured in 3D constructs. Validation of qPCR data on the expression of extracellular matrix genes collagen I (A), collagen III (B) and fibronectin (C) compared to 2D cultures [30]. Data are shown as mean fold changes $\pm$ SD. * $P < 0.05$ vs day 1 in 2D cultures, ** $P < 0.01$ vs day 1 in 2D cultures, *** $P < 0.001$ vs day 1 in 2D cultures, \$ $P < 0.05$ vs day 1 3D constrained construct, # $P < 0.05$ vs day 1 3D stress-free construct, ## $P < 0.01$ vs day 1 3D stress-free construct.

(Table 3, Fig. 5). When compared to 2D cultures, the expression of collagen I significantly decreased in a 3D environment (Table 3, Fig. 5A [30]). In constrained constructs expression decreased 30 fold ($P < 0.001$) compared to 2D. In stress-free constructs expression of collagen I was even further reduced to 72 fold ($P < 0.001$) compared to 2D. Extension of culture time to 9 days in both types of media resulted in slight increase in expression of collagen I ($P < 0.05$ stress-free d9) (Fig. 5A). Expression of collagen III showed induction in both constructs in time and by exposure to the differentiation protocol ($P < 0.01$ stress-free d9DM) (Table 3, Fig. 5B). In a 3D environment, the expression of fibronectin was induced as shown by an average Ct value of approximately 9 compared to 16 in 2D culture conditions (Table 3, Fig. 5C [30]). The additional effect of exposure to the differentiation protocol even increased the expression of fibronectin 10 fold in the constrained constructs ($P < 0.05$). Interestingly, the expression of elastin was only induced in both constrained and stress-free in the presence of

differentiation medium (3D constrained d9 DM: Ct = 28.8 ± 0.23 ; 3D stress-free d9 DM: Ct = 27.61 ± 1.64 ; day 1 and day 9 GM: Ct > 34).

Analysis of ECM proteins by immunofluorescent staining revealed presence of collagen I and elastin in CMPCs in both constrained as well as in stress-free constructs at day 1 (Fig. 6). By using an antibody for collagen I that recognized specifically human collagen I combined with an antibody that recognized multispecies collagen, it was possible to distinguish between the collagen I present in the collagen/Matrigel hydrogel and the collagen I produced by the CMPCs (Fig. 6A–F). At day one, collagen I was expressed by CMPCs in both constrained and stress-free constructs (Fig. 6A,D). In time, the number of CMPCs that expressed collagen I increased in both constructs cultured in growth media (Fig. 6B,E). At day 9, an increase in the number of CMPCs expressing collagen I cultured in constrained constructs with differentiation media is visible (Fig. 6C) as well as the surrounding matrix. The staining of the surrounding matrix is also present in the stress-free constructs

Fig. 6. Extracellular matrix (ECM) expression by CMPCs cultured in 3D constructs. Representative fluorescent images of ECM proteins collagen I (A–F), collagen III (G–L) (red), fibronectin (M–R) (red) and elastin (S–X) (red). To distinguish between human collagen I produced by the CMPCs (red) and the collagen I present in the collagen/Matrigel hydrogel (green) antibodies against collagen I with different species recognition were used. Nuclei are stained with DAPI (blue). Scale bar: 50 μ m.

with differentiation media, but the increased number of CMPCs expressing collagen I in this construct is less clear (Fig. 6F).

At the protein level, presence of collagen III (Fig. 6G–L) and fibronectin (Fig. 6M, P–R) was also visible in the collagen/Matrigel hydrogels. This staining of the hydrogel could be caused by specific binding of the antibodies to the Matrigel or by de novo synthesis by the CMPCs. Actually, the intensity of collagen III in the hydrogel seems to increase in the constrained constructs at day 9 and with exposure to the differentiation protocol (Fig. 6I), indicative of de novo protein synthesis. At day 9 in constrained hydrogels, expression of fibronectin is visible in CMPCs in both culture media (Fig. 6N,O).

In contrast to the very low expression of elastin at gene level in CMPCs in both constructs at day 1, at the protein level elastin was detected inside CMPCs at day 1 (Fig. 6S,V). In constrained hydrogels the expression of elastin increased when hydrogels were cultured in

differentiation media, while the expression pattern in growth media was comparable to day 1 (Fig. 6T,U). The stress-free hydrogels were more difficult to interpret due to staining of the collagen/Matrigel hydrogel (Fig. 6W,X).

Investigation of the matrix remodeling proteins, the MMPs and TIMPs, showed that MMP-1 was moderately expressed and that the expression was not affected by the environment or culture protocol (Table 3, Fig. 7A [30]). The expression of MMP-2 was induced in a 3D environment, while there was no significant difference between constrained or stress-free constructs. In constrained constructs, the additional effect of exposure to the differentiation protocol increased the expression of MMP-2 with 3.3 fold ($P < 0.005$), this was almost twice as high as the induction of differentiation in stress-free constructs (1.6 fold, $P < 0.05$) (Fig. 7B). The additional effect of differentiation on the expression of MMP-9 showed an 178 fold increase (not significant)

Fig. 7. Expression of matrix modulators in CMPCs cultured in constrained and stress-free 3D constructs. The mRNA levels of MMP-1 (A), MMP-2 (B) and MMP-9 (C) are evaluated with qPCR analysis using TOP as internal control and previous 2D-data [30] as a reference. Zymography results of MMP-2 (D) and MMP-9 (E) from media samples are normalized to the intensity (INT) of the constrained construct at day 1. The changes in expression of inhibitors of matrix remodeling TIMP-1 (I) and TIMP-2 (J) are also revealed by qPCR analysis. Data are shown as mean fold changes $\pm$ SD. * $P < 0.05$ vs day 1 in 2D cultures, ** $P < 0.01$ vs day 1 in 2D cultures, *** $P < 0.001$ vs day 1 in 2D cultures, \$ $P < 0.05$ vs day 1 3D constrained construct, # $P < 0.05$ vs day 1 3D stress-free construct, ## $P < 0.01$ vs day 1 3D stress-free construct.

in constrained constructs while in stress-free constructs exposed to differentiation media the expression only increased 8 fold ($P < 0.05$) (Fig. 7C).

Enzymatic activity of MMP-2 and -9 in media samples were determined by gelatin zymography (Fig. 7D,E). Zymography on media samples showed previously that SP++ media and IFDIFF media already express latent MMP-2 and -9 at low levels [34]. Expression of latent MMP-2 (72 kDa) was determined in all culture conditions at both time points (Fig. 7D). The levels of latent MMP-2 at day 1 were comparable between constrained and stress-free constructs (Fig. 7F). In time, levels of latent MMP-2 were increased in growth media in both constructs with 2.2 fold (constrained) and 1.3 fold (stress-free), respectively (Fig. 7F). In differentiation media, the expression of latent MMP-2 increased 2.1 fold in constrained constructs while in stress-free constructs no induction of latent MMP-2 was observed compared to day 1 (Fig. 7F). The active form of MMP-2 (66 kDa) was also present in media samples at day 1 in both constructs (Fig. 7D). The levels of active MMP-2 in media increased over time in both constrained and stress-free constructs. The induction of active MMP-2 was higher in both media conditions in the constrained constructs (20.1 fold (growth media) and 19.8 fold (differentiation media), respectively) compared to the 2.3 fold (growth media) and 4.7 fold (differentiation media) induction in stress-free constructs (Fig. 7G).

MMP-9 was also detected in media samples (Fig. 7E). The expression of latent MMP-9 (92 kDa) decreased over time for constrained constructs with 2.0 fold in growth media and 1.3 fold in differentiation media (Fig. 7H). In stress-free constructs the expression of latent MMP-9 also decreased over time with 1.3 fold in growth media and 1.7 fold in differentiation media (Fig. 7H). Active MMP-9 could not be detected.

Tissue inhibitors of MMPs, TIMP-1 and -2, were highly expressed in 2D culture environments (Table 3, Fig. 7I,J [30]). In 3D, the expression of TIMP-1 was increased in constrained (not significant) and stress-free ($P < 0.05$) constructs in time. The protocol of differentiation had no significant effect on the expression of both TIMP-1 and -2 (Fig. 7I,J).

3.4. CMPCs 3D align in the direction of the constraint

To investigate whether CMPCs embedded in a 3D construct are able to sense the unidirectional static strain imposed by construct constraint in the longitudinal direction, actin stress fiber formation was studied. After 1 day of culture, CMPC encapsulated in constrained constructs showed cytoplasmic staining of actin, but also stress fibers could be observed (Fig. 8A). After 9 days of culture in both culture media, the cytoplasmic actin staining was diminished and actin stress fibers were observed throughout the cells (Fig. 8C,D), suggesting that the cells have become mechanosensitive. Contrarily, CMPCs encapsulated

in stress-free constructs showed either cytoplasmic or cortical staining of actin and no stress fibers were developed (Fig. 8E–G). Only cells at the border of the stress-free gels showed some formation of actin stress fibers, probably because of stress in the gels generated by gel compaction. Others have also observed this phenomenon in compacting gel systems [40].

In constrained constructs, actin fiber formation correlated with construct retraction. After loosening the constructs from the constraint at one side, gels retracted from $19.6 \pm 5.0\%$ at 1 day to $27.0 \pm 9.2\%$ at day 9 of culture under normal culture conditions, and even up to $51.6 \pm 10.9\%$ in differentiation medium (Fig. 8D). This suggests that CMPCs in constrained constructs are able to sense and respond to mechanical strain, and that differentiation results in an enhanced development of their mechanosensing apparatus. Indeed, CMPCs encapsulated in constrained constructs had a dominant orientation along the direction of the constraint (90°), which becomes more pronounced with culture time in both types of media (Fig. 9A–F). CMPCs encapsulated in stress-free constructs showed a random orientation (Fig. 9G–L).

4. Discussion

The beneficial effects of current cell based therapy for cardiac regeneration are limited due to low cell retention. Since encapsulating of cells in a biomaterial may improve effectiveness of stem cell therapy, we investigated whether the contribution of CMPCs to the cardiac regeneration process can be improved by culturing them in unidirectional constrained or stress free 3D hydrogels. The 3D culture system used in this study, composed of a collagen/Matrigel hydrogel constrained between 2 Velcro attachments, is based on a well-developed model for skeletal muscle [31–33]. The system, which is based on a combination of collagen type I and Matrigel, allows for the use of high cell densities, and provides a biological and natural environment to the cells. Moreover, by applying a constraint to the gel, the cells are exposed to intrinsically generated stress, the stress generated by the cells themselves, which results in cell alignment. The composition of the hydrogel resembles that of the native healthy cardiac ECM, which is composed of collagen types I, III and IV, laminin, fibronectin, elastin and fibrillin [41]. Next to their proven value in skeletal muscle tissue engineering, collagen/Matrigel based hydrogels are also used for cardiac tissue engineering in combination with neonatal chicken or rat cardiomyocytes [22] and neonatal rat heart cells [23,42]. The system supports cellular differentiation, cardiomyocyte maturation, sarcomere development, and contractility. Therefore, collagen/Matrigel represents a promising 3D culture environment for use in cardiac regeneration in combination with stem cells [43].

Our data shows that culture of CMPCs in a 3D environment stabilizes viability and proliferation of these cells and indeed enhances their differentiation and remodeling capacity.

Fig. 8. Actin stress fiber development and internal stress formation. Immunofluorescent images of the actin cytoskeleton (green) in constrained (A) and stress free constructs after 1 day in culture (E) and culture for 9 days in growth media (B,F) and differentiation media (C,G). Cell nuclei are stained with DAPI (blue). Arrows denote the direction of constraint in the constrained constructs. Bar plots show the % of retraction of constrained constructs over time and subjection to differentiation (D). Data is shown as mean $\pm$ SD. $N = 6$. *** $P < 0.001$. Scale bar: 50 μ m.

Fig. 9. Orientation of CMCPs in constrained and stress-free constructs. Confocal images show cell tracker green stained CMCPs in constrained (A–C) and stress-free constructs (G–I) after 1 and 9 days in culture. Arrows denote the constrained direction (90°). Fiber fraction (cellular structures per orientation angle) in constrained (D–F) and stress-free constructs (J–L) after 1 and 9 days of culture are represented in the histograms. Data is represented as mean $\pm$ SD, N = 4–6. Scale bar: 100 μ m.

4.1. Differentiation and proliferation

We observed that culture of CMCPs in a 3D hydrogel environment does not affect their viability and improved the cardiomyogenic differentiation capacity of CMCPs. An increased differentiation capacity has previously been described by Dawson et al. [16] and Liu et al. [17] for (embryonic) stem cells cultured in 3D. The increased gene expression of the cardiac transcription factor Nkx2.5 is consistent with the results of Gaetani et al. [44], who cultured CMCPs in an alginate scaffold. However, this study is the first to investigate not only the impact of a 3D environment but also the effect of mechanical load on 3D embedded CMCPs. The application of static strain in our study provides insight into the behavior of CMCPs when injected in the rhythmic beating heart. We show for the first time that 1) the induction of cardiomyogenic differentiation related genes in a 3D environment is

independent of the application of static strain and 2) construct constraint resulted in an increased sarcomeric protein α -actinin expression and the expression and organization of the cardiac marker cTnI. Cardiomyocyte maturation was however not yet observed. To exclude the possibility that CMCPs encapsulated in 3D constructs differentiate towards fibroblast-like cells, we show that in both constrained and stress-free hydrogel constructs, vimentin was expressed in a dotted pattern or cytoplasmic, but not as fiber-like structures, indicating that the CMCPs were not de-differentiating towards fibroblast-like cells [45].

Finally, proliferation in constrained constructs diminished with culture time as shown by a reduction of Ki-67 positive cells. The decrease in proliferation is in line with data by Pontes Soares et al. which show that proliferation of cardiac cells decreases in 3D culture compared to 2D culture [46]. Withdrawal from the cell cycle, as observed in stress-free

hydrogels, can be a sign of terminal differentiation [47], although this was not supported by the expression of cardiac genes and proteins detected in CMPCs in the stress-free constructs. More likely, the stress-free environment causes the cells to stay in a more quiescent state, similar to the descriptions by Li et al. for mesenchymal stem cells encapsulated in thermosensitive synthetic hydrogels [48].

4.2. Extracellular matrix production and remodeling

The present data show that CMPCs in a 3D environment were able to contribute to matrix production and remodeling. Reconstruction of the damaged myocardium and preventing or reversing fibrotic tissue formation by matrix production and remodeling of the heart is an important feature in cardiac regeneration. Previous studies have shown that 3D culture of stem cells increase the expression and production of ECM proteins [17,49]. This is in line with the increased expression of elastin, collagen III and fibronectin, but in contradiction with the collagen I expression we observed in 3D cultures. Interestingly, although the gene expression of collagen I decreased over time, on protein level, the number of collagen I positive CMPCs increased. In the presence of differentiation media even the surrounding matrix became positive for human collagen I suggesting excretion of collagen I by CMPCs in both constrained and stress-free constructs. The 3D tissue culture system used in the present study is composed with a hydrogel which is for 50% composed of collagen I and furthermore with Matrigel. The presence of collagen type I in the hydrogel may cause the reduction in gene expression of collagen type I in 3D. Although, previous studies showed the ability of cardiac stem cells and CMPCs to produce collagens [30,50], in the heart collagens are generally considered to be produced by cardiac fibroblasts, and as mentioned earlier, CMPCs do not de-differentiate towards fibroblast-like cells. Finally, in a 3D environment, cell–matrix interaction is of great importance and fibronectin as an adhesive protein of the supporting extracellular matrix might therefore be induced in 3D, to improve cell–matrix interactions [51]. Moreover, fibronectin can provide a temporary matrix for the deposition and remodeling of other matrix proteins, such as collagen [52], which is more important in 3D as in 2D.

Next to ECM production, remodeling of both the matrix proteins present in the hydrogel as well as the newly formed ECM is essential for cardiac regeneration. We showed that encapsulation of CMPCs in 3D hydrogels induced the expression of matrix remodeling enzymes MMP-1 and MMP-2, while the expression of MMP-9, TIMP-1 and -2 was not affected. Like in 2D, exposure to differentiation medium decreased the expression of MMP-1 whereas expression of MMP-2 and -9, TIMP-1 and -2 were increased [30]. While also enzymatic activity of both latent and active MMP-2 is present upon encapsulation and increased with time and the exposure to differentiation media, activity of latent MMP-9 is not affected. The increased remodeling capacity in 3D culture compared to 2D had also been described by Liu et al. in mouse embryonic stem cells [17]. The expression of MMPs and TIMPs by CMPCs in 3D culture indicates that the cells participate in the remodeling of their own environment [53].

4.3. Mechanosensitivity

For stem cell integration into the host tissue, response to mechanical signals is important. In this study, we show that the application of static strain had a large effect on CMPC morphology. Cells in stress-free constructs were small and had a rounded morphology, whereas in the constrained constructs cells elongated and had a more rod shaped morphology, which is a characteristic of maturing cardiomyocytes [47]. After release from the constraint, constructs retracted 20%–52% depending on culture time and medium used, which demonstrates that the cells were actively pulling on the matrix components in the tissue formed and indeed are exposed to a static strain situation. The development of actin stress fibers, as characteristic of mechanosensitive cells

[54], in combination with construct retraction was investigated to study whether the cells were able to sense this strain. Here, we show that the mechanosensitivity of undifferentiated CMPCs is developed upon encapsulation in 3D collagen/Matrigel hydrogels. Although actin stress fiber development was independent of culture media, construct retraction was higher in constructs cultured in differentiation media, suggesting an increased development of the actin cytoskeleton. In stress-free constructs, actin staining was either cytoplasmic or cortical, indicating that the cells were not actively pulling on the gel, and had not a developed mechanosensing system.

Interestingly, application of strain also resulted in cell alignment, which is typical of myocardial tissue and demonstrating that CMPCs were not just able to sense but in fact were able to respond to mechanical strain. CMPCs encapsulated in constrained constructs oriented in the direction of the constraint. These data correspond to the study of Hansen et al. [23], who showed that neonatal rat heart cells encapsulated in collagen/Matrigel aligned in the direction of the constraint. Fink et al. [22] also showed alignment of neonatal cardiomyocytes (chicken and rat) in constrained collagen/Matrigel hydrogels. However, they only reported alignment at the free lateral edges and not in the center of the hydrogel, whereas this study also showed alignment in the center of the constructs. In contrast to constrained constructs, CMPCs encapsulated in stress-free constructs did not show a preferred orientation.

4.4. Implications for cardiac therapy

The challenge in cardiac regeneration therapy is to reconstruct the original composition and structure of the heart. Current stem cell therapies aim at creating de novo cardiac tissue and preventing scar formation, unfortunately with modest and temporary beneficial effects due to low cell retention [3,6,7]. Encapsulation of CMPCs in a biomaterial might increase cell retention and thereby already improve cardiac regeneration. Our data show that encapsulation of stem cells stabilize cell viability and proliferation and moreover induce the mechanosensitivity of the cells, which might contribute to improvement of the efficacy of cardiac repair. Furthermore, the induction of cell differentiation capacity upon encapsulation may improve the formation of new cardiac cells by CMPCs. The increased ability of CMPCs to produce ECM proteins and modulators in a 3D environment suggest that CMPCs can create de novo matrix and can break down the hydrogel used for encapsulation, thereby creating their own niche which may be beneficial for survival and differentiation. Overall, the combined observation of the induced differentiation and matrix remodeling capacity after CMPC encapsulation and the fact that CMPCs develop a mechanosensing apparatus and align in the direction of static strain suggests that a combination of cells and a biomaterial may result in an enhanced efficacy of cell therapy. However, to ensure that the mechanical signals of the heart are also transmitted to the biomaterial and the cells after injection, the biomaterial should adhere to, and integrate into the host heart tissue. Future research should focus on the contribution and ability of encapsulated progenitors in modulating the cardiac extracellular matrix in vivo.

4.5. Conclusion

CMPCs can be cultured in 3D collagen/Matrigel hydrogels thereby keeping their early cardiomyogenic profile and increasing their cardiomyogenic differentiation capacity. Furthermore, enhanced expression of the ECM proteins and matrix remodeling enzymes is indicative of enhanced matrix remodeling capacity of CMPCs in a 3D environment. Moreover, static strain, as imposed by hydrogel constraint, results in stabilized cell viability and proliferation and enhanced cardiac marker protein expression and appeared crucial for cell, organization and morphology. CMPCs cultured in 3D collagen/Matrigel become readily mechanosensitive and respond to hydrogel constraint by orienting in the direction of the constraint which is crucial for cell integration into the host tissue. Overall, our results suggest that CMPC encapsulation in a

biomaterial stabilize viability and proliferation of these cells, enhance their differentiation and remodeling capacity and can induce cellular reorganization, which may result in an increased cell retention and improved efficiency of cardiac stem cell therapy.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jymcc.2015.08.010>.

Acknowledgments

The authors gratefully acknowledge F. Kanter for the development of the algorithm for the orientation analysis, and M. van Doeselaar for performing the qPCR analysis. This research forms part of the project P1.04 SMARTCARE of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation. The financial contribution of the Nederlandse Hartstichting is gratefully acknowledged.

Author disclosure statement

No competing financial interests exist.

References

- [1] T.I. van der Spoel, S.J. Jansen of Lorkeers, P. Agostoni, E. van Belle, M. Gyongyosi, et al., Human relevance of pre-clinical studies in stem cell therapy: systematic review and meta-analysis of large animal models of ischaemic heart disease, *Cardiovasc. Res.* 91 (4) (2011) 649–658, <http://dx.doi.org/10.1093/cvr/cvr113>.
- [2] F. Sharif, J. Bartunek, M. Vanderheyden, Adult stem cells in the treatment of acute myocardial infarction, *Catheter. Cardiovasc. Interv.* 77 (1) (2011) 72–83, <http://dx.doi.org/10.1002/ccd22620>.
- [3] N.M. Novotny, R. Ray, T.A. Markel, P.R. Crisostomo, M. Wang, et al., Stem cell therapy in myocardial repair and remodeling, *J. Am. Coll. Surg.* 207 (3) (2008) 429–434, <http://dx.doi.org/10.1016/j.jamcollsurg.2008.04.013>.
- [4] J.H. Traverse, T.D. Henry, S.G. Ellis, C.J. Pepine, J.T. Willerson, et al., Effect of intracoronary delivery of autologous bone marrow mononuclear cells 2 to 3 weeks following acute myocardial infarction on left ventricular function: the LateTIME randomized trial, *JAMA* 306 (19) (2011) 2110–2119, <http://dx.doi.org/10.1001/jama.2011.1670>.
- [5] E.C. Perin, J.T. Willerson, C.J. Pepine, T.D. Henry, S.G. Ellis, et al., Effect of transcatheter delivery of autologous bone marrow mononuclear cells on functional capacity, left ventricular function, and perfusion in chronic heart failure: the FOCUS-CCTRN trial, *JAMA* 307 (16) (2012) 1717–1726, <http://dx.doi.org/10.1001/jama.2012.418>.
- [6] A.R. Chugh, G.M. Beache, J.H. Loughran, N. Mewton, J.B. Elmore, et al., Administration of cardiac stem cells in patients with ischemic cardiomyopathy: the SCPIO trial: surgical aspects and interim analysis of myocardial function and viability by magnetic resonance, *Circulation* 126 (11 Suppl. 1) (2012) S54–S64.
- [7] R. Bolli, A.R. Chugh, D. D'Amario, J.H. Loughran, M.F. Stoddard, et al., Cardiac stem cells in patients with ischemic cardiomyopathy (SCPIO): initial results of a randomised phase 1 trial, *Lancet* 378 (9806) (2011) 1847–1857, [http://dx.doi.org/10.1016/S0140-6736\(11\)61590-0](http://dx.doi.org/10.1016/S0140-6736(11)61590-0).
- [8] V.F. Segers, R.T. Lee, Biomaterials to enhance stem cell function in the heart, *Circ. Res.* 109 (8) (2011) 910–922, <http://dx.doi.org/10.1161/CIRCRESAHA.111.249052>.
- [9] J.M. Singelyn, J.A. DeQuach, S.B. Seif-Naraghi, R.B. Littlefield, P.J. Schup-Magoffin, et al., Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering, *Biomaterials* 30 (29) (2009) 5409–5416, <http://dx.doi.org/10.1016/j.biomaterials.2009.06.045>.
- [10] J. Leor, Y. Amsalem, S. Cohen, Cells, scaffolds, and molecules for myocardial tissue engineering, *Pharmacol. Ther.* 105 (2) (2005) 151–163.
- [11] T. Eschenhagen, W.H. Zimmermann, Engineering myocardial tissue, *Circ. Res.* 97 (12) (2005) 1220–1231.
- [12] J.R. Venugopal, M.P. Prabhakaran, S. Mukherjee, R. Ravichandran, K. Dan, et al., Biomaterial strategies for alleviation of myocardial infarction, *J. R. Soc. Interface* 9 (66) (2012) 1–19, <http://dx.doi.org/10.1098/rsif.2011.0301>.
- [13] K. Yu, K.T. Du, Q. Fang, Y. Gu, S.S. Mihadja, et al., The use of human mesenchymal cells encapsulated in RGD modified alginate microspheres in the repair of myocardial infarction in rat, *Biomaterials* 31 (27) (2010) 7012–7020, <http://dx.doi.org/10.1016/j.biomaterials.2010.05.078>.
- [14] E. Tous, B. Purcell, J.L. Ifkovits, J.A. Burdick, Injectable acellular hydrogels for cardiac repair, *J. Cardiovasc. Transl. Res.* 4 (5) (2011) 528–542, <http://dx.doi.org/10.1007/s12265-011-9291-1>.
- [15] N.T. Elliott, F. Yuan, A review of three-dimensional in vitro tissue models for drug discovery and transport studies, *J. Pharm. Sci.* 100 (1) (2011) 59–74, <http://dx.doi.org/10.1002/jps.22257>.
- [16] E. Dawson, G. Mapili, K. Erickson, S. Taqvi, K. Roy, Biomaterials for stem cell differentiation, *Adv. Drug Deliv. Rev.* 60 (2) (2008) 215–228.
- [17] H. Liu, J. Lin, K. Roy, Effect of 3D scaffold and dynamic culture condition on the global gene expression profile of mouse embryonic stem cells, *Biomaterials* 27 (36) (2006) 5978–5989.
- [18] Y. Duan, Z. Liu, J. O'Neill, L.Q. Wan, D.O. Freytes, G. Vunjak-Novakovic, Hybrid gel composed of native heart matrix and collagen induces cardiac differentiation of human embryonic stem cells without supplemental growth factors, *J. Cardiovasc. Transl. Res.* 4 (5) (2011) 605–615, <http://dx.doi.org/10.1007/s12265-011-9304-0>.
- [19] M.W. Curtis, B. Russell, Micromechanical regulation in cardiac myocytes and fibroblasts: implications for tissue remodeling, *Pflügers Arch.* 462 (1) (2011) 105–117, <http://dx.doi.org/10.1007/s00424-011-0931-8>.
- [20] D. Shav, S. Einav, The effect of mechanical loads in the differentiation of precursor cells into mature cells, *Ann. N. Y. Acad. Sci.* 1188 (2010) 25–31, <http://dx.doi.org/10.1111/j.1749-6632.2009.05079.x>.
- [21] Y. Huang, L. Zheng, X. Gong, X. Jia, W. Song, et al., Effect of cyclic strain on cardiomyogenic differentiation of rat bone marrow derived mesenchymal stem cells, *PLoS One* 7 (4) (2012), e34960, <http://dx.doi.org/10.1371/journal.pone.0034960>.
- [22] C. Fink, S. Ergun, D. Kralisch, U. Remmers, J. Weil, T. Eschenhagen, Chronic stretch of engineered heart tissue induces hypertrophy and functional improvement, *FASEB J.* 14 (5) (2000) 669–679.
- [23] A. Hansen, A. Eder, M. Bonstrup, M. Flato, M. Mewe, et al., Development of a drug screening platform based on engineered heart tissue, *Circ. Res.* 107 (1) (2010) 35–44, <http://dx.doi.org/10.1161/CIRCRESAHA.109.211458>.
- [24] E. Bellas, C.S. Chen, Forms, forces, and stem cell fate, *Curr. Opin. Cell Biol.* (2014) 92–97, <http://dx.doi.org/10.1016/j.ceb.2014.09.006>.
- [25] M.C. den Haan, R.W. Grauss, A.M. Smits, E.M. Winter, J. van Tuyn, et al., Cardiomyogenic differentiation-independent improvement of cardiac function by human cardiomyocyte progenitor cell injection in ischaemic mouse hearts, *J. Cell. Mol. Med.* 16 (7) (2012) 1508–1521, <http://dx.doi.org/10.1111/j.1582-4934.2011.01468.x>.
- [26] E.M. Winter, A.A. van Oorschot, B. Hogers, L.M. van der Graaf, P.A. Doevendans, et al., A new direction for cardiac regeneration therapy: application of synergistically acting epicardium-derived cells and cardiomyocyte progenitor cells, *Circ. Heart Fail.* 2 (6) (2009) 643–653, <http://dx.doi.org/10.1161/CIRCHEARTFAILURE.108.843722>.
- [27] M.J. Goumans, T.P. de Boer, A.M. Smits, L.W. van Laake, P. van Vliet, et al., TGF-beta1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro, *Stem Cell Res.* 1 (2) (2007) 138–149, <http://dx.doi.org/10.1016/j.scr.2008.02.003>.
- [28] P. van Vliet, M. Rocco, A.M. Smits, A.A.M. van Oorschot, C.H.G. Metz, et al., Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy, *Neth. Heart J.* 16 (5) (2008) 163–169.
- [29] A.M. Smits, P. van Vliet, C.H. Metz, T. Korfage, J.P. Sluijter, et al., Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an in vitro model for studying human cardiac physiology and pathophysiology, *Nat. Protoc.* 4 (2) (2009) 232–243, <http://dx.doi.org/10.1038/nprot.2008.229>.
- [30] N.A. Bax, M.H. van Marion, B. Shah, M.J. Goumans, C.V. Bouten, D.W. van der Schaft, Matrix production and remodeling capacity of cardiomyocyte progenitor cells during in vitro differentiation, *J. Mol. Cell. Cardiol.* 53 (4) (2012) 497–508, <http://dx.doi.org/10.1016/j.jymcc.2012.07.003>.
- [31] D. Gawlitta, K.J. Boonen, C.W. Oomens, F.P. Baaijens, C.V. Bouten, The influence of serum-free culture conditions on skeletal muscle differentiation in a tissue-engineered model, *Tissue Eng. Part A* 14 (1) (2008) 161–171, <http://dx.doi.org/10.1089/ten.a.2007.0095>.
- [32] M.L. Langelaan, K.J. Boonen, K.Y. Rosaria-Chak, D.W. van der Schaft, M.J. Post, F.P. Baaijens, Advanced maturation by electrical stimulation: Differences in response between C2C12 and primary muscle progenitor cells, *J. Tissue Eng. Regen. Med.* 5 (7) (2011) 529–539, <http://dx.doi.org/10.1002/term.345>.
- [33] D.W. Van der Schaft, A.C. van Spreeuwel, K.J. Boonen, M.L. Langelaan, C.V. Bouten, F.P. Baaijens, Engineering skeletal muscle tissue from murine myoblast progenitor cells and application of electrical stimulation, *J. Vis. Exp.* 73 (2013), e4267, <http://dx.doi.org/10.3791/4267>.
- [34] S.A. De Vries, M.C. van Turnhout, C.W. Oomens, A. Erdemir, K. Ito, C.C. van Donkelaar, Deformation thresholds for chondrocyte death and the protective effect of the pericellular matrix, *Tissue Eng. Part A* 20 (13–14) (2014) 1870–1876, <http://dx.doi.org/10.1089/ten.TEA.2013.0436>.
- [35] D.P. De Kleijn, J.P. Sluijter, E. Smit, W. Velema, A.H. Richard, et al., Furin and membrane type-1 metalloproteinase mRNA levels and activation of metalloproteinase-2 are associated with arterial remodeling, *FEBS Lett.* 501 (2001) 37–43, [http://dx.doi.org/10.1016/S0014-5793\(01\)02622-9](http://dx.doi.org/10.1016/S0014-5793(01)02622-9).
- [36] A.F. Frangi, W.J. Niessen, K.L. Vincken, M.A. Viergever, Medical image computing and computer-assisted intervention, in: A. Wells, A. Colchester, S.L. Delp (Eds.), *Multiscale Vessel Enhancement Filtering*, 1496, Springer Verlag, Berlin, Germany, 1998, pp. 130–137.
- [37] D.W. van der Schaft, A.C. van Spreeuwel, H.C. van Assen, F.P. Baaijens, Mechanoregulation of vascularization in aligned tissue-engineered muscle: a role for vascular endothelial growth factor, *Tissue Eng. Part A* 17 (21–22) (2011) 2857–2865, <http://dx.doi.org/10.1089/ten.TEA.2011.0214>.
- [38] N. de Jonge, F.M. Kanter, F.P. Baaijens, C.V. Bouten, Strain-induced collagen organization at the micro-level in fibrin-based engineered tissue constructs, *Ann. Biomed. Eng.* 41 (4) (2013) 763–774, <http://dx.doi.org/10.1007/s10439-012-0704-3>.
- [39] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} method, *Methods* 25 (4) (2001) 402–408.
- [40] N.C. Rivron, E.J. Vrij, J. Rouwkema, S. Le Gac, A. van den Berg, et al., Tissue deformation spatially modulates VEGF signaling and angiogenesis, *Proc. Natl. Acad. Sci. U. S. A.* 109 (18) (2012) 6886–6891, <http://dx.doi.org/10.1073/pnas.1201626109>.
- [41] L. Moore, D. Fan, R. Basu, V. Kandam, Z. Kassiri, Tissue inhibitor of metalloproteinases (TIMPs) in heart failure, *Heart Fail. Rev.* 17 (4–5) (2012) 693–706, <http://dx.doi.org/10.1007/s10741-011-9266-y>.
- [42] W.H. Zimmermann, I. Melnychenko, T. Eschenhagen, Engineered heart tissue for regeneration of diseased hearts, *Biomaterials* 25 (9) (2004) 1639–1647.

- [43] P.Y. Dankers, T.M. Hermans, T.W. Baughman, Y. Kamikawa, R.E. Kieltyka, et al., Hierarchical formation of supramolecular transient networks in water: a modular injectable delivery system, *Adv. Mater.* 24 (20) (2012) 2703–2709, <http://dx.doi.org/10.1002/adma.201104072>.
- [44] R. Gaetani, P.A. Doevendans, C.H. Metz, J. Alblas, E. Messina, et al., Cardiac tissue engineering using tissue printing technology and human cardiac progenitor cells, *Biomaterials* 33 (6) (2012) 1782–1790, <http://dx.doi.org/10.1016/j.biomaterials.2011.11.003>.
- [45] A. Grigore, D. Arsene, F. Filipoiu, F. Cionca, S. Enache, et al., Cellular immunophenotypes in human embryonic, fetal and adult heart, *Rom. J. Morphol. Embryol.* 53 (2) (2012) 299–311.
- [46] C. Pontes Soares, V. Midlej, M.E. de Oliveira, M. Benchimol, M.L. Costa, C. Mermelstein, 2D and 3D-organized cardiac cells shows differences in cellular morphology, adhesion junctions, presence of myofibrils and protein expression, *PLoS One* 7 (5) (2012), e38147, <http://dx.doi.org/10.1371/journal.pone.0038147>.
- [47] M. Tiburcy, M. Didie, O. Boy, P. Christalla, S. Doker, et al., Terminal differentiation, advanced organotypic maturation, and modeling of hypertrophic growth in engineered heart tissue, *Circ. Res.* 109 (10) (2011) 1105–1114, <http://dx.doi.org/10.1161/CIRCRESAHA.111.251842>.
- [48] Z. Li, X. Guo, A.F. Palmer, H. Das, J. Guan, High-efficiency matrix modulus-induced cardiac differentiation of human mesenchymal stem cells inside a thermosensitive hydrogel, *Acta Biomater.* 8 (10) (2012) 3586–3595, <http://dx.doi.org/10.1016/j.actbio.2012.06.024>.
- [49] W.L. Grayson, T. Ma, B. Bunnell, Human mesenchymal stem cells tissue development in 3D PET matrices, *Biotechnol. Prog.* 20 (3) (2004) 905–912, <http://dx.doi.org/10.1021/bp034296z>.
- [50] H. Kurazumi, M. Kubo, M. Ohshima, Y. Yamamoto, Y. Takemoto, et al., The effects of mechanical stress on the growth, differentiation, and paracrine factor production of cardiac stem cells, *PLoS One* 6 (12) (2011), e28890, <http://dx.doi.org/10.1371/journal.pone.0028890>.
- [51] M.M. Ulrich, A.M. Janssen, M.J. Daemen, L. Rappaport, J.L. Samuel, et al., Increased expression of fibronectin isoforms after myocardial infarction in rats, *J. Mol. Cell. Cardiol.* 29 (9) (1997) 2533–2543, <http://dx.doi.org/10.1006/jmcc.1997.0486>.
- [52] X. Wu, Z. Sun, A. Foskett, J.P. Trzeciakowski, G.A. Meininger, M. Muthuchamy, Cardiomyocyte contractile status is associated with differences in fibronectin and integrin interactions, *Am. J. Physiol. Heart Circ. Physiol.* 298 (6) (2010) H2071–H2081, <http://dx.doi.org/10.1152/ajpheart.01156.2009>.
- [53] M.L. Coker, M.A. Doscher, C.V. Thomas, Z.S. Galis, F.G. Spinale, Matrix metalloproteinase synthesis and expression in isolated LV myocyte preparations, *Am. J. Physiol.* 277 (2 Pt 2) (1999) H777–H787.
- [54] S. Nemir, J.L. West, Synthetic materials in the study of cell response to substrate rigidity, *Ann. Biomed. Eng.* 38 (1) (2010) 2–20, <http://dx.doi.org/10.1007/s10439-009-9811-1>.