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Improved targeting and enhanced retention of the human, autologous, fibroblast-derived, induced, pluripotent stem cells to the sarcomeres of the infarcted myocardium with the aid of the bioengineered, heterospecific, tetravalent antibodies**

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Abstract

Clinical trials, to regenerate the human heart injured by myocardial infarction, involve the delivery of stem cells to the site of the injury. However, only a small fraction of the introduced stem cells are detected at the site of the injury, merely two weeks after this therapeutic intervention. This significantly hampers the effectiveness of the stem cell therapy.

To resolve the aforementioned problem, we genetically and molecularly bioengineered heterospecific, tetravalent antibodies (htAbs), which have both exquisite specificity and high affinity towards human, pluripotent, stem cells through the htAbs' domains binding SSEA-4, SSEA-3, TRA-1-60, and TRA-1-81, as well as towards the injured cardiac muscle through the htAbs' domains binding human cardiac myosin, α -actinin, actin, and titin. The cardiac tissue was acquired from the patients, who were receiving heart transplants. The autologous, human, induced, pluripotent stem cells (hiPSCs) were generated from the patients' fibroblasts by non-viral delivery and transient expression of the DNA constructs for: Oct4, Nanog, Sox2, Lin28, Klf4, c-Myc. In the trials involving the htAbs, the human, induced, pluripotent stem cells anchored to the myocardial sarcomeres with the efficiency, statistically, significantly higher, than in the trials with non-specific or without antibodies ($p < 0.0003$). Moreover, application of the htAbs resulted in cross-linking of the sarcomeric proteins to create the stable scaffolds for anchoring of the stem cells. Thereafter, these human, induced pluripotent stem cells differentiated into cardiomyocytes at their anchorage sites.

By bioengineering of these novel heterospecific, tetravalent antibodies and using them to guide and to anchor the stem cells specifically to the stabilized sarcomeric scaffolds, we demonstrated the proof of concept *in vitro* for improving effectiveness of regenerative therapy of myocardial infarction and created the foundations for the trials *in vivo*.

Keywords

Myocardial infarction; heart attack; ischemic heart disease; stem cell therapy; cardiac regeneration; myofibril; SSEA-4; SSEA-3; TRA-1-60; TRA-1-81; myosin; α -actinin; actin; titin; desmin; troponin; bi-specific antibody; heterospecific tetravalent antibody; tetrabody

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Conflict of interest statement

Dr Marek Malecki owns the IP for the Fv gene coding sequences, gene transcripts, and gene expression products used in this work, as well as their streamlining to diagnosis and therapy, all protected at the USPTO and the WIPO.

Introduction

Stem cell therapy, aimed at regeneration of the hearts injured by myocardial infarctions, has become a subject of major clinical trials [1–9]. The main strategies of delivering stem cells to the treated hearts include intravenous and intracoronary infusions, myocardial injections, and bio-scaffold patching. Reaching the sites of injury by the stem cells is further refined by their natural tropism towards injured tissues. Nevertheless, the inaccuracy of targeting and the low retention of the stem cells to the site of cardiac infarction are the real problems, with detection of only 3–6% of the infused or 6–12% of the injected cells, merely two weeks from the procedure [10–14]. These problems dramatically hamper therapeutic effectiveness. Previous attempts of improving retention of the cells to the site of the cardiac injury involved applying anti-CD45 x anti-myosin light chain bispecific antibodies or scaffolding gels withholding the stem cells [15–21].

The specific aim of this work was to create molecules, which would anchor autologous, human, induced pluripotent stem cells (hiPSCs) to a stabilized structure of the infarction. That would be followed by the induction of the myocardial differentiation of the anchored hiPSCs *in situ*.

SSEA-4, SSEA-3, TRA-1-60, TRA-1-81 – biomarkers displayed on surfaces of the undifferentiated stem cells are considered the hallmarks of pluripotency. Efficient methods of obtaining high viability batches of the pluripotent biomarkers' displaying stem cells, by magnetic or fluorescent sorting, have been developed [22–25]. Moreover, some of the main obstacles for streamlining of the pluripotent stem cells into regenerative medicine, including the ethical constraints for using pluripotent embryonic stem cells, incompatibility of human pluripotency stem cells' biomarkers with those of other species, controversies surrounding trans-differentiation, and rejection of foreign stem cells by the immune systems of the patients, have recently been addressed by developing human induced pluripotent stem cells (hiPSCs), strongly displaying the aforementioned biomarkers [26–37]. Thereafter, these hiPSCs have been induced *in vitro* to differentiate into cardiac myocytes with fully functional contractile sarcomeres [38–46].

Myosin, α -actinin, actin, and titin are the major proteins of the cardiac sarcomere's contractile and skeletal apparatus. In the healthy hearts, these proteins are covered by the cell membranes - sarcolemmas. However, myocardial infarctions result in the cardiomyocytes' death, sarcolemmas' damage, and sarcomeres' exposure. Subsequently, some of the cardiac muscle proteins are quickly released to the circulation, while the others are retained on the site of the injury. The former have become diagnostic laboratory biomarkers of the cardiac damage detected in blood and urine (e.g., troponin, myosin light chains). The latter have become landmarks of the location and extent of the cardiac damage determined by molecular imaging (e.g., α -actinin, myosin) [47–50].

Therefore, trials of cardiac regenerative therapy may consist of the four main phases: (1) bioengineering batches of autologous, human, induced pluripotent stem cells (hiPSCs), for the particular patient, ahead of the scheduled stem cell therapy procedure; (2) stabilizing the patient's cardiac sarcomeres, as a potential scaffold for harboring therapeutic stem cells; (3) delivering of the autologous human induced pluripotent stem cells (hiPSCs) to the site of injury and retaining them there; (4) inducing cardiac differentiation of the anchored induced pluripotent stem cells *in situ*.

The novelty of our work presented herein relies on bioengineering of the entirely new class of biomolecules – hetero-specific tetravalent antibodies (htAbs) targeting simultaneously the unique biomarkers displayed on the human induced pluripotent stem cells (hiPSCs) and the unique molecules of the cardiac sarcomeres [51]. Moreover, it further relies on the

anchoring of the hiPSCs to the stabilized sarcomeric scaffolds and inducing the hiPSCs' myocardial differentiation at the anchorage sites.

Patients. Materials. Methods

Bioengineering of heterospecific tetravalent antibodies (htAbs)

The genetically engineered, variable fragment antibodies (Fvs) were prepared as described [24–25, 51], thus only briefly outlined here. The Fvs for SSEA-4, SSEA-3, TRA-1-60, TRA-1-81, CD-45, CD-34, CD-19, and CD-20 were genetically engineered from the B cells of patients suffering cancers. The anti-DOTA, anti-DTPA, and anti-TETA Fvs were genetically engineered from the B cells of patients undergoing multiple rounds of imaging and therapy involving chelates as the contrast agents. The Fvs for myosin, actin, titin, troponin, and α -actinin were genetically engineered from the B cells of patients suffering myocardial infarctions and receiving heart transplants. The pooled B cells from these patients were used to isolate mRNA, reverse transcribe, and create the cDNA libraries of complementarity determining regions (CDR) and framework regions (FWR) for anti-cancer-antibodies (ACA) coding sequences and for anti-heart muscle antibodies (AHA). The cds, after insertion into the plasmids containing chelates' harboring coding sequences under the CMV promoters and terminated with polyA, were propagated and expressed in human myelomas. The native SSEA-4, SSEA-3, TRA-1-60, TRA-1-81, CD-45, CD-34, CD-19, CD-20, myosin, actin, titin, and α -actinin were purified by immunoprecipitation with monoclonal antibodies, which followed by their modification with biotin, digoxigenin, or fluorescein [52]. They were anchored onto anti-biotin, anti-dig, or anti-FITC saturated pans and served as baits for selection of the Fv clones from the ACA and AHA expression libraries. The genetically engineered antibodies for sarcomeric proteins were manufactured as described [47]. The chelates were saturated with Gd, Tb, and Eu [52]. The specificity and sensitivity were determined based upon elemental compositions with EDXS (Noran, Middleton, WI, USA), EELS (Zeiss, Oberkochen, D, EU), or TRXFS (Bruker AXS, Fitchburg, WI, USA). The fluorescent properties were measured with the RF-5301PC spectrofluorometer (Shimadzu, Tokyo, Japan). The specificity and sensitivity of the Fvs were tested with the EELS and EDXS. The magnetic relaxivities were measured on the DMX 400 WB or AVANCE II NMR spectrometers (Bruker Optics, Dallas, TX, USA). The monoclonal antibodies for these antigens served as the positive controls, and antibodies towards 6His, FLAG, DOTA, TETA, and DTPA served as the negative controls. For preparing tetravalent antibodies, the first batch of monovalent Fvs was sprayed from air-brush with a single pulse over the pan filled with the 0.01–0.001mg/mL recombinant avidin (rA) in PIPES buffer in a saturated humidity chamber maintained at room temperature. Upon completion of binding, the fractions of resulting solution were separated by the size exclusion chromatography on either gravity or through high pressure liquid chromatography (HPLC) (Pharmacia, S, EU) columns. The fractions were collected on the fraction collector (Pharmacia, S, EU). The fractions detected at the ~70kD peak and containing rA linked with the single monovalent Fv were pooled together and sprayed over the new pan. The procedure was repeated for all the Fvs, one Fv at a time, in a random order. In the final separation tetrabodies were collected at the peak of ~150kDa. The system was calibrated using classic IgG, Fab, Fv, and avidin, as the references. Specificity and sensitivity of the bioengineered tetravalent antibodies were tested by immunoblotting (IB), multiphoton fluorescence spectroscopy (MPFS), EDXS, and EELS.

Patients. Cardiac tissues

All the samples were obtained in accordance with the Declaration of Helsinki with the Patients' Informed Consent and with the Institutional Review Boards' approval. The cardiac tissue samples were obtained from the patients undergoing heart transplants. The cardiac

tissues were processed on different ways depending on their further applications, as described, thus only briefly outlined here [24–25,51]: (1) as native myofibrils to retain their antigenicity; (2) dissociated in single molecules for native electrophoresis and blotting; (3) retained in the Wisconsin solution for primary cultures or gradually frozen; (4) rapidly cryoimmobilized for retaining structural live like architecture and antigenicity on cryosections; (5) snap frozen, crushed, homogenized, and lyophilized to be used for cultures or denaturing electrophoresis.

The myofibrils were prepared on the ways, which assured retention of the native antigenicity. Strips of cardiac muscle tissue were brought to a stretched or contracted state and clamped with the U shaped vascular surgery forceps. They were immersed in the buffer solution (75mM KCl, 10mM Tris pH6.8, 2mM EGTA, 2mM MgCl₂, 0.1mM PMSF, 0.1% TritonX-100). Thereafter, the tissues were homogenized in a Polytron (Brinkman Instruments Co., Westbury, NY, USA) and a Teflon glass homogenizer. The myofibrils were collected by centrifugation at 1,000 g for 5 min. The pellets were washed by cycles of suspension and centrifugation. Finally, they were infused with the fresh buffer containing 50% glycerol and frozen at –20°C for storage. They were thawed and rinsed with the fresh buffer before use.

Small cubes of the fresh cardiac tissues were disintegrated with the sterile, surgical scalpel and plated onto the Petri dishes with the bottoms covered by matrigel or cardiac tissue sections and filled with the DMEM supplemented with serum and antibiotics. The primary cultures were grown in the incubators maintaining 37°C, 10% CO₂, and saturated humidity. For storage, the tissue cultures were infused with DMSO or glycerol and frozen gradually to retain their viability.

Alternatively, the cardiac tissue was inserted into the gold planchettes. They were rapidly cryoimmobilized in the HPM 010 (Balzers, Lichtenstein, EU). The frozen muscles were either sectioned in the frozen hydrated state or cryo-substituted, infused with 2.3M sucrose, refrozen, and sectioned on the cryoultramicrotome (Leica, Vienna, A, EU).

Alternatively, the rapidly cryoimmobilized samples were crushed and homogenized. They were resuspended in the buffers and frozen or lyophilized to the powders.

All these approaches assured preservation of native state of the cardiac muscle protein antigenicity and architecture. All specimens were examined by MPFS, IB, EELS, and EDXS [52].

Cultures. Inducing Pluripotency. Inducing Differentiation

The cell lines were cultured as originally described and only briefly outlined here [25, 31]. The cell line of human fibroblasts derived from the lungs IMR90 (Cat# CCL-186) (ATCC, Manassas, VA) was cultured in Eagle Minimum Essential Medium (ATCC, Manassas, VA) supplemented with 10% heat-inactivated donor calf serum, 0.1 mM non-essential amino acids, and 1.0 mM Sodium pyruvate. The cell lines of human embryonic stem cells H1, H9 were used as the controls. They were grown in a mixture of Dulbecco Modified Essential Medium and F12 supplemented with 20% KnockOut, 0.1 mM non-essential amino acids, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 100 ng/ml zebra fish basic fibroblast growth factor (zbFGF). These cell lines were grown on matrigel (Becton Dickinson, Bedford, MA).

Inducing pluripotency of the patients' and cultured cells was performed by transgenic expression of the transcription factors as described, but with some essential differences [31]. Coding sequences for OCT4, SOX2, NANOG, LIN28, KLF4, cMYC were generated by mRNA isolation followed by reverse transcription, and polymerase chain reaction

amplification with restriction sites' extensions. The amplicons were incorporated in the non-viral plasmid vectors. The cds, after insertion into the plasmids containing chelates' harboring coding sequences under the CMV promoters with the enhancers, and terminated with polyA, were cloned, and purified. These plasmids were used to continuously transfect the cells by adding them embedded into liposomes into the media, while also supplementing the mixes with 1 mM valproic acid. The sustained cultures of the human induced pluripotent stem cells were grown the same way as the embryonic stem cells outlined above. Within two weeks, the cells were either grown as embryoid bodies and induced to differentiate or sorted by MACS or FACS based upon cell surface displayed biomarkers as described and applied to myocardial injury sarcomeric models [22,23].

Pluripotency was verified by inducing differentiation towards the three germ layers of the embryoid bodies' derived lineages by 1% dimethyl sulfoxide (DMSO), 10^{-5} M retinoic acid, 3 mM hexamethylene bisacetamide (HMBA) (Sigma-Aldrich, Saint Louis, MO, USA). The evaluation was determined based upon labeling with the Fvs for myosin, keratin, and neurofilaments for x-ray, magnetic resonance, or fluorescence spectroscopy [22–23].

The autologous, human, induced pluripotent stem cells, pluripotent embryonic stem cells, and induced cultured fibroblasts were subjected to fraction enrichment by labeling with two of the superparamagnetic antibodies and magnetic activated cell sorting up to > 99.5% purity as described [22–25]. The enriched fractions were spiked into the solution flowing over the human cardiac sarcomeres attached to the bottoms of the Petri dishes. The anchored hiPSCs were induced to differentiate into cardiomyocytes by media supplemented with 20% human serum, 1% lyophilized cardiac tissue, 10 ng/ml BMP2, 10 mM NAM [51]. Progression of differentiation was determined by quantitative reverse transcription and amplification by polymerase chain reaction of the transcripts unique for the cardiac myogenesis: GATA4 and MEF-2c, while also probing the transcripts for the key genes of pluripotency: OCT4 and NANOG.

Flow cytometry (FCM). Fluorescently activated cell sorting (FACS). Multiphoton Fluorescence Spectroscopy (MPFS)

The cell clusters were thoroughly disintegrated into single cell suspension by short treatment with the PIPES buffered DNase, RNase, trypsin, collagenase, or dispase as described [22,23, 26–30]. The negative selection involved depletion of hematopoietic progenitor cells with the Fvs anti-CD34; differentiated hematopoietic cells with the Fvs anti-CD45, apoptotic cells with the Fvs anti-PS, the dead cells with the Fvs anti-DNA to reach above 99.5% of purity. The enriched populations of the cells labeled with the fluorescent Fvs targeting TRA-1-60 and SSEA-4 were measured with the Calibur, Vantage SE, or Aria (Becton-Dickinson, Franklin Lakes, NJ, USA) or the FC500 (Beckman-Coulter, Brea, CA, USA). The fluorescently labeled cells were imaged with the Axiovert (Zeiss, Oberkochen, D, EU) equipped with the Enterprise argon ion (457 nm, 488 nm, 529 nm lines) and ultraviolet (UV) (364 nm line) lasers; Odyssey XL digital video-rate confocal laser scanning imaging system operated up to 240 frames/s under control of Intervision software (Noran, Madison, WI, USA), and the Diaphot (Nikon, Tokyo, Japan) equipped with the Microlase diode-pumped Nd:YLF solid state laser (1048 nm line), the pulse compressor with the pulses' rate 300 fs at 120 MHz and the MRC600 scanning system under control of Comos software (the multi-photon fluorescence station built based upon the NIH funds – PI: Dr J. White). Deconvolution of images was done on the Indy workstation (Silicon Graphics, Fremont, CA, USA).

Nuclear Magnetic Resonance (NMR). Magnetic Activated Cell Sorting (MACS)

The cells were labeled for positive selection with the superparamagnetic Fvs targeting TRA-1-60 and SSEA-4, and for the negative selection targeting CD45, CD34, dsDNA, and PS, while suspended in the physiological buffer supplemented with serum and glucose. The small aliquots were dispensed into the magnetism-free NMR tubes (Shigemi, Tokyo, Japan). The relaxation times T1 were measured in resonance to the applied FLAIR pulse sequences on the NMR spectrometers: DMX 400 WB or AVANCE II NMR (Bruker, Billerica, MA) or the Signa clinical scanners (GE, Milwaukee, WI, USA). The superparamagnetic Fvs were also used to isolate the labeled cells from the solution using the magnetic sorter to reach above 99.5% of purity (the sorter designed and built based upon the NSF funds – PI: Dr M. Malecki).

Electron Energy Loss Spectroscopy (EELS). Energy Dispersive X-Ray Spectroscopy (EDXS). Total Reflection X-ray Fluorescence Spectroscopy (TRXFS)

The samples, which were cryo-immobilized, presented the life-like supramolecular organization. Molecular imaging was pursued as described [52]. The field emission, scanning transmission, electron microscope FSTEM HB501 (Vacuum Generators, Kirkland, WA, USA) was equipped with the energy dispersive x-ray spectrometer (EDXS) (Noran, Middleton, WI, USA) and post-column electron energy loss spectrometer (EELS) (Gatan, Pleasanton, CA). The cryo-energy filtering transmission electron microscope 912 Omega was equipped with the in-column, electron energy loss spectrometer (EELS) (Zeiss, Oberkochen, D, EU). The cryo-energy filtering transmission electron microscopes 410 and 430 Phillips were equipped with the post-column, electron energy loss spectrometers (EELS) (Noran, Middleton, WI, USA). The field emission, scanning electron microscope SEM1530 (Zeiss, Oberkochen, D, EU) was equipped with the energy dispersive x-ray spectrometer (EDXS) (Noran, Middleton, WI, USA). The field emission, scanning electron microscope 3400 was equipped with the energy dispersive x-ray spectrometer (EDXS) (Hitachi, Tokyo, Japan). The images and spectra were acquired using the ccd camera operating under the image acquisition and processing software (SIS, Herzogenrath, D, EU or Emispec Systems, Tempe, AZ, USA). In this study, the ICP standard of 1000 mg/l of mono-element Gallium (CPI International, Denver, CO, USA) was added to 500 microL of each sample to the final concentration of 10 mg/l. The data were generated from the S2 Picofox TXRF spectrometer equipped with a molybdenum (Mo) X-ray target and the Peltier cooled Xflash Silicon Drift Detector (Bruker AXS, Fitchburg, WI, USA). Scan times ranged up to 1000 seconds. The automatic sample changer, which can hold up to 25 samples, was also used along with the SPECTRA 7 software for the instrument control, data collection, and analysis (Bruker AXS, Fitchburg, WI, USA).

Targeting and retention of pluripotent stem cells to the cardiac sarcomeres. Directed differentiation

Simulation of the therapeutic application of the stem cells was based upon the environmental chambers. The chambers bottoms were covered with firmly attached myofibrils or sectioned cardiac tissues. In both cases the sarcomeres were exposed to the over-flowing solution as they would be in the myocardial infarctions. The buffered culture medium was flown over these sarcomeres, while propelled by the peristaltic pump (Flowrox, Linthicum, MD, USA). The chambers were tightly sealed and connected with the environmental incubator through flexible Tygon hoses. They assured maintaining the myofibrils and cells at 37°C, pH 7.3, 120/80 mmHg, and 330 mOsm. Cardiac actin was labeled with phalloidin modified with FITC or Gd, Tb, or Eu chelates. Genomic DNA of the stem cells was stained with bisbenzimidazole (Sigma Aldrich, St Louis, MO, USA). Under continuous flow, the buffer was spiked with the human induced pluripotent stem cells. At various time intervals, the flow was stopped and the number of cells quantified based upon the ratios of fluorescence or x-

ray scintillations. Directed differentiation of the hiPSCs towards cardiomyocytes was pursued as originally described for *Xenopus laevis*, but modified for stem cells.

Immunoblotting (IB)

The cells and tissues were either frozen in liquid nitrogen, crushed, and thawed or/and disintegrated with ultrasonicator (Branson Ultrasonic, Danbury, CT, USA) within the sample buffers for native protein analysis. They were stored in liquid nitrogen or electrophoresed in the native buffer (Invitrogen, Carlsbad, CA, USA). They were vacuum- or electro-transferred onto the PVDF membranes (Amersham, Buckinghamshire, UK, EU). The membranes carrying the transferred proteins were soaked within human serum and labeled with the Fvs. The samples of purified cardiac muscle myosin, actin, α -actinin, titin served as the controls. The monoclonal antibodies against myosin, actin, α -actinin, titin, SSEA-4, SSEA-3, TRA-1-60, TRA-1-81, CD34, and CD45 served as the controls. The images of the blots were acquired and quantified with Fluoroimager (Molecular Dynamics, Sunnyvale, CA, USA) or Storm 840 (Amersham, Buckinghamshire, UK, EU). The levels of the gene expression products were also calculated, as the ratio between the protein concentration in the examined patient's cells and the controls.

Quantitative Reverse Transcription and Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated with TRIzol (MRC, Cincinnati, OH, USA). In addition to the patients' cardiac tissues, the fibroblasts, peripheral blood cells, and bone marrow cells were acquired. The cultured fibroblasts (IMR90), human embryonic stem cells (H1, H9), and blood from the healthy volunteers served as the controls. For all, RNA served as the template to generate cDNA through reverse transcription using random hexamers and reverse transcriptase (ABI, Foster City, CA, USA) as described [24–25]. The primers' sequences and cycling settings were modified from those published [27, 33]. The transcripts for GAPDH and actin served as the internal controls (ABI, Foster City, CA, USA). They were synthesized on the 380A DNA Synthesizer (ABI, Foster City, CA, USA). The PCR reactions were carried using the mix of the cDNA, the synthesized primers, dNTPs, and Taq DNA polymerase (Hoffmann–La Roche, Basel, H) on the Robocycler (Stratagene, San Diego, CA, USA), Mastercycler (Eppendorf, Hamburg, D, EU), or 7500, 7900 systems (ABI, Foster City, CA, USA). The images of the gels were acquired and quantified with Fluoroimager (Molecular Dynamics, Sunnyvale, CA, USA) or Storm 840 (Amersham, Buckinghamshire, UK, EU). The levels of the transcripts were all normalized against GAPDH or actin, and thereafter calculated as the ratios between the transcript concentration in the examined patient's cells versus the cells from the healthy control tissues and cultures.

Statistical analysis

Quantification of the differences between gene expression in the patients' hiPSCs and differentiating hiPSCs was performed against GAPDH and actin as reference genes, versus the patients' cardiac and skin fibroblasts, cultured pluripotent ESCs (H1, H9) and cultured human fibroblasts (IMR90). Average gene expression measurements were run in triplicates for each patient, which were used for gene expression statistical analysis. The data were analyzed and displayed using GraphPad software (GraphPad Software, Inc, La Jolla, CA). APC was accepted as statistically significant with $p < 0.05$ or better.

Results

The novel concept for anchoring of the bioengineered, autologous, pluripotent stem cells to the cardiac sarcomeres by heterospecific tetravalent antibodies is illustrated in the figure 1. The genetically engineered antibodies against SSEA-4, SSEA-3, TRA-1-60, TRA-1-81, myosin, α -actinin, actin, and titin were each modified to carry the single avidin-docking

group at the carboxyl terminus. One at a time, these antibodies were docked into avidin, thus forming sequentially mono-, bi-, tri-, or tetra-valent antibody. If each of the incorporated monovalent antibodies was different, then the resulted, final antibody was the hetero-tetra-specific, tetravalent antibody (htAb). However, monospecific or bispecific, tetravalent antibodies were also generated, as the sarcomeric protein scaffolding molecules. They were collected as the initial fractions from chromatography. Moreover, the initial experiments involved modifications of IgGs, Fabs, and dcFvs according to the same principles.

The heterospecific, tetravalent antibodies (htAbs) were injected into the solution flown over the sarcomeres (a). That was a model of the therapeutic, stem cell infusion. The htAbs locked onto the bare sarcomeres. This was a model of the sarcomeres exposed by the damaged sarcolemmas due to the myocardial infarction (b). The htAbs were cleared from the circulation, so that only those bound firmly to the sarcomeres retained. This was done to make sure, that they would not cluster the cells. Thereafter, the human induced pluripotent stem cells were spiked into the flow (hiPSCs) (c). They promptly anchored through heterospecific tetravalent antibodies already locked onto the sarcomeres (d). The anchored stem cells were firmly retained on site. The anchored stem cells were exposed to the epigenetic factors inducing their differentiation towards cardiac muscles.

A molecular architecture of the human cardiac muscle was revealed by electron energy loss spectroscopy (EELS) as shown in figure 2. Its analysis was essential for designing the efficient strategy for anchoring of the therapeutic stem cells. This ultrastructure was captured in the life-like conformation due to the rapid cryo-immobilization protocols followed by low temperature preparation including cryo-sectioning. All the samples were analyzed in triplicates. This image was representative for all the patients' samples. The Z-line was the site in which actin filaments bind to α -actinin, which appears as the dense, stable structure well outlined due to efficient stain exclusion. The A-band was formed by thick myosin filaments. It outlined also the cross-bridges between myosin heads and thin actin filaments. It was the most prominent and rigid structure of the sarcomeres. The H-zone was the central portion of thick myosin filaments with the M line in its center. The H-zone, within A-band, contained space between filaments, which absorbed and retained a lot of contrast, therefore appeared as the very dark structure. The I-band consisting of the thin, actin filaments had more spaces between the filaments, thus capturing the stain. It was appearing as the dense band between the A-band and the Z-line. The length of the I-band changed depending on the state of the contraction, thus the potential surface for binding the stem cells to actin. Thin titin filaments stretched along the entire sarcomeres to join the Z-lines. The densely packed biomolecular clusters of Z-line and A-band excluded the molecules of the negative stains, hence they appeared with light contrast.

An ultrafine specificity of the genetically engineered Fv antibodies for the analysis of the molecular architecture of the human cardiac muscle was pursued by immunolabeling on the cryo-immobilized whole mounts as illustrated in the figure 3. In this approach, cryo-immobilization was not only important for retaining architecture, but also for preservation of antigenicity of the cardiac muscle molecules the same as in the living state, so that the data from these experiments, could be extrapolated on the *in vivo* trials. All the samples were analyzed in triplicates. This image is representative for all of the patients' samples and all of the Fvs tested. The immunolabeling of α -actinin in the Z-line was performed with the Fvs tagged with core-shell nanoparticles and revealed by EELS. The labeling of α -actinin was very specific, while restricted to the Z-line and absent from the background.

The bioengineered heterospecific, tetravalent antibodies (htAbs) were validated by comparison, side-by-side, of their specificity with the well known commercially available, monoclonal IgG antibodies (mAbs), as illustrated in the figures 4–5. Specificity and

sensitivity of the bioengineered antibodies towards cardiac α -actinin, titin, myosin, and actin were highlighted on human cardiac myofibrils by multiphoton fluorescence spectroscopy (MPFS) and epi-fluorescence microscopy (EFM), while projecting the patterns of fluorescence onto the phase-contrast overviews of the cardiac myofibrils as the references. The cardiac myofibrils were double-labeled with the pairs of the htAbs. Various pairs of htAbs were applied depending on the purpose. The combinations of α -actinin and titin (domains close to the Z-line) were used to stabilize the portions of the sarcomeres close to the Z-lines. It was illustrated in the figure 4. The combinations of myosin and actin were used to stabilize the portions of sarcomeres around A-band. It was illustrated in the figure 5. These and other combinations were applied for anchoring the hiPSCs through the htAbs. They were labeled with the various fractions of the htAbs coming into the collectors. They were modified with chelates coordinating Eu and Tb, thus were emitting the unique and stable fluorescence. All the samples were run in triplicates. The images were representative for all the patients' samples studied. The htAbs were exquisitely specific to the main components of the sarcomeres. The Z-line was revealed by the strong band of the labeling on α -actinin in the figure 4. It was flanked on both sides of the Z-line by the labeling of the sequences of titin near Z-lines, which further confirmed the specificity of labeling. The labeling of the sarcomeres with the novel htAb was identical to that obtained with the mAbs. The prominent band of labeling was specific to A-band in the figure 5. The labeling was more prominent on M-line of creatine kinase due to the reduced steric hindrance as expected from the figure 2. The heavy htAb labeling of myosin is intercalated by the bright labeling of I-band. The htAbs labeling was verified with the mAbs.

Specificity and sensitivity of the htAbs, which were demonstrated in the images of immunolabeling by fluorescence microscopy, were also validated with the immunoblots shown in the figure 6. For that purpose, the homogenized human cardiac tissues were electrophoresed, transferred onto PVDF, and labeled with the first fractions of the htAbs towards cardiac α -actinin, titin, actin, and myosin. They were compared side-by-side with the mAbs as the controls. Both labeling approaches were projecting the patterns of labeling onto the patterns of the electrophoresed cardiac muscle molecules. As demonstrated by the single bands of labeling and clean backgrounds, the htAbs were exquisitely specific to the myosin. All tests were run in triplicates. The images were representative for all the patients' samples, antigens, and htAbs studied.

Specificity and sensitivity of the htAbs towards human cardiac α -actinin and myosin were tested on cryo-sections of the cryo-immobilized cardiac tissues as illustrated in the figure 7. These serial cryo-sections were labeled with the htAbs and imaged by multiphoton fluorescence spectroscopy (MPFS). All samples were run in triplicates. The images were representative for all the patients' samples studied. The htAbs were exquisitely specific to α -actinin and myosin, as demonstrated by heavy labeling of the Z-lines and the A-bands. Moreover, they were demonstrated by the entire absence of any labels in the background (figure 7a). The highlighting the sarcomeric structures were much stronger with the htAbs, than with the mAbs (figure 7b). The labeling was very specific, sensitive, and sustained.

Stability of the sarcomeres was essential for them to serve as the anchoring scaffolds. It was reinforced by labeling of the most prominent structures of the sarcomeric architecture with the htAbs and mAbs against α -actinin and myosin as documented in the figure 7c-f. This stability was tested in the time-lapse study, in which the cryosections of the cardiac sarcomeres were maintained in the environmental chambers simulating *in vivo* conditions. After prolonged incubation times in those environments, the sarcomeric patterns started to diffuse. It was revealed, when the sections were incubated first and labeled with the fluorescent htAbs afterwards (figure 7c). However, if the sarcomeres were stabilized by introducing the tetravalent antibodies, which effectively crosslinked the elements of the

sarcomeres, prior to inserting into the incubators, then the stability of the sarcomeric pattern was much better (figure 7d–f).

Testing the transcription levels of the genes for the transcription factors OCT4 and NANOG was the way to probe the pluripotent potential of the induced cardiac and skin fibroblasts obtained from the patients, who were also the donors of the cardiac tissues. These were compared to the transcription levels of these genes in the cultured human, embryonic stem cells H1, H9 (hESC) and the cultured, human fibroblasts IMR90 (hF). For this analysis, the cells were labeled with the superparamagnetic htAbs targeting SSEA-4, SSEA-3, TRA-1-60, and TRA-1-81 and isolated by magnetic activated cell sorting (MACS). This resulted in gaining of the highly enriched fraction of the cells expressing the biomarkers of pluripotency. Total quantified RNA from these cells was isolated and reverse transcribed into cDNA. Thereafter, the specific sequences were amplified by polymerase chain reaction and electrophoresed as documented in the figure 8. Each of the patients' and control samples was measured in triplicates. The presented read-outs are representative for all the samples studied. Expression of the OCT4 and NANOG genes was highly up regulated in the hiPSCs.

Differentiation of the hiPSCs into embryoid bodies (EBs) with formation of the three germ layers, which continued through myogenesis to the contracting myocytes was an important functional test. From the contracting areas, the samples were taken to determine changing levels of gene expression for the transcription factors as documented in the figure 9. The OCT4 gene expression was down regulated in the non-induced fibroblasts. Induction of pluripotency in the human cultured skin and cardiac fibroblasts led to the genes' up regulation, while creating human induced pluripotent stem cells (hiPSCs). Myogenesis was reflected by the down regulation of OCT4 in the hiPSCs, as well as in the human embryonic stem cells used for reference.

Cell surface display of biomarkers facilitated determination of the state of pluripotency of the studied cells. The display of the biomarkers on surfaces of the living human cells was studied after labeling with fluorescent chelates tagged htAbs and mAbs by multiphoton fluorescence and epifluorescence spectroscopy as illustrated in the figure 10. The biopsy-derived, primary culture skin and cardiac fibroblasts were efficiently labeled with the bioengineered antibodies targeting fibroblast growth factor receptors (FGFR). They were not labeled with the bioengineered htAbs targeting SSEA-4, SSEA-3, TRA-1-60, and TRA-1-81. Identically, the control cultured human fibroblasts were not displaying the biomarkers of pluripotency. The induction of the gene expression for the six factors changed that status. The labeling with the fluorescent htAbs highlighted the cell surface displayed SSEA-4, SSEA-3, TRA-1-60, and TRA-1-81 of the hiPSCs. Specificity and sensitivity of the genetically engineered antibodies targeting these biomarkers of pluripotency was already previously validated and compared to monoclonal antibodies (mAbs) on the human totipotent embryonic stem cells and human embryonal carcinoma cells of the ovaries and testes, as in the details described elsewhere [24–25]. Each of the samples was run in triplicates. The presented images are representative for all the ones studied. Saturation of the chelating domains of the htAbs with Eu and Tb assured strong and sustained fluorescence. The human induced pluripotent stem cells were displaying the biomarkers of pluripotency strongly. This cell surface display was equivalent to that of the embryonic stem cells used as the controls.

Further testing of the induced pluripotency was performed by immunoblotting with the bioengineered antibodies against the key cell surface biomarkers of pluripotency, as shown in the figure 11. For this study, the cryo-immobilized cells were homogenized, electrophoresed, transferred and labeled with the htAbs. The hiPSCs' labeling was restricted to the single bands on the blots. The blots of the hESCs' showed the identical pattern. The

labeling was absent on the blots of the human cultured skin and cardiac fibroblasts (hpcF). Each of the samples was run in triplicates. The presented blots are representative for all the samples studied. Therefore, immunoblotting revealed high levels of the gene expression products displayed on the surface of the hiPSCs.

Effectiveness of the htAbs to anchor pluripotent stem cells to the human cardiac sarcomeric scaffolds was quantified by energy dispersive x-ray spectroscopy (EDXS), total reflection x-ray fluorescence spectroscopy (TRXRFS), and fluorescence spectroscopy (FS) as presented in the figure 12. This effectiveness was measured on various ways. The fluorescent tagging of the sarcomeres, the htAbs, mAbs, and the hiPSCs' genomic DNA facilitated measurements of the ratios between fluorescent intensities emitted from these entities: htAbs, hiPSCs, and sarcomeres or endothelia. The elemental tagging of the sarcomeres, antibodies, and cells facilitated measurements of the scintillations emitted from these structures. Each of the samples was run in triplicates. The measurements documented in the figure were representative for all the ones studied. These measurements facilitated calculations of the ratios between the sarcomeres, htAbs, and hiPSCs. These measurements facilitated comparisons between the number of pluripotent cells anchored and retained to the sarcomeres under different conditions.

Injection of the hiPSCs, hESCs, and fibroblasts into the buffers flowing over the sarcomeres or human endothelial monolayers was aimed as a model of interactions between the htAbs and hiPSCs with the sarcomeres of the myocardial infarction or the blood vessels' endothelia. It was preceded by labeling with the bioengineered, heterospecific, tetravalent antibodies. The hiPSCs and hESCs with the strong cell surface display of SSEA-4, SSEA-3, TRA-1-60, and TRA-1-81 very efficiently were binding to sarcomeres through the htAbs, but not to the endothelia.

Retention of the anchored cells was tested by incubation in the *in vivo* like environment for different periods of time. The anchored cells were retained with minimal losses. An important factors contributing to the efficient retention was stabilization of the sarcomeres with the htAbs cross-linking the molecules of the sarcomeres.

Anchoring of the hiPSCs to the sarcomeres was primarily contingent upon specificity of the bioengineered antibodies. To test this notion, various tests included mono-specific, selectively blocking antibodies, non-specific antibodies, and those without antibodies at all. These antibodies were used to block the antigens on the hiPSCs or on the sarcomeres.

Importantly, the htAbs used for stabilizing the sarcomeres had to target different antigens than those in the htAbs aimed at anchoring of the hiPSCs. The most often used combination of the genetically engineered Fvs used to bioengineer htAbs was: anti-SSEA-4, anti-TRA-1-60, anti-myosin, anti- α -actinin. The htAbs targeting the pluripotency biomarkers on the human induced pluripotent stem cells, SSEA-4, SSEA-3, TRA-1-60, and TRA-1-81, were described in the details earlier [24,25]. The totipotent hESCs, as well as the embryonal carcinomas of the ovaries and testes strongly displaying these biomarkers, were the controls. The number of the anchored hiPSCs after initial treatment with the htAbs was very high. Blocking of the sarcomeric proteins and/or pluripotency biomarkers with the monospecific antibodies reduced significantly the number of the cells attached. The genetically engineered antibodies against the cardiac sarcomere proteins were described earlier [47]. Omitting the htAbs from the procedure or using non-specific antibodies practically eliminated binding of the hiPSCs to the sarcomeres. The contributions of the different elements of the htAbs towards anchoring events were demonstrated by blocking the antigens on the sarcomeres with the monoclonal antibodies for a single sarcomeric protein, e.g., myosin, actin, or α -actinin.

The ultimate goal for the induced pluripotent stem cell therapy of the patients suffering myocardial infarctions was to provide the contracting myocytes; thus to improve the effectiveness of the cardiac muscle. The tests of pluripotency and differentiation were performed on the cells by qRT-PCR as documented in the figure 13. The only living cells present in the environmental chambers were the autologous, human, induced pluripotent stem cells (hiPSCs). Any risks of cross-contaminations were eliminated. The total RNA was isolated from these cells, reverse transcribed, and quantitatively amplified by PCR (qRT-PCR). Each of the patients' and controls' samples was run in triplicates. The measurements presented in the figures were representative to all the samples studied.

To test pluripotency of the autologous, human, induced pluripotent stem cells, we quantified the transcripts for the genes: OCT4 and NANOG. Their levels of expression were falling down already during the course of two weeks from the start of differentiation.

To test initiation of myogenesis, we quantified the transcripts for the genes: GATA4 and MEF-2c. The levels of transcripts for these genes were increasing with progressing differentiation. This was synchronous with the down regulation of the gene expression for OCT4 and NANOG.

From these tests, we concluded that the patients' autologous, human, induced pluripotent stem cells anchored to the sarcomeres with the aid of the htAbs, did differentiate into the myocytes. The onset of the differentiation was reflected by the suppressed levels of the transcripts for the transcription factors unique for pluripotency. The robust, specific, induced differentiation was demonstrated by the increasing levels of the transcripts unique for the myogenesis.

Discussion

Herein, we report the proof of concept *in vitro* for development of a novel strategy, for the stem cell therapy of myocardial infarction, aimed at the improving targeting and retention of the stem cells to the site of the injury. It is based upon the bioengineering of a novel class of biomolecules – heterospecific tetravalent antibodies (htAbs), alternatively known as tetrabodies (tAbs). Furthermore, it is based upon using the htAbs for crosslinking of the components of the cardiac sarcomeres in order to stabilize them. Moreover, it is based upon anchoring of the human induced pluripotent stem cells (hiPSCs) to the stabilized sarcomeres used as the anchoring scaffolds. These phases are followed by the induction of myocardial differentiation of the anchored hiPSCs.

We attribute the effectiveness of the htAbs in anchoring of the stem cells to the human cardiac muscle sarcomeres to several factors. (1) They have exquisite specificity and high sensitivity towards SSEA-4, SSEA-3, TRA-1-60, TRA-1-81, which are uniquely present on the human pluripotent stem cells, but entirely absent on differentiating cells. At the same time, they have exquisite specificity and high sensitivity towards cardiac myosin, α -actinin, titin, and actin, which are uniquely accessible in the cardiac sarcomeres in the aftermath of the myocardial infarction, but entirely absent in on the surfaces of any other anatomical structures. (2) They have four different binding domains, which enhance the probability of binding to targeted antigens and increase the binding forces. (3) Flexible linkers, between the central hosting core and different antigen binding domains, add the ability of htAbs to bind to a few antigens in various spatial configurations and at different distances at the same time, which is a strong advantage over the rigid IgG, scFv, and their combinations based molecules having fixed, limited range between the antigen binding domains. (4) The htAbs are manufactured, to the high grade purity and sterility, through very simple purification process by high pressure liquid chromatography (HPLC), so that they are easy for

streamlining to cGMP. (5) The hiPSCs' isolation is based upon a simple procedure of MACS or FACS, which are available in the clinical laboratories. (6) The htAbs are very stable in solutions due to the high affinity of biotin towards recombinant avidin, which makes this platform suitable for a long shelf-life. (7) Total molecular weight of the bioengineered htAbs in this study is equivalent to one full IgG molecule, what helps with predicting and modifying their approximate pharmacokinetics.

The step-wise, one-antibody-at-a-time saturation of biotinylated antibodies to biotin binding domains of avidin, which we used, creates a universal system for constructing other tetravalent antibodies. In fact, in our initial attempts, we were using carboxyl terminus biotinylated Fab fragments prepared by enzymatic digestion. Moreover, this approach also facilitates the preparation of other mono-, bi-, or poly-specific polyvalent antibodies. It is easily accomplished by selection of the desired fraction collected from the high pressure liquid chromatography unit.

The observations, that applying the htAbs to the cardiac muscles' sarcomeric stabilized them, may have practical clinical consequences. The htAbs, which were attached to and spanned between the various sarcomeric molecules, were *de facto* strengthening stability of the sarcomeres' three-dimensional architecture. These muscle molecules, enforced and stabilized *in situ* by the htAbs, created the solid scaffolds capable for anchoring and harboring the pluripotent stem cells. This is formation of the biogels *in situ*, rather than delivering such synthetic gels. These scaffolds can be also used to incorporate other elements stimulating differentiation of the anchored stem cells in the desired lineage.

Streamlining this novel therapeutic strategy of regenerative medicine requires some precautions. All of the components of tetravalent antibodies are potential immunogens, which may elicit immune response. Therefore, the same precautions, as those undertaken in immunotherapy, are necessary. The tetra-specific antibodies, similarly to bi- or polyvalent antibodies are capable for linking different cells and/or molecules, which creates risks of microemboli *in vivo*. Therefore, free, circulating htAbs have to be cleared from the circulation after infusion or injection, but prior to infusion of the stem cells.

Nevertheless, with this novel approach, we demonstrated a statistically significant increase in the number of the autologous, human, induced pluripotent, stem cells (hiPSCs) anchored to the human cardiac muscle sarcomeres with the aid of the htAbs over the number of these hiPSCs detected without using the htAbs or with using non-specific antibodies. In this approach, targeting and retention of the hiPSCs to the sarcomeres relied upon the htAbs functioning as the direct cross-linking interface between them. Importantly, this anchoring took place under the continuous flow of the buffer carrying pluripotent stem cells over the cardiac muscles' sarcomeres, thus closely imitating conditions *in vivo*, when the stem cells are delivered in the physiological buffers by infusion or injections. However, the capabilities of the htAbs can also be utilized for cross-linking of the molecules at the site of the myocardial infarction, present physiologically or supplied artificially, to create biogels holding the stem cells in the site of the delivery. In all cases, the high number of the autologous, human, induced, pluripotent stem cells, anchored to the infarcted cardiac muscle sarcomeres, was a very encouraging factor for us undertaking of the trials *in vivo*.

Conclusion

By bioengineering of the new class of biomolecules - hetero-specific tetravalent antibodies and using them to anchor the patients' autologous, human, induced pluripotent stem cells to the human, infarcted, cardiac muscle sarcomeres, we created foundations for the trials aimed at improving the effectiveness of regenerative therapy of myocardial infarction.

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Abbreviations

htAb	heterospecific tetravalent antibody
mAb	monoclonal antibody
Fab	antigen binding fragment antibody
scFv	single chain variable fragment antibody
biAb	bispecific antibody
diFv	diabody
dcFv	dual chain variable fragment antibody
Oct4 aka POU5F1	gene for octamer-binding transcription factor 4A aka POU-type homeodomain – containing DNA binding protein
Sox2	for SRY (sex determining region Y)-box 2
Nanog	for homeobox transcription factor
Lin28	for cytoplasmic microRNA binding protein
Klf 4	Kruppel-like factor 4
Sv40	Simian vacuolating virus 40
FCM	flow cytometry
IB	immunoblotting
MACS	magnetically activated cell sorting
FACS	fluorescently activated cell sorting
NMRS	nuclear magnetic resonance spectroscopy
TRXFS	total reflection x-ray fluorescence spectroscopy
MPFS	multiphoton fluorescence spectroscopy
EELS	electron energy loss spectroscopy
EDXS	energy dispersive x-ray spectroscopy

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Figure 1.

The concept of anchoring of the human, induced, pluripotent stem cells (hiPSCs) to the cardiac sarcomeres (A: A-band; Z: Z-line) with the aid of the heterospecific, tetraivalent antibodies (htAbs) is presented at this figure. Icons for some of the elements are explained on the left side of this diagram. The htAbs are first spiked into the solution flowing over the sarcomeres (a). The htAbs anchor onto the sarcomeres by the myosin (A-band), α -actinin (Z-line), actin, titin binding domains of the htAbs (only the combination of anti-myosin and anti- α -actinin is shown for clarity here) (b). After clearing out the unbound htAbs, the solution is spiked with the hiPSCs (c). The hiPSCs, with the cell surface display of SSEA-4, SSEA-3, TRA-1-81, and TRA-1-60 (only the combination of the SSEA-4 and TRA-1-60 is shown for the clarity here) dock into the binding domains of the htAbs anchored onto the sarcomeres (d).

Figure 2.

The molecular architecture of the human cardiac muscle is revealed by electron energy loss spectroscopy (EELS). The fresh cardiac muscle was cryoimmobilized and cryosectioned followed by embedding/negative staining. The image was acquired at the zero energy loss, with the contrast tuned setting of the energy filter at the accelerating voltage 300keV. All the samples were prepared in triplicates. This image is representative for all the samples tested. The Z-line (Z) is the part of the sarcomeres in which actin filaments bind to its α -actinin. The A-band is formed by thick myosin filaments. It also includes the cross-bridges between myosin heads and thin actin filaments; the H-zone is a bare portion of thick myosin filaments; the M line in the H-zone's center. I-band, consisting of the thin, actin filaments, is the part of the sarcomere between the Z-line and A-band. The densely packed biomolecular clusters of Z-line and A-band exclude the molecules of the negative stain; hence they appear with light contrast. The H-zone and I-band contain more space between filaments, which absorb and retain a lot of contrast, therefore appear as the very dark structures. HFW: 3750 nm.

Figure 3.

The localization of α -actinin in the Z-line is revealed by electron energy loss spectroscopy (EELS). The fresh cardiac myofibrils were stained with the bioengineered, monospecific tetravalent antibodies anti- α -actinin covalently linked to the core-shell nanoparticles. The image was acquired at the zero energy loss, with the contrast tuned setting of the energy filter at the 300keV accelerating voltage. The Z-line's α -actinin is heavily labeled (black dots), while the background is label-free. Vanadate-PVP based negative stain/embedment offered lower contrast of the imaged cardiac muscle myofibril, but promoted a better contrast for the nanoparticles. The threshold driven nanoparticles' counts facilitated determination of specificity of the labeling, which was accepted at the statistical significance $p < 0.001$. The high specificity of this and other Fvs was required for bioengineering of the highly specific htAbs with no cross-reactivity. All the samples were measured in triplicates. This image is representative for all of the samples and all of the Fvs tested. HFW: 390 nm.

Figure 4.

Specificity and sensitivity of the bioengineered, tetraivalent bodies, towards cardiac α -actinin and titin, were highlighted on the human cardiac myofibrils by multiphoton fluorescence spectroscopy (MPFS). The cardiac myofibrils were labeled with the monospecific tetraivalent antibodies (htAbs) or monoclonal antibodies (mAbs) targeting α -actinin and titin, which were modified with chelates coordinating Eu and Tb with their unique emission spectra, thus providing opportunities for double labeling. The images of the whole mounts by Zernicke's contrast were added for the structural reference (wm). All samples were run in triplicates. The images are representative for all the patients' samples studied. The htAbs are exquisitely specific to the main components of the sarcomeres revealed in the figure 2: α -actinin – appearing as the bright line of the Z-line and two bright lines parallel to the Z-line of the titin's elastic domains. Pixel brightness compared between the specific labels and the backgrounds was accepted at the statistical significance $p < 0.001$. HFW: $4.8 \mu\text{m}$.

Figure 5.

Specificity and sensitivity of the bioengineered, tetraivalent antibodies towards cardiac myosin and actin were highlighted on human cardiac myofibrils by multiphoton fluorescence spectroscopy (MPFS). The cardiac myofibrils were labeled with the monospecific tetraivalent antibodies (htAbs) or monoclonal antibodies (mAbs) targeting myosin and actin, which were modified with chelates coordinating Eu and Tb, thus providing opportunities for double labeling. The images of the whole mounts by Zernicke's contrast were added for the structural reference (wm). All samples were run in triplicates. The images are representative for all the patients' samples studied. Pixel brightness compared between the specific labels and the backgrounds was accepted at the statistical significance $p < 0.001$. HFW: 5.1 μm .

Figure 6.

Specificity and sensitivity of the bioengineered, tetraivalent antibodies towards cardiac myosin was determined by immunoblotting (IB). The homogenized human cardiac sarcomeres were electrophoresed on polyacrylamide gels (EP), transferred onto PVDF membranes, and labeled with the HPLC fraction of the monospecific tetraivalent antibodies binding myosin (htAb), as well as with the monoclonal antibodies against myosin (mAbs) as the controls. All tests were run in triplicates. The images are representative for all the patients' samples studied. The htAbs are exquisitely specific to the cardiac myosin as demonstrated by the single bands and clean backgrounds; therefore the entire lanes are shown. Pixel brightness compared between the bands and the backgrounds were accepted at the statistical significance $p < 0.001$.

Figure 7.

Specificity and sensitivity of the bioengineered, bispecific tetraivalent antibodies towards cardiac α -actinin and myosin was highlighted on the cryosections by multiphoton fluorescence spectroscopy (MPFS). Immediately after the surgery, the cardiac muscles were cryoimmobilized and cryosectioned. Thereafter, the cryosections were labeled with the bioengineered tetraivalent antibodies targeting α -actinin and myosin (a) or mixtures of the monoclonal antibodies against α -actinin and myosin (mAbs) both tagged with the same chelates as fluorochromes. The patterns A-bands (A) and Z-lines (Z) are clearly highlighted in both approaches. Stability of the sarcomeres was tested by incubation at 37°C for different durations. The sarcomeres were labeled with the htAbs after (c) and before the 24h incubation (d). Without stabilization of the sarcomeres with the aid of the htAbs, outlines of the A-band and Z-line start to diffuse, as the signs of deterioration (c). Presence of cross-linking htAbs assures preservation of the sarcomeric architecture (d). This result reinforces the concept of using the myocardial infarction sarcomeres as the anchoring scaffold for the hiPSCs. However, the prolonged incubation leads to the gradual deterioration of the sarcomeric architecture (e, f). Pixel brightness compared between the specific labels and the backgrounds were accepted at the statistical significance $p < 0.001$. HFW: 9 μm .

Figure 8.

Gene expression of OCT4 and NANOG of the human induced pluripotent stem cells was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The cells expressing SSEA-4, SSEA-3, TRA-1-60, and TRA-1-81 were isolated from the induced cultures by magnetic activated cell sorting. The patients' (encoded C001–005) cardiac or skin fibroblasts (lanes 3–7) were cultured (Primary) and induced into pluripotency (hiPSCs), while the human embryonic stem cells H1 (lane 1) and human fibroblasts IMR90 (lane 2) were the controls. All the samples were run in triplicates. The data are representative to all the samples run. The total RNA was isolated, reverse transcribed to cDNA, amplified by PCR, adjusted, and the amplicons electrophoresed. The human induced pluripotent stem cells (hiPSCs) were expressing the transcription factors, which are unique for undifferentiated pluripotent stem cells. These genes were not expressed in the primary culture human fibroblasts.

Expression of OCT4 in hiPSCs

Figure 9.

Expression of the OCT4 gene in the embryoid bodies (EB) differentiating for over sixty days from the human induced pluripotent stem cells was studied by quantitative polymerase chain reaction after reverse transcription (qRT-PCR). At that time point, in the EBs, there were detectable contractions. The expression values were calculated with the reference to GAPDH, while relative to the cultured undifferentiated embryonic stem cells. The samples were run in triplicates. The graph is representative for all the samples studied. Differentiation of these cells resulted in down regulated gene expression for the OCT4. The statistical significance was accepted at $p < 0.001$.

hpcF

hiPSC

Figure 10.

Cell surface display of the biomarkers SSEA-4, SSEA-3, TRA-1-60, and TRA-1-81, as the hallmark of pluripotency, was studied with the confocal fluorescence. The human primary culture fibroblast (hpcF) of this patient (encoded C003) expressed fibroblast growth factor receptor (FGFR), but none of the pluripotency receptors (blank fields of view not shown). HFW: 60 μm . However upon induction, the fibroblasts from these cultures changed their geometry, as the first sign of the successful induction, and expressed on their surfaces these pluripotent biomarkers (SSEA-4, TRA-1-60), highlighted by labeling with the htAbs. The staining of the genomic DNA (gDNA) served as the internal reference of the nucleus. The samples were run in triplicates. The data are representative for all the samples studied. HFW: 20 μm .

Figure 11.

Specificity and sensitivity of the htAbs towards the pluripotency biomarkers displayed on the hiPSCs were tested on blots. The patients' human induced pluripotent stem cells (hiPSCs), as well as the patients human primary skin or cardiac fibroblasts (hpcF) and human embryonic stem cells were as the controls, were cryoimmobilized, homogenized, electrophoresed, transferred, and labeled with the htAbs. Presence of the chelated metals results in distinctive purple coloration of the blots. The blots were performed in triplicates. The blots in this figure are representative to all the studied. The hiPSCs displayed the same biomarkers as the totipotent ESCs, which were detected efficiently with the htAbs. The primary fibroblasts did not express the biomarkers of pluripotency.

Figure 12.

Effectiveness of the heterospecific tetraivalent antibodies to anchor the human induced pluripotent stem cells to the cardiac muscle sarcomeres (hiPSCs sarc) and to the endothelial cell monolayers (hiPSCs EC) were measured by x-ray energy dispersive spectroscopy (EDXS). The data for the bioengineered htAbs constructed of: anti-SSEA-4, anti-TRA-1-60, anti-myosin, anti-actinin are presented in these graphs. The measurements for each patient were conducted in triplicates. The data presented here are representative to all the samples studied. (a) Anchoring of the patients' unmodified human primary skin or cardiac fibroblasts anchored to the sarcomeres (hpcF sarc) and to the endothelium (hpcF EC) served as the reference. Human cultured embryonic stem cells H1, H9 anchored to the sarcomeres or endothelium (hESCs sarc or hESC EC respectively) and human cultured fibroblasts IMR 90 anchored to the sarcomeres or endothelium (hF sarc or hF EC respectively) were the positive and negative controls. The statistical significance was accepted for the hiPSCs and hESCs onto the sarcomeres versus onto the endothelium at $p < 0.0003$. (b) Retention of the anchored hiPSCs onto the sarcomeres upon the start day (hiPSC sarc 1d) was compared with that on the day 12 (hiPSC sarc 12 d). For reference, retention onto the sarcomeres of the human primary fibroblasts (hpcF sarc 1d and hpcF sarc 12d), human embryonic stem cells (hESCs sarc 1d and hESCs sarc 12d), and cultured human fibroblasts (hF sarc 1d and hF sarc 12d) were measured at the start of the trial (1d) and on the day 12 (12d). (c) Ability of the htAbs to anchor the hiPSCs to sarcomeres was measured (hiPSCs sarc). It was validated by attempts of anchoring with the non-specific tetrabodies (hiPSC ntAb block) (anti-EGFRvIII, anti-EGFRvIV, anti-CEA, anti-PSMA), by blocking with the mixtures of monospecific tetrabodies (hiPSC ST ntAb block) (SSEA-4, SSEA-3, TRA-1-60, TRA-1-81), the mixtures of monoclonal monospecific antibodies (hiPSC ST mAb block) (SSEA-4, SSEA-3,

TRA-1-60, TRA-1-81), and by omitting the antibodies (hiPSC no Ab). (d) Specificity of htAbs to anchor the hiPSCs to sarcomeres was measured (hiPSCs sarc). It was validated by attempts of binding with the non-specific tetravalent antibodies (hiPSC ntAb) (anti-EGFRvIII, antiEGFRvIV, anti-CEA, anti-PSMA), with the mixtures of monospecific tetrabodies against myosin, actin, actinin, titin (hiPSC MA mtAb), with the mixtures of monospecific monoclonal antibodies against myosin, actin, actinin, titin (hiPSC MA mAb), or by omitting the antibodies (hiPSC no Ab). The statistical significance was accepted based upon comparisons of the measurements of the numbers of human autologous pluripotent induced stem cells anchored to the sarcomeres with the aid of the htAb versus with no antibodies at $p < 0.0003$.

Figure 13.

Directed differentiation of the anchored hiPSCs towards cardiomyocytes was demonstrated by qRT-PCR. The human autologous induced pluripotent stem cells (hiPSCs) anchored onto the sarcomeres with the heterospecific, tetravalent antibodies (htAbs) targeting SSEA-4, TRA-1-60, myosin, actinin were induced into cardiomyogenesis. Expression of the OCT4 and NANOG genes for the transcription factors involved in pluripotency were down regulated over the two week period. Expression of the GATA4 and MEF-2c genes involved in myogenesis were up regulated over the same period of time. The statistical significance was accepted for $p < 0.0003$.