Isolation and culture of murine fibroblasts from cardiac and skeletal muscle tissue for co-culture experiments with murine embryonic stem cells; influence of co-culture on cardiac differentiation

An extensive report of an internship

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Abstract: Although it is known that fibroblasts do initiate MES cell differentiation, it is not known whether this signaling is due to biochemical, cell-cell interaction or both factors. Forward to this research, it is shown that fibroblasts from murine cardiac and skeletal tissue can be isolated and cultured by using a “halo of cell” technique. Second, a suggestion was made that CD34 staining could prove the presence of some fibroblastic types by using immunofluorescence. And third, a new approach of EB culturing showed that 50 ml tubes would work probably better for bringing EB’s in suspension than easy grip petri dishes. Further no MES differentiation was observed because of unsuitable EB’s or fibroblastic monolayer formation.

Introduction

Heart failure is one of the most frequent cardiovascular diseases in the world. Most of these problems are caused by a decrease of cardiomyocyte functioning. Because adult cardiomyocytes are unable to regenerate, the only effective treatment is heart transplantation.

Newer and better insights in the promising field of regenerative medicine could hopefully solve this issue. In the last decades, several animal studies demonstrated that transplantation of isolated cardiomyocytes might offer an alternative approach for the treatment of severe heart failure. In this context, several animal studies demonstrate a successful engraftment of cardiac myocytes into the adult heart. (1)

Cells that are capable for differentiation into cardiomyocytes are embryonic stem cells (ES cells). ES cells are pluripotent, which means that they are able to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. These include each of the more than 220 cell types in the adult body, under which cardiomyocytes. (2)

What determines the chosen differentiation path of the cells is probably dependent on their environment. This includes a combination of matrix stiffness, extracellular matrix composition, neighbouring cells and biochemical factors. The hypothesis for this study is that the fibroblasts in different tissues differ. The ES cells that will interact with these different types of fibroblasts should therefore choose a different path of differentiation.

In this project, the influence of murine cardiac and skeletal muscle fibroblasts on the differentiation of murine ES cells (MES cells) will be investigated. The bulk of the project went to the purification of fibroblast isolation from murine tissues.

Methods and Materials

The study can be subdivided into individual projects that will come together when all pieces are finished. These are: 1) Fibroblast isolation, 2) Immunofluorescence, 3) qPCR preparation 4) Formation of embryoid body’s (EB’s), 5) Apply the EB’s to the fibroblast monolayer.

Now, step by step each part will be investigated thoroughly. Also the results of placing individual parts together will be discussed.
Fibroblast Isolation

The goal was to isolate the fibroblasts from murine tissue, and culture them in monolayers.

Murine tissue isolation
Murine cardiac and skeletal tissue was isolated from laboratory mice. These mice were involved in another experiment and therefore sacrificed.

Beforehand, two 50 ml tubes were filled with 30 ml phosphate buffered saline (PBS) and 2 ml of the broad spectrum antibiotic combination of penicillin and streptomycin (penstreps).

After isolation of the heart and some skeletal muscle, both tissue types were separately stored in the tubes and transported to the laboratory for further treatment.

Method 1: Separate tissue into single cells

After isolating the tissue, it should be separated into individual cells. The idea was to culture the individual cells, and by letting the fibroblasts overgrow the other cell types, a single monolayer, which consists of only fibroblasts, would be obtained.

A 6 wells plate was coated with a 0.1% gelatin solution and then incubated for 20 min. The tissue parts were put in separated large petri dishes and a scalpel was used to cut pieces as small as possible. Hereafter, the pieces were carried over to a new 10 ml tube, and 2.5 ml collagenase solution was added to it. Now this was incubate for 60 min. at 37 °C. 5 ml trypsin was added in the last 10 min. The tubes were centrifuged for 5 min. at 300 g with the break set on 9. After resuspension in 5 ml PBS, different sizes of pipettes were used for cell loosening. If first a larger pipette was used to suck the tissue pieces up and down with heavy force, cells will detach from the tissue part. When repeating these steps by using smaller tips each time, single cell fragments will remain. After this, the tubes were centrifuged again with the same settings and the sediment was resuspended in 4 ml PMEF medium. The gelatin was removed from the dish, and the volume was distributed equally over 2 coated wells and placed in the incubator for 2 days.

Passaging of the cells was done when the culture plate grown confluent. A small plane of 0.25% trypsin was added to the cells after removing the medium and placed in the incubator at 37 °C for 10 minutes. Now the loosen cells were put in a tube. The culture plate was washed with PBS and added to the cells. After centrifugation for 5 minutes at 1000 RPM, cells where resuspended and seeded at 1:3 dilution on a new coated plate which surface is now approximately 3 times larger. New culture medium was added and the cells were placed in the incubator.

Method 2: Halo of cells

The second attempt for fibroblast isolation was based on the article of Sharon Etzion et al. (3). They stated the possibility to let fibroblasts proliferate from the margin of tissue fragments.

Tissue parts were placed in separated large petri dishes and a scalpel was used to cut pieces of 1 mm³. Now the 4 pieces were distributed equally in 1 small uncoated petri dish, like there is no contact to the wall or other present pieces. The previous step was repeated for petri dishes coated with 0.1% gelatin. Fragments were incubated without any further additives for 2 hours. 3 droplets of PMEF medium was added and incubated for 1 day. Here after, enough medium was added to cover the soil. Another day later, enough medium was added to cover the whole fragments. After 4 to 5 days, outgrow of fibroblast should be seen.

Carrying out this experiment, 3 mice were available. These were of the same type as in method 1. From mouse 1, 4 petri dishes were obtained: 1 uncoated and 1 coated dish containing heart tissue and 1 uncoated and 1 coated dish containing skeletal muscle tissue. For mouse number 2 and 3, the experiment setup was doubled, resulting in 8 dishes each.

Culturing fibroblast monolayer

Now all fibroblasts isolated from the cardiac tissue were brought together and all the fibroblasts originating from the skeletal tissue. This was done by gently removing the tissue fragments. After loosening the cells with trypsin and PBS, cells were transported to a 10 ml tube and centrifuged for 7 minutes at 1000 RPM. Hereafter cells were counted by using the nucleon counter (Automated Mammalian Cell Counter, New Brunswick Scientific). Cells were cultured at the density of 5*10³ cells per cm².
Formation of embryoid body's

The goal was to see whether ES cells differentiate into cardiomyocytes. Here for the article of Agapios Sachinidis et al. (4) was used as a guideline for cardiac specific differentiation of mouse embryonic stem cells.

Primary mouse embryonic fibroblast feeder layer

The article of Izhak Kehat et al. (5) stated that when ES cells were brought into contact with inactivated primary mouse embryonic fibroblasts (iPMEFs), they maintain their undifferentiated state for prolonged periods.

PMEFs were taken out of the liquid nitrogen, thawed and then the fibroblasts were grown confluent. Inactivation of the fibroblasts will occur while using mitomycin C. (5) Take 80 µl of mitomycin C out of the fridge. Remove all the medium of the T75 flask and add the mitomycin to the cells. Incubate this for 6 hours. Hereafter, transfer the inactivated cells into 2 wells of a 6 well plate. After 2 days, refresh the medium.

Now take the MES out of the liquid nitrogen and put the content in a 10 ml tube. Add another 9 ml MES medium to it, centrifuge it during 5 minutes at 1000 RPM and resuspend it in 8 ml MES medium. Replace the iPMEF medium by the MES suspension. Now they are available for further use.

Creating hanging drops

From the article of Agapios Sachinidis et al. (4), a protocol was found which made clear how MES could differentiate into beating cardiomyocytes. For this, hanging drops of 20 µl containing 400 MES cells should be made. This was done by taking the MES up from the iPMEF layer, counting the cells and diluted the solution in a sufficient way. After 2 to 4 days, the MES started to aggregate inside the wells of a 6 well plate. After 2 days, refresh the medium.

Now they are ready for further use. (36x50)

Immunofluorescence

In order to see if the fibroblast isolation succeeded, immunofluorescence was used to visualize the cell types. Because no fibroblast markers could be found during the study (although it turned out they do exist (6), (7)), it was thought of another approach. Instead of proving the presence of fibroblast, other cell types could be excluded.

Antigens

α-actinin: contains two actin-binding domains that are around 30 nm separated from each other for forming a more loosely packed actin bundle. It is concentrated in stress fibers, where it is responsible for the relatively loose cross linking of actin filaments in these contractile bundles. It also helps forming the structure that holds stress fiber ends in focal contacts at the plasma membrane. (6)

α-SMA: alpha smooth muscle actin (α-SMA). These are actin bundling proteins. It contains two actin-binding domains and is concentrated is stress fibers where it is responsible for the relatively loose cross linking of actin filaments in these contractile bundles. They are largely present in muscular cells. (8)

CD34: This cadherin is a heavily glycosylated, (105-120) kDa, transmembrane glycoprotein expressed on hematopoietic progenitor cells, vascular endothelial cells and some fibroblasts. It may also play a role as a cell-cell adhesion factor of certain antigens to endothelium. (9)

CD144: This cluster of differentiation is a calcium-independent epitope (antigenic determinant) on cadherin 5 and is a calcium-dependent adhesion molecule. It is expressed on endothelial cells in vivo and in vitro. It may play a role in the organization of lateral endothelial junctions and in the control of permeability properties of vascular endothelium. (10)

Primary Antibodies

The primary antibodies used in these are: monoclonal IgG1 from Sigma (1:800, nr A7811) against α-actinin, IgG2a from Sigma (unknown dilution, nr A2547) against α-SMA, anti-CD34 IgG from Santa Cruz (1:100, SC7045) and anti-CD144 IgG1k from BD Pharmingen (unknown dilution, nr 555661).

Secondary Antibodies

For CD 144 and α-actinin GaM IgG1 Alexa 488 from Molecular Probes (1:300, nr: A21121) was used. For α-SMA GaM IgG2a Alexa 488 from Molecular Probes (1:300, nr: A21131). And for CD 34, RaG IgG FITC from Sigma (1:400, nr: F7367) was used.
Staining protocol
First place 24 small round glasses in a 24 wells plate after thoroughly cleaning with acetic acid and 70% EtOH and applying a gelatin coating to it. The cells from the isolation were grown on these glasses for 1 day.

For a positive check of the antibodies, frozen tissue sections of 10 µm thickness were cut from the skeletal muscle, (Microm HM 550, Adamas). These had the same staining protocol.

Two small glasses were placed on cover plates which were wrapped in parafilm. One glass was used for the real measurement, and the second one for the conjugate control. The cells were fixated with 100 µl 1% formalin for 10 minutes. For antigens lying inside the cells like α-actinin and α-SMA, cells needed to be permeabilized by adding 100 µl 0.5% Triton X-100 for 30 minutes. Every sample was blocked twice, 15 minutes with 1% Horse Serum (HS) 100 µl each and twice with 100 µl NET-gel for 15 minutes. Then the primary antibody was added on only 1 glass of the cover plate and incubated at room temperature for 2 hours. The samples were washed 6 times with 100 µl for 5 minutes with NET-gel. Then the secondary antibody was added for 1 hour and incubated at room temperature. Then washed two times for 5 minutes with NET-gel and added 75 µl DAPI staining (1:500 diluted) for 5 minutes to every sample for visualizing the cell nuclei. They were washed 4 times 5 minutes with 100 µl PBS. Hereafter, 2 drops of 20 µl Mowiol were placed onto a new cover slip and the round glasses were placed upside down in these drops. They were stored in a dark box at -4 °C. Now photos were taken with a fluorescence microscope (Zeiss axiovert 200 MOT fluorescence microscope with axiocam & environmental control unit).

Preparations for qPCR analysis
qPCR can give information on the gene expression in a sample. Although the qPCR reaction itself was not executed, preparations were made for future studies.

Placing EB’s in RLT buffer
After the first set of EB’s did not differentiate into cardiomyocytes, an attempt was made to isolate its RNA. For storage of RNA, the EB’s should be placed into RLT buffer (lysis buffer), (Qiagen, Leusden, The Netherlands) which contains guanidine thiocyanate. Complete disruption of cell membranes and organelles is absolutely required to release all the RNA contained in the sample.

In addition to the RLT buffer, β-mercaptoethanol was added in a 1:100 ratio to eliminate ribonucleases, which are released during cell lysis. Otherwise it would result in very stable enzymes. This irreversible denaturing of the proteins prevents them from digesting the RNA during its extraction procedure. (11) Now the sample was stored at -80 °C.

Purification of the RNA
For the isolation of RNA an RNeasy Mini Kit (Qiagen, Leusden, The Netherlands) was used. Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Centrifuge for 15 s at 10,000 RPM. Discard the flow-through. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 10,000 RPM to wash the spin column membrane. Discard the flow-through. Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix gently by inverting the tube and centrifuge briefly to collect residual liquid from the sides of the tube. Add the DNase I incubation mix (80 µl) directly to the RNeasy spin column membrane, and place on the bench top (20–30 °C) for 15 min. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 10,000 RPM. Discard the flow-through. Add 500 µl Buffer RPE to the RNeasy spin column and centrifuge. Discard the flow-through. Add 500 µl Buffer RPE to the RNeasy spin column. Place the RNeasy spin column membrane. Add 30-50 µl RNase free water directly to the spin column membrane. Centrifuge for 1 min at full speed to elude the RNA.

RNA quantification
The quantity of the RNA in the sample can be measured by using a spectrophotometer such as the Nanodrop (ND-1000, Isogen Life Sciences, IJsselstein, The Netherlands). If known that the stock solution of the RNA sample has an absorption level of 230 nm, RNA (as well as genomic DNA and some plasmids) has their absorption at the wave length of 260 nm and that other important compounds (known as tyrosine and tryptophan residues) which are used in protein formation have their absorption
level at 280 nm, an RNA concentration can be measured. By using the formula of Lambert-Beer, which makes use of the incoming light intensity compared to the outgoing intensity and taking into account the absorption level of the RNA, the RNA concentration can be calculated. (Formula not worked out) (12). When calculating the $A_{260}/A_{280}$ ratio, the proportion of protein contamination can be calculated. A rule of thumb is that this number should be between the 1.7 and 2 for having a workable solution. The $A_{260}/A_{230}$ proportion gives knowledge about the proportion of the solution contamination. As a guideline, this number should be around the 1.5 when having a good sample.

Gel electrophoresis
A drawback on spectrophotometric measurements is that DNA as well as ribosomal RNA (rRNA) and some plasmids all contribute to the absorption around the 260 nm. So it is not known what you are really measuring. Now gel electrophoresis, selecting on molecular weight, can give more information. When having a pure rRNA isolation, 2 bands should be detected, representing the two subunits (the small 18S and bigger 28S) of the rRNA.

The RNA was put in a 1% agarose gel and stained with Ethidium Bromide. This will visualize the RNA after UV exposure with fluorescent light. In this study, the Versa Doc (Bio-Rad) was used. The electrophoresis was executed at 100 Volt for 20 minutes.

cDNA synthesis and qPCR analysis
Up to here, the purity of the RNA was checked. Further treatments towards the cDNA synthesis or even qPCR itself were not made. Although it was seen that others synthesizing cDNA from the sample, no results were available.

Results

Fibroblast isolation
During the study, different isolations were done to improve results by learning from the previous isolation. The alterations will be chronically described.

Isolation 1
This pilot experiment seemed to work well. Isolation of the fibroblast using method 1 succeeded and different passages were done. After obtaining 2 confluent grown T75 flasks of fibroblasts, samples were taken for further immunofluorescence microscopy. After 2 weeks of culturing, cells stopped growing and started to die after 9 weeks.

Isolation 2
During this isolation, a cell strainer was used. Several passages worked well, only once placed in a T75 flask, cells started to die.

Isolation 3
In an attempt to copy the results from isolation 1, the same protocol was repeated. Besides this experiment, a side project was done by spitting the fibroblast isolation into two groups. One was treated with medium and one with medium in addition of β-mercaptoethanol.

One week after the isolation, the cells could still not be transferred to a bigger environment and started to die. These were the results in both skeletal and cardiac isolation with and without treatment of β-mercaptoethanol; many small straight unmoving particles attached to the bottom, which did not loosen with trypsin. (Figure 1, Figure 2)

Figure 1: Results from skeletal isolation 3, one week after isolation.

Figure 2: Results from cardiac isolation 3, last few cells surrounded by undefined noise.
The medium was checked on the presence of contamination, but after incubation of several days, nothing was found.

**Isolation 4**
For this isolation, the same protocol was continued but now again in the presence of the cell strain. Only after 2 days, the same results as from isolation 3 were obtained. Because of the fear of contamination, 50 µg/ml of the antibiotic kanamycin was added to the medium.

**Isolation 5**
A new isolation, treated according to the standard protocol without the usage of a cell strain but including kanamycin in the medium, was done. Only one hour after the isolation was done, the following pictures were taken. (Figure 3, Figure 4)

As can be seen, not every cell is separated from each other, resulting in rectangular pieces of skeletal tissue. For the cardiac isolation, a lot of red blood cells were present, leading to a blurred image. One week after isolation, the results were similar to those of isolation 3.

**Figure 3**: Skeletal tissue isolation 5 one hour after finishing the protocol.

**Isolation 6**
Now a new approach of fibroblast isolation was tested. This approach is described as method 2 in the materials and methods section. The isolation went well and resulted in one coated and one uncoated petri dish of cardiac fragments containing 4 pieces each. Also 1 coated dish of skeleton muscle containing 4 fragments was obtained.

**Isolation 7 and 8**
Because isolation 6 went well, more results were generated by isolating tissue from 2 more mice treated according to the same protocol as in isolation 6. Now from each mouse 1 dish with 4 fragments of cardiac tissue were prepared, on both a coated as an uncoated dish. The same was done from the skeletal muscle.

1 week after isolation, fibroblast started to migrate from the tissue. (Figure 5, Figure 6)

**Figure 5**: Fibroblast migration from undefined tissue of isolation 7 or 8

**Figure 6**: Close-up of fibroblast migration out of undefined tissue isolation 7 or 8.
Figure 7: Loosening of a tissue fragment reveals a nicely equal distribution of fibroblasts.

Figure 8: Fibroblasts escaping the circular “fence” which surrounds every tissue fragment.

Figure 9: A fanned shaped orientation of fibroblast migration.

4 days after observation of the migration of fibroblasts, each fragment was investigated very carefully. If a fragment contained any fibroblast, estimation was made if it were 0, 1-10, 10-100 or 100-1000 fibroblasts present. Respectively they were assigned with labels 0, 1, 2 or 3. This gave the following results (Table 1).

Also three interesting observations were made. Of these, pictures were taken and will be discussed later on. There will be a reference of the picture numbers in the table (Table 1).

**Quantity estimation of fibroblast outgrow**

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**Table 1:** Labels 0, 1, 2, 3 means respectively that there are 0, 1-10, 10-100 or 100-1000 fibroblasts present.

**Culturing fibroblast monolayer**

Now all fibroblasts isolated from the cardiac tissue were brought together and all the fibroblasts originating from the skeletal tissue were brought together. After counting with the nuclei counter, it appears that the fibroblast isolated from the heart contained $15\times10^3$ cells/ml and the skeletal muscle fibroblasts contained $60\times10^3$ cells/ml.

After passage 1, cardiac as well as skeletal fibroblasts started to die. Unfortunately all the cardiac fibroblasts died so fast, that no monolayer could be formed. For the skeletal fibroblasts, it turned out that only a small amount of fibroblasts could be applied to half of a 96 wells plate and even these did not grow confluent.
Formation of embryoid body’s

As stated in the Materials and Methods section, EB’s should be formed, before placing them on the fibroblastic monolayers. This requires a nice piece of time management. The creation of EB’s and monolayers separately was one thing, but finishing them together for further use was another.

Spontaneous differentiation

To see if EB’s were able to differentiate without having a fibroblastic monolayer, EB’s were just put on a 0.1% gelatin coated wells plate. This was directly done after the creation of hanging drops, so without first bringing the EB’s in suspension. After 7 days of culturing, no beating cardiomyocytes were obtained. For further investigation, these EB’s were put in RTL buffer for qPCR analysis.

New EB’s

Because it seems that EB’s do not differentiate without the presence fibroblasts, new EB’s were formed. This time the suspension step was included. After 5 days of culturing, the EB’s did not look nicely round shaped, and a lot of debris was present in the medium. (Figure 10) After keeping them for a while, no healthy EB’s were left.

From these cells, 4 different dilution series were made for the creation of hanging drops, namely: 1000, 600, 400 and 200 MES in each drop of 20 µl. Each petri dish consisted approximately 20 drops. For each dilution, 3 dishes were made.

Another important difference is that instead of bringing the EB’s in an easy grip dish in suspension, 1 dish of each dilution was brought in suspension in a 50 ml tube, added with 4 ml MES medium. This was for checking the growth capacity in both media.

As a result, the following pictures were taken from both EB’s cultured in an easy grip dish and from those in a 50 ml tube.

4 different MES concentrations for the hanging drops

In this last attempt to create EB’s, a new layer of PMEFs was grown confluent in 1 well of a 6 wells plate. After putting the whole highly concentrated tube of MES cells on the cultured layer, (probably around the 5*10³ MES cells) after 4 days the medium was found yellow. After counting the MES cells, there appeared to be approximately 75400 cells in 20 µl.

Figure 10: Death EB’s surrounded by debris. Magn: 5X

Figure 11: EB’s in suspension originating from a 50 ml tube

Figure 12: EB’s in suspension originating from an easy grip dish
Although it was not understood what particles in the media the EB’s were and what debris, one thing that can be seen is that there are larger clustered particles in the culture media of a 50 ml tube (Figure 11) compared to those from an easy grip dish (Figure 12). Magnified pictures of these particles were taken. (Figure 13, Figure 14)

**Immunofluorescence**

To check the presence of cells other than fibroblasts, immunofluorescence was used. When no other cells than fibroblasts are present, only the DAPI staining would light up. Otherwise, the second antibody would light up in green. Except CD34, what can visualize some fibroblast types.

**Isolation staining**

Cells that were isolated using method 1, in the very first stage of the research, were stained according to the protocol.

As a result, only the CD34 staining lighted up in green in both cardiac and skeletal tissue. The rest of the stained samples visualized blue DAPI fluorescence. (Figure 15, Figure 16)

The pictures were taken after seeding the EB’s in half of a 96 wells plate, (see fibroblast isolation part) in an attempt to culture the EB’s on the leftover isolated fibroblasts. This observation was the same for every well in the plate. But after culturing of the EB’s, no beating cardiomyocytes were obtained.
Positive control
For this purpose, the antibodies for α-actinin, α-SMA and CD34 were checked. CD144 was not checked, because of absent information about the usable dilution in the staining protocol. The following results about CD34 were obtained (Figure 17). Here can be seen that slight green fluorescence is visible at the edges of the cell and that this green staining is absent at Figure 18.

Although still no green fluorescence of α-actinin, α-SMA was detected. It would be useful to repeat this staining protocol once more, because there might be a problem when making the cells permeable and that therefore the antibodies for α-actinin, α-SMA could not enter the cell. Unfortunately, there was no more time left to check this.

Preparations for qPCR analysis
As described above, several steps are needed to take, before one can start analyzing with qPCR.

Gel electrophoresis
During electrophoresis, the sample didn’t get the time to fully extend because it loosened its fixed position before the test was finished. Still two clear bands were visible, indicating the two subunits of the rRNA (Figure 199).

RNA quantification
These are the results coming from the nanodrop experiment.

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Table 2: Measured absorbance of purified RNA at corresponding wavelengths

This spectrometric analysis of the RNA resulted in an \( A_{260/280} \) ratio of 2.08 and an \( A_{260/230} \) ratio of 1.76. The total concentration of the RNA was 892.7 ng/µl.
Discussion

Fibroblast isolation

In general it can be said that isolation method 2 works well, although it is not clear why method 1 failed several times. For getting a much better result when using method 2, it should be investigated why not every tissue fragment resulted in a migration of fibroblasts.

Death cell results as in isolation 3

What causes the death cells in isolation 3 (Figure 1) is not exactly know. These undefined so called “Black Cells" did not move, where straight and did not loosen their ground surface when using trypsin. A possible solution to this observation could be found in the following explanation.

What might be the case is that these so called “Black Cells" are the fragments of deceased isolated cells. After applying an overload of cells to the wells plate (Figure 3), waste products of the cells will end up in the medium, resulting in a lowered pH value. When this happens, cell adhesion will become unstable and eventually, cells detach from the surface and round themselves up or totally lose contact.

Another effect of having an overload of cells in culture is that there could be a lack of oxygen supply (hypoxic event), due to extreme consumption. Now cells started to die in a so called coagulative necrosis way. This type of necrosis, as a result of hypoxia, leaves microscopically visible fragments of cells (13).

Halo of cells

The second method of fibroblast isolation worked well for some of the tissue fragments. Now the question that still remains is why not every particle resulted in the same amount of fibroblast migration.

What appears was that tissue fragments that came off the plate (this happened twice) (Figure 7), had an enormous amount of fibroblasts underneath it. Also both tissue fragments were relatively small (less than 0.5 mm²).

Hypothetically speaking, it can be said that, there is a relation between the loosening of the fragments and the enormous amount of fibroblasts. What could be is that these small tissue parts had not enough surface contact to resist slight fluidal movement when placing the dishes from the lab cabinet into the incubator. Because of the shear stress from the fluid, the tissue fragments started to loosen. As a result of these stresses, maybe the cell triggered some signals that activate the fibroblasts to create a more stable foundation.

A different approach is that there are so many fibroblasts surrounding and lying underneath the tissue parts that those bulk of cells eventually pushed the fragments of from the dish. Although the question than still remains why exactly these two small fragments had by far the most fibroblast formation compared to the much bigger parts.

Different fibroblast outgrows

As described in the method, first the tissue fragments needed to dry out on the dish surface, for creating a stable adhesion between the fragments and the surface. Later on, a drop of medium was added to supply the parts with nutrients. This resulted in two circular shaped fences surrounding the fragment (as can be seen in Figure 6 and Figure 8). This was believed to impede further fibroblast formation. As can be seen in figure 8, it seems that the fibroblast formation is not affected by the medium remaining because they just grow out underneath these remains.

Fibroblast orientation

As known from the principles of wound healing, fibroblasts line themselves up in the orientation of the injury. Now it seems that there was some kind of fibroblastic orientation obtained as can be seen in Figure 9. What causes the alignment is not known.

Quantity estimation of fibroblast outgrows

It seems that fibroblast outgrow from skeletal tissue is easier to obtain than those isolated from the cardiac tissue. Also both coated as uncoated plates resulted in fibroblast outgrows. So it seems that extra coating will not amplify fibroblast formation.

Culturing fibroblast monolayer

Although it is known that fibroblasts are easily cultured cell types because of their high dividing rate, still they can generate a limited number of cells. So when a small piece of tissue contains a few fibroblasts, after several days these single cells duplicated themselves many times. After bringing them into a bigger culture medium, it is understandable that the division rate is not as high as expected. Therefore it is needed to culture a lot more of separated fragments for the creation of a monolayer.
Formation of embryoid body's

Formation of hanging drops
The size of the EB's created in the hanging drops, highly depended on the total length of time they were in the drop and the amount of MES cells present in the drop. The longer they are in, the more time the cells got to clot together. On the other hand, the more cells are present, the bigger the EB’s will be. Although different dilutions were made in the last experiment, no differentiation could be observed.

No good fibroblastic monolayer
During the experiment, there was not a moment in which both fibroblast monolayer and the EB’s were brought perfectly together. This could be of great influence on the results. Even in the last experiment where we put the EB’s on the fibroblasts, these monolayers had a bad condition and probably started to die. Therefore, next time, the two parts should be brought together when both being in a more perfect condition.

EB’s suspension medium
What stands out was that EB’s that were brought together in a 50 ml tube, were larger clustered particles, compared to those placed in an easy grip petri dish. If these particles are indeed EB’s or debris is not known.

Looking at the structure of the magnified pictures (Figure 13, Figure 14); there are indeed clumps of cells visible. They also have the appropriate size of an EB looking at the 40 times magnified images. This was most clear for the EB in the 50 ml tube suspension.

A possible explanation could be that the EB’s in the tube had more free space for movement and therefore could have a better interaction. Also when fluid is spread out on a bigger surface (as in a petri dish), due to hydrostatic interaction, less fluidal movement is possible, resulting in less EB interaction.

Immunofluorescence
It seems that the staining protocol only worked well for CD34. Here, green fluorescence was observed in the positive check as well as in the isolation, concluding that probably vascular endothelial cells and some fibroblasts may be present in the isolation. Looking at their morphology, these cells are probably fibroblasts. (Figure 15)

Because the other antigens were not visible on the positive check, therefore the given results in the first isolation are not reliable. It is nearly impossible to have perfect fibroblast isolation without the presence of any other cell types. Most likely, the staining protocol didn’t worked well, or some antibody combinations were mixed up. Further investigation on this is needed.

Also this kind of approach; to exclude cell types instead of identify the fibroblastic cells, is very impractical. It would be much better to investigate further isolations, using a fibroblastic marker.

Preparations for qPCR analysis

RNA quantification
The proportions for protein and solution contamination, calculated from the absorbance, are within the given boundaries which were stated in the Material and Method subsection. A RNA concentration of 892.7 ng/µl is enough for further qPCR measurements. (Reference not given)

Gel electrophoresis
The next time gel electrophoreses is used. One should be sure about the stability of the gel before starting it to run. Now it cannot 100% be guaranteed that there were only 2 bands in the sample present. Maybe when this experiment had a longer duration, different results were obtained. Although it can be seen that the reference sample is nicely stretched out and therefore makes this result still acceptable.
Conclusion

In this study, no results were obtained from which can be concluded, or excluded, that fibroblasts do initiate any sort of MES cell differentiation.

Fibroblasts can be obtained from a halo of cell method whereby fibroblasts migrate out of small tissue parts, although it is not yet demonstrated by immunofluorescence that these outgrowing cells are indeed fibroblasts.

CD34 is a cell marker that can be used in fibroblast isolation. This will indicate the presence of hematopoietic progenitor cells, vascular endothelial cells and some fibroblasts. A better way when using immunofluorescence is not to exclude other cell types, but to identify the fibroblasts.

Looking at the morphology of EB’s, it seems that they can be better put in suspension in 50 ml tubes rather than in easy grip petri dishes. Although more debris in the tubes was observed, larger EB’s were formed, making them more appropriate for further use.

It is not clear why there is no EB differentiation observed when putting them on a fibroblastic monolayer. This could be both caused by the EB’s as well as the fibroblastic monolayers used so far. Both were not ideally cultured before usage.

For further research, it is important to culture the EB’s as well as the monolayers in such way, that both are ready for usage at the same time. Also larger amounts of fibroblasts should be obtained before culturing them into monolayers. Also further process must be made to see if EB’s differentiation is triggered by biochemical, cell-cell interaction or both factors.
References


