Implication of SPARC in the modulation of the extracellular matrix and mitochondrial function in muscle cells Version 2

Aicha Melouane,Mayumi Yoshioka,Jonny St-Amand

Abstract

Secreted protein, acidic and rich in cysteine (SPARC) is differentially associated with cell proliferation and extracellular matrix (ECM) assembly. We show here the effect of exogenous SPARC inhibition/induction on ECM and mitochondrial proteins expression and on the differentiation of C2C12 cells. The cells were cultured in growth medium (GM) supplemented with different experimental conditions. The differentiation of myoblasts was studied for 5 days, the expressions of ECM and mitochondrial proteins were measured and the formation of the myotubes was quantified after exogenous induction/inhibition of SPARC. The results indicate that the addition of recombinant SPARC protein (rSPARC) in cell culture medium increased the differentiation of C2C12 myoblasts and myogenin expression during the myotube formation. However, the treatment with antibody specific for SPARC (anti-SPARC) prevented the differentiation and decreased myogenin expression. The induction of SPARC in the proliferating and differentiating C2C12 cells increased collagen 1a1 protein expression, whereas the inhibition decreased it. The effects on fibronectin protein expression were opposite. Furthermore, the addition of rSPARC in C2C12 myoblast increased the expression of mitochondrial proteins, ubiquinol-cytochrome c reductase core protein II (UQCRC2) and succinate dehydrogenase iron-sulfur subunit (SDHB), whereas the anti-SPARC decreased them. During the differentiation, only the anti-SPARC had the effects on mitochondrial proteins, NADH dehydrogenase ubiquinone 1 beta subcomplex subunit 8 (NADHB8), SDHB and cytochrome c oxidase 1 (MTCO1). Thus, SPARC plays a crucial role in the proliferation and differentiation of C2C12 and may be involved in the link between the ECM remodeling and mitochondrial function.

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Protocol

Step 1.

Before starting cell culture : General considerations

ANNOTATIONS

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- Before starting any experiment, washthe hood with70% ethanol.
- Neveruse glovesthat were usedin themainlab. In caseyou have to leavethe hood, alwaysspray thegloves with70% ethanolbefore startingany manipulation.
- Clean withethanoleverything thatgoes in and outof the hood:boxof tips, culture flask, bottleof medium,trypsintube, .For hematocymeter:It mustberigorouslycleanedwith ethanolbetween

uses.
- Manipulatemorepossible depthof the hood
- Don’t cough or sneezefacing the hood. Also, don’t avoid or talkingfacingan incubatorwhen it isopened.
- Nevermovethe armsor equipmentover abottleor a plateopen.
- After your manipulation don’t forget to :
    - Wash the surface of the hood with the « Vim ».
    - Rinse thoroughly with water.
    - Rinse thoroughly with ethanol and dry.

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Step 2.
C2C12 cell line

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- The cell line C2C12 is an immortal line of mouse skeletal myoblasts originally derived from satellite cells from the thigh muscle of a two month old female C3H mouse donor 70h after a crush injury (*Yaffe and Saxel., 1977, Karyotyping., 2011*). From the C2s the immortal subline C2C12 was selected (*Blau et al., 1985*).
- C2C12 myoblasts are often used in vitro to study the muscle development and differentiation because they have all the characteristics needed for the study of myogenesis: differentiates rapidly, forming contractile skeletal myotubes and producing characteristic muscle proteins and the androhen receptor (AR) (*Bains et al., 1984, Bennett et al., 1997*).
- The ability of C2C12 cells to differentiate *in vitro* means that these cells represent a valuable source of material for a wide variety of studies examining skeletal myoblasts and myotubes in muscle biology. In addition to studies directly examining myogenesis and gene regulation (*Iyer et al., 2006, Ono et al., 2006*) , applications of C2C12 cells and differentiated skeletal myotubes include studies examining insulin signaling (*Kumar et al., 2002)*, metabolic activity (*Weber et al., 2002*) electrophysiology (*Fioretti et al., 2005*), oxidative stress (*Rohrbach et al., 2006*), cell migration (*Goetsch et al., 2005*) signal transduction (*Ko WC et al., 2006*), hypertrophy (*Cross-Doersen et al., 2003***)** and tissue replacement (*Laino et al., 2006*).

Step 3.
C2C12 cell culture

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- C2C12 is a fairly quickly growing cell line (**doubling time is approximately 12h**); **we will need to passage them every 1-2 days.**

Step 4.
Medium and reagents preparation under clean bench

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- **Fetal Bovine Serum (FBS)**
- Defreeze 500 ml of a FBS bottle (Hyclone, stored at -80ºC) in 37ºC water bath.

- Prepare 10 x 50 ml Falcon tubes (FBS, 1/10-10/10, date) and 50 ml pipette.
- Aliquot 50 ml with a 50 ml pipette to 50 ml Falcon tubes (total 10 tubes).
- Store at -20ºC.
- **Penicillin-Storeptomycin solution**
- Defreeze 100 ml of a 100X Penicillin-Storeptomycin bottle (Sigma Cat# P0781, Stored at -20ºC) in 37ºC water bath.
- Prepare 10 x 15 ml Falcon tubes (100X PS, 1/10, date) and 50 ml Eppendorf Conbitip Plus Tip.
- Aliquot 10 ml with a 50 ml Eppendorf Conbitip Plus Tip (Set channel at 5) to 15 ml Falcon tubes (total 21 tubes).
- Store at -20ºC.
- **10% FBS Dulbecco's Modification of Eagle's Medium (10% FBS-DMEM, myoblast growth medium) for 100 ml :**
- Incubate 89 ml of DMEM, 10 ml of FBS (Stored at –20ºC in a 50 ml Falcon tube), and 1 ml of 100X Penicillin-Storeptomycin Solution (Store at –20ºC in a 15 ml Falcon tube) in 37ºC water bath.
- Add 10 ml of FBS and 1 ml of 100X Penicillin-Storeptomycin Solution into 89 ml of DMEM (10%FBS-DMEM, date).
- **Medium for freeze: 90% FBS + 10% Dimethyl sulfoxide (DMSO), for 10 ml**
- Add 9 ml of FBS + 1 ml of DMSO (Sigma Cat# D2650)

*Cells can be frozen for several months at –80ºC or for years in liquid N_2.

**Using the Cell Banker (ZENOAQ Cat# BLC-1), cells can be frozen for 2 years at –80ºC (Freezing medium, date).

- **1X Phosphate buffer solution (PBS)**
- Prepare each 1000 ml bottle and 1000 ml cylinder (500 ml can be used), and 2 bottles of 500 ml bottle with filter 0.22 µm (1X PBS, 1/2-2/2, date).
- Add 100 ml of 10X PBS (Invitrogen Cat# 70011-044) + 900 ml of DW in a 1000 ml bottle.
- Filter with 2 bottles of 500 ml bottle with filter.
- **05% Tripsin-Ethylenediaminetetraacetic acid (EDTA) solution**
- Prepare 1000 ml bottle and 1000 ml cylinder (500 ml can be used).
- Defreeze 100 ml of a 0.5% Tripsin-EDTA bottle (Sigma Cat# T4174, store at -20ºC) in 37ºC water bath.
- Prepare 7 x 15 ml Falcon tubes (0.05% Tripsin-EDTA, 1/7-7/7, date) and 50 ml pipette.
- Add 100 ml of 0.5% Tripsin-EDTA (Sigma Cat# T4174, store at -20ºC) + 900 ml of 1X PBS in a 1000 ml bottle.
- Aliquot 13 ml with a 50 ml pipette to 15 ml Falcon tubes (total 7 tubes).

- **Diluted Vim**

- Take 500 ml of DW.
- Add 1 plug of Vim solution.

Step 5.
Maintenance of CO2 incubator

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- **Change water each 2 wks.**
- Trash water in the bat and clean the bat with 70% ethanol.
- Add 1 l of DW from 1 l bottle into the bat.
- Add 10 ml Lysol with a 15 ml Falcon tube into 1 l of DW.
- **Check CO_2 concentration every wk.**
- Stand up CO_2 meter straight.
- Connect a black rubber pump to sample plug of the incubator.
- Squeeze the pump 3X.
- Incline CO_2 meter upside down at 45 degree and then stand up CO_2 meter straight.
- Push the top of the CO_2 meter and check if the level of red liquid is at 0.
- Connect pump and the top of the CO_2 meter (Push the top of the CO_2 meter down with the pump connection part).
- Squeeze the pump 18X while holding down the connection part.
- Check the level of red liquid (CO_2 level should be in the range of 4.5 to 5.5).
- Remove the connection.
- Repeat from “inclining the CO_2 meter”.
- If CO_2 level is not in a range, ask for repare.

Step 6.

Preparation of clean bench before use

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- Wet a paper towel with water and put Vim.
- Wipe surface of clean bench with Vim side of the paper towel.
- Wipe surface of clean bench with water side of the paper towel.
- Spray 70% ethanol to the surface of clean bench.
- Wipe all materials which will use in the clean bench with 70% ethanol.

Step 7.

Washing glass wares

ANNOTATIONS

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- After use, rinse with hot water.
- Place glass wares in a washing machine.
- Put 20 ml Glass-Klenz (STERIS Cat# 1114-08) in the washing machine.
- Start the machine.
- Rinse with DW 20X.

Step 8.

C2C12 cell culture thaw

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- **It is important to avoid excessive alkalinity of the medium during recovery of the cells.**
- **Place DMEM and FBS in 37°C water bath for 30 min.**
- **Two culture vessels containing the growth medium be placed into the incubator for at least 15 min to allow the medium to reach its normal pH (7.0 to 7.6).**
- **Make 5 ml of warm 10% FBS-DMEM in a 15 ml flacon tube**.
- **Take out cells from liquid N_2**.
- Underthe hood, **unscrew the cap and then screw the tube** (This can leave evacuate the stored pressure in the tube).
- **Quickly place in the 37ºC water bath** (disposed to the half of the tube in the bath at 37 °C, to reduce the possibility of contamination, keep the O-ring and cap out of the water). **Shake the tube quickly in the bath**.
- **Under the hood, immediately transfer all the contents of the cryovial tube** (1ml) **to the 15 ml flacon tube contains 5 ml of warm medium**.
- **Centrifuge at 500 G for 5 min**.
- **Aspirate the medium**.
- **Resuspend the pellet with approximately 2 ml of warm 10% FBS-DMEM and transferred into two flasks T-75 containing each 10 ml of warm 10% FBS-DMEM.**
- **Incubate cells** at 37°C **(5% CO_2).**
- **Change medium after all cells have adhered** (After 48 h).

Note: Thawing should be rapid (Approximately 2 min).

Step 9.

C2C12 cell culture passage10. Viability Test

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- **Put medium and trypsin-EDTA in 37°C water bath for 30 min**.
- **Prepare flasks and 50 ml Falcon tubes as needed (name of cells, number of passage, date)**.
- **Check the amount of 2.5, 5** and **10 ml pipettes** and **Pasteur pipettes**.
- **Observe cells under an inverted microscope**to evaluate the

Note: It is important to not let the cells become fully confluent because they can begin to fuse and partially differentiate upon cell-cell contact.

- **To passage the cells**: **aspirate the culture medium in the flask**.

- **Rinse twice with PBS**.
- Subculture the cells using standard tryspinization techniques : **add 3 ml of 0.05% trypsin-EDTA solution**.**Trash the pipette** in the autoclaving bag in the clean bench.
- **Incubate at 37°C for 2-10 min** (not more than 10 min).
- **Observe cells under an inverted microscope until cell aspect changes to round** (Usually within 60-90 sec).
- **To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.**
- **Add 9 ml of new myoblast growth medium** (3 times the volume of the enzyme) **in the flask. Mix by pipetting** with the 10 ml pipette.
- **Collect cells by gently pipetting.**
- **Transfer the medium containing cells into a 15 ml Flacon tube**.
- **Take a small volume of cells** (Usually we take 10 µl × 2= 20 µl) and **count cells** using a hemocytometer (Step I.10).
- **Centrifuge for 5 min at 500G**.
- **Standup a new flask** and **add 15 ml** (10-20 ml) **of new medium into the flask**.
- **Transfer 2 ml of medium containing cells into the flask containing new medium**. (In order to have 1×10^6 cells/mel means 10^6 viable cells/75 cm^2).
- **Incubate at 37°C**.
- If cells have been growing well for a few days but are not yet confluent, then they will require media changing to replenish nutrients and keep correct pH. If there are a lot of cells in suspension (attached cell lines) or the media is staring to go orange rather than pinky orange then media change them as soon as possible. **To change the medium, remove the medium and add fresh media**:
- Put medium in 37ºC water bath for 30 min.
- Prepare Pasteur and 10 ml pipettes.
- Aspirate the medium.
- Add 15 ml (10-20 ml) of new medium.

Note:

- **Mycoblasma test must be done**: **using Mycoplasma detection kit conventional PCR (VenorGem**) (See Appendix 1).
- **If the cells are still attached,** aseptically remove all but 5 to 10 ml of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.
- **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 xg for 5 to 10 min. Remove shipping medium and save. Resuspend the pelleted cells in 15 ml of this medium and add to 75 cm^2 Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.
- **Medium renewal:** Every 2 or 3 days (ATCC recommendation).

Trypan Blue is a vital dye. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable.

Note: Trypan Blue has a greater affinity for serum proteins than for cellular protein. If the background is too dark, cells should be pelleted and resuspended in protein-free medium or salt solution prior to counting.

Staining facilitates the visualization of cell morphology.

- **Reagents preparation**
- **4% Trypan Blue solution** (Sigma Cat# T8154)
- **1X PBS** (Step I.4)
- **Prepare a cell suspension in PBS.**
- **Transfer 0.5 ml of 0.4% Trypan Blue solution** (Sigma Cat# T8154) **(w/v) to a test tube.**
- **Add 0.3 ml of PBS and 0.2 ml of the cell suspension (dilution factor = 5) and mix thoroughly.**
- **Allow to stand for 5 to 15 min at RT.**

Note: If cells are exposed to Trypan Blue for extended periods of time, viable cells, as well as non-viable cells, may begin to take up dye.

- With the cover-slip in place, **transfer 20 µl of the mixture cell suspension- trypan blue to both chambers of the hemocytometer** (10 µl in each chamber).
- **Carefully touch the edge of the cover-slip with the pipette tip** and **allow each chamber to fill by capillary action**. Do not overfill or underfill the chambers.
- **Incubate the hemocytometer and cells for 1 – 2 min at RT**. For longer incubations, please use a humid chamber. (Incubations exceeding 30 min may cause decreased cell viability due to Trypan toxicity).
- **Load a hemacytometer and examine it under a microscope at low magnification.**
- **Starting with chamber 1 of the hemacytometer**, **count all the cells in the 1 mm center square and four 1 mm corner squares** (see **pictures** below). Non-viable cells will stain blue. Keep a separate count of viable and non-viable cells.

Note: Count cells on top and left touching middle line of the perimeter of each square. Do not count cells touching the middle line at bottom and right sides.

- **Repeat this procedure for chamber 2.**

Note:

- If greater than 10% of the cells appear clustered, repeat entire procedure making sure the cells are dispersed by vigorous pipetting in the original cell suspension as well as the Trypan Blue-cell suspension mixture. If less than 200 or greater than 500 cells (i.e., 20–50 cells/square) are observed in the 10 squares, repeat the procedure adjusting to an appropriate dilution factor.
- Cell viability should be at least 95% for healthy log-phase cultures (count the number of all the cells on 4 corners in each chamber).

- **Calculations**

- **Cell Counts**– Each square of the hemacytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the subsequent cell concentration per ml (and the total number of cells) will be determined using the following calculations:
- **Cells/ml =** (average count of 2 chambers/4 corners) 10^4
- **Cells Per ml** = the average count per square × dilution factor × 10^4 (count 10 squares).
- **Total Cells** = cells per ml × the original volume of fluid from which cell sample was removed.

Cell Viability (%) = total viable cells (unstained) ÷ total cells (stained and unstained) × 100.

Step 10.
Cleaning of clean bench after use

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- Put aseptone in all dispensable tubes and flasks which contain cells. Then trash as an usual trash.
- Aspirate aseptone, then 70% ethanol. Trash the liquid in the flask connected to aspirator to sink.
- Wet a paper towel with water and put Vim.
- Wipe surface of clean bench with Vim side of the paper towel.
- Wipe surface of clean bench with water side of the paper towel.
- Spray 70% ethanol to the surface of clean bench.
- Wipe all materials which have been used in the clean bench with 70% ethanol.

Step 11.
Storage of cellsII. Effects of SPARC on C2C12 cells differentiation, ECM remodeling andmitochondrial function

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- Can store the leftover cells from the Step I.9
- Transfer 4 ml of the leftover into a 15 ml Falcon tube.
- Centrifuge at 500 G for 5 min and remove supernatant carefully by aspiration.
- Add 1 ml of Cell Banker (ZENOAQ Cat# BLC-1) for 2.0 × 10^6
- Transfer 1 ml of the medium containing cells to a 1.0 ml Nunc cryo tube vial.
- Store the tube at -80ºC (Can be stored for 2 yrs).

If using medium for freeze, place the tube at -80ºC for 24 h – 1 wk (Can be stored for 2-3 months), then place the tube in liquid N_2 (Can be stored for 10 years).

Step 12.
Number of cells needed

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- We want to study if induction or inhibition of SPARC will affect the extracellular matrix (ECM) and mitochondrial function. Thus, we will measure C2C12 cells differentiation (morphology) and study the expression of 6 proteins by western blot: fibronectin (Fn), Collagen (Col) type I, Col III and Col IV, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1a) and nuclear respiratory factor 1 (NRF1).
- For each well (protein) in western blot we need 30 µg of proteins so in total: **30 µg × 6 proteins = 180 µg.**
- A 'typical' mammalian cell, for example a liver hepatocyte, is thought to contain 10 000–20 000 different proteins, about 8×10^9 individual molecules in all, representing approximately **5 ng of protein** or 18–20% of the total cell weight (*Alberts et al., 1994*, *Lodish et al., 2000*) so :
- 1 cell 5 ng of protein= 5×10^{-4} µg
- X cell 180 µg of proteins.
- We need to plate in each well: **number of cells = 3.6 × 10^5 cells/well.**

Http://www.invitrogen.com/etc/medialib/en/filelibrary/pdf.Par.4786.File.dat/Useful_Numbers_Y14472_Useful_Nmbrs.pdf

- **12-well plates can be used because we can plate 4 × 10^5 cells at confluency/well.**

Step 13.
Differentiation treatment

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- Cells will be washed with PBS, and the culture medium will be replaced with differentiation medium.
- Differentiate for 24 hours to 7 days **by rinsing fully confluent cells once with PBS and adding low-serum differentiation medium**.
- Feed with fresh differentiation medium every **24 hours up to the 72h** timepoint and after that, **every 24 hours** (as these cells differentiate, they begin to deplete and acidify the medium more quickly)
- **Myotube formation is enhanced when the differenciation medium is supplemented with 10% horse serum (HS) instead of FBS.**

Step 14.
Differentiation Medium and reagents

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- Hourse serum (HS)
- Defreeze 500 ml of a HS bottle in 37ºC water bath.
- Prepare 21 x 15 ml Falcon tubes (HS, 1/10-10/10, date) and 50 ml Eppendorf Conbitip Plus Tip.

- Aliquot 10 ml with a 50 ml Eppendorf Conbitip Plus Tip (Set channel at 5) to 15 ml Falcon tubes (total 21 tubes).
- Store at -20ºC.
- **2% HS-DMEM: For 100 ml**
- Incubate 97 ml of DMEM, 2 ml of HS and 1 ml of 100X Penicillin-Storeptomycin Solution (Store at –20ºC in a 15 ml Falcon tube) in 37 º C water baths.
- Add 2 ml of HS and 1 ml of 100X Penicillin-Storeptomycin Solution into 97 ml of DMEM (2 % HS-DMEM, date).
- 10% HS-DMEM: (ATCC recommendation)
- Incubate 445 ml of DMEM, 50 ml of HS and 5 ml of 100X Penicillin-Storeptomycin Solution (Store at –20ºC in a 15 ml Falcon tube) in 37 º C water baths.
- Add 10 ml of horse serum and 5 ml of 100X Penicillin-Storeptomycin Solution into 445 ml of DMEM (10 % HS-DMEM, date).
- 10%FBS-DMEM (Step I.4)
- 1X PBS (Step I.4)
- Methanol
- **Hematoxylin solution according to Delafield** (Sigma Cat#**03971).**
- Glycerol in PBS (v/v) :
- Add 5 ml of glycerol in tube.
- Add 5 ml of 1X PBS.
- Vortex
- Make appropriate label (50% Glycerol in PBS (v/v), date).

Step 15.
General procedure for differentiation

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- **Prepare differenciation medium (2% HS-DMEM).**
- **Aspirate growth medium**, when cells reach about 50-80% confluence,
- **Switch cells to differenciation medium.**
- **Incubate at 37°C and 5% CO_2.**
- Visualization under light microscopy should reveal myotube formation after **24 h** in differenciation medium.
- Differenciation medium must be changed **each 2 days**.

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Step 16.
Doubling time of C2C12 and myoblast fusion

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In order to confirm the doubling time of our cell line C2C12, its attachment, confluency and myoblast fusion, we will do this experiment.

Appropriate seeding density: Start with an optimal seeding density is also very important for adherent cells.

Fast attachment of adherent cells: Preheating of the medium and the use of large volume flask containing already CO_2 in the medium were recommander in order that the medium will quickly reache its optimum pH, this step can cause a faster attachment.

Fusion: After staining with haematoxylin, the nuclei present in myoblasts and in myotubes were counted.

Fusion (%) = Number of nuclei present in myotubes /Total number of nuclei (myoblasts + myotubes) x 100.

Reagents preparation

- 10%FBS-DMEM (Step I.4)
- 1X PBS (Step I.4)
- 05% trypsin-EDTA solution (Step I.4)
- **2% HS-DMEM** (Step II.3)
- Methanol
- **Hematoxylin solution according to Delafield** (Sigma Cat#**03971).**
- **Glycerol in PBS (v/v)** (Step II.3)

- **Procedure of doubling time of C2C12 and myoblast fusion :**

[Day 1]: Seed cells

- **Trypsinize cells.**
- **Plate different number of cells** (in growth medium: 10% FBS-DMEM) **in 3X 12-well plates**. (See picture below).

Well	Cells
1	4×10^3
2	4×10^4
3	1×10^5
4	2×10^5
5	3×10^5
6	4×10^5

[Day 2]: Add d**ifferenciation medium to plate nº2 and plate nº3**

- **After 24h** (3h or 4h for its attachment + 12h doubling time), **Observe cells under an inverted microscope**to evaluate the
- **For plate nº1 :**
- **Put medium and trypsin-EDTA in 37°C water bath for 30 min**.
- **Aspirate the culture medium in the plate**.
- **Rinse twice with PBS**.
- **Add 0.5 ml of 0.05% trypsin-EDTA solution**. **Trash the pipette** in the autoclaving bag in the clean bench.
- **Incubate at 37°C for 2-10 min** (not more than 10 min).
- **Observe cells under an inverted microscope until cell aspect changes to round** (usually within 60-90 sec).
- **To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.**
- **Add 1.5 ml of new myoblast growth medium** (3 times the voulme of the enzyme) **in each well of the plate. Mix by pipetting** with the 10 ml pipette.
- **Collect cells by gently pipetting.**
- **Take a small volume of cells** (usually we take 10 µl × 2= 20 µl) and **count cells using a hemocytometer** (Step I.10).
- **Transfer the medium containing cells into a 15 ml tubes**.
- **Centrifuge for 5 min at 500G**.
- **Freez cells**

- **For plate nº2 and plate nº3, when cells are at 70-80% confluent transfer cells into differenciation medium (2% HS-DMEM):**
- Put differenciation medium in 37ºC water bath for 30 min.
- Prepare Pasteur pipettes.
- Aspirate the growth medium by using a pasture pipette.
- Rinse the cells with cold PBS (2 ml).
- Add 2 ml of differenciation medium in each well.
- Incubate cells at 37°C and 5% CO_2.

[Day 3]: Change d**ifferenciation medium for plate nº 3**

- Put differenciation medium in 37ºC water bath for 30 min.
- Prepare Pasteur and 10 ml pipettes.
- Aspirate the differenciation medium.
- Add 2 ml of new differenciation medium in each well.
- Incubate cells at 37°C and 5% CO_2.

[Day 4]: Change d**ifferenciation medium for plate nº 2 and plate nº 3.**

- For steps: see day 3.

[Day 5]: Change d**ifferenciation medium for plate nº 3.**

- For steps: see day 3.

[Day 6]: Cell fixation and observation under light microscopy

- **Rinse the cells twice with could PBS buffer**.
- **Fix the cells for 5 min in methanol.**
- **Stain in hematoxylin for 5 -15 min.**
- **Rinse cells with DW.**
- **Fix the cells by adding some drops of PBS-glycerol (v / v)** (Face that contains cells must be below).
- **Observe the preparations under the light microscopy.**

Step 17.
Effects of exogenous SPARC inhibition/addition on morphological analysis

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Reagents preparation

- 10%FBS-DMEM (Step I.4)
- 1X gelatin (LifeTechnology cat#S-006-100)
- **2% HS-DMEM** (Step II.3)
- **1X PBS** (Step I.4)
- **Anti-SPARC antibody:** (R&D system Cat#**MAB942) (0.5 mg/ml PBS)**
- **Stock solution of recombinant SPARC protein (rSPARC):** (R&D system Cat#**942-SP-050) (100 µg/ml PBS)**
- **Methanol**
- **Hematoxylin solution according to Delafield** (Sigma Cat#**03971)**
- **Glycerol in PBS (v/v)** (Step II.3)

- **Procedure of morphological analysis:**

[Day 1]: Seed cells

- **One day before adding anti-SPARC antibody or rSPARC**, seed **4 X 10^4 cells in 24-well plates** as following :
- **Take small slide**
- **Make it in wells**
- **Add 1 ml of 1X gelatin**
- **Incubate at 37°C for 30 min**
- **Aspirate gelatin**
- **Add growth medium and cells**
- **Incubate over night**

►We need in total : 28 **X 10^4 cells**

[Day 2]: Culture cells in different conditions

- **In 24-well plates, culture cells in different conditions** (See table below).
- **For well 1 to 6 : when cells are at 70-80% confluent transfer cells into differenciation medium (2% HS-DMEM):**
- Put differenciation medium in 37ºC water bath for 30 min.
- Prepare Pasteur pipettes.
- Aspirate the growth medium by using a pasture pipette.
- Rinse the cells with cold PBS (1 ml).
- Add 1 ml of differenciation medium in each well with different conditions (See table).
- Incubate cells at 37°C and 5% CO_2.

	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7
Growth medium (ml)	-	-	-	-	-	-	1
Differenciation medium (DM) (ml)	1	1	1	1	1	1	-
PBS (µl)	-	40	-	30	20	-	-
Anti-SPARC antibody (µl) **at 0.5 mg/ml**	-	-	20	-	-	-	-
rSPARC (µl) **at 100 µg/ml**	-	-	-	10	20	40	-
Final concentration of anti-SPARC antibody (µg/ml)	-	-	10	-	-	-	-
Final concentration of rSPARC (µg/ml)	-	-	-	1	2	4	-

For 1 experiment: **10 µg × 3 changes of medium= 30 µg** of anti-SPARC antibody.

For 3 repetitions: **30 µg × 3 repetitions = 90 µg** of anti-SPARC antibody.

For 1 experiment: **7 µg × 2 changes of medium = 14 µg** of rSPARC.

For 3 repetitions: **14 µg × 3 = 42 µg** of rSPARC.

[Day 4]: Change growth medium and differenciation medium

- Put growth medium (10% FBS-DMEM) and differenciation medium (2% HS-DMEM) in 37ºC water bath for 30 min.
- Prepare Pasteur pipettes.
- Aspirate growth medium using Pasteur pipettes.
- Aspirate differenciation medium using an other Pasteur pipettes.
- Add 1 ml of new growth medium or differenciation medium with other reagents in each well. (As mentioned in the table above).
- Incubate cells at 37°C and 5% CO_2.
- **Culture cells for 4 days** (*Lim et al., 2000*) to 6 days.

[Day 6]: Change growth medium and differenciation medium

- **Change differenciation medium and growth medium.**

[Day 7]: Cell fixation and observation under light microscopy

- **Rinse the cells twice with could PBS buffer**.
- **Fix the cells for 5 min in methanol.**
- **Stain in hematoxylin for 5 -15 min.**
- **Rinse cells with DW.**
- **Fix the cells by adding some drops of PBS-glycerol (v / v).**
- **Observe the preparations under the light microscope**

Fusion:

- Afterstaining withhaematoxylin, thenuclei presentin myoblastsandinmyotubeswere counted.

Fusion (%) = Number of nuclei present in myotubes /Total number of nuclei (myoblasts

+ myotubes) x 100.

Step 18.
Effect of induction / inhibition of SPARC on ECM and mitochondrial proteins

ANNOTATIONS
rimca@live.fr mouloudia87 10 Sep 2017

-

- **Reagents preparation**

- **10% FBS-DMEM** (Step I.4)
- **2% HS-DMEM** (Step II.3)
- 1X PBS (Step I.4)
- 05% trypsin-EDTA solution (Step I.4)
- **Anti-SPARC antibody:** (R&D system Cat#**MAB942) (0.5 mg/ml PBS)**
- **Stock solution of recombinant SPARC protein (rSPARC):** (R&D system Cat#**942-SP-050) (100 µg/ml PBS)**
- **Protease inhibitors cocktail** (Sigma, Cat# R0278 store at – 20 ºC)
- **RIPA buffer** (Sigma, Cat# R0278 store at 4 ºC)
- **Protein Assay Kit II** (Bio-Rad, Cat# 500-0002)
- **COL1A1** (Santa Cruz Biotechnology, Cat# sc-8784-R store at 4 ºC)
- **COL1A2** (Santa Cruz Biotechnology, Cat# sc-28654 store at 4 ºC)
- **Fibronectin** (Santa Cruz Biotechnology, Cat# sc-9068 store at 4 ºC)
- **Myogenin** (Santa Cruz Biotechnology, Cat# sc-12732 store at 4 ºC)
- **Reagents for usual western**
- **MitoProfile Total OXPHOS** (Abcam, Cat# ab110413 store at 4 ºC)
- **Mouse TrueBlot Western Blot Kit** (Cedarlane, Cat# 88-8887-31 store at 4 ºC)
- **TrueBlot Assay (20X):**

♠ For 100 ml of TrueBlot Assay 1X: take 5 ml of TrueBlot Assay 20X + 95 ml DW.

- **TrueBlot Blocker (5% milk):**

♠ Take 5 g of TrueBlot Blocker.

♠ Add 100 ml of of TrueBlot Assay 1X.

- **CAPS transfer buffer** (See bellow)

- **Procedure**

[Day 1]: Seed cells

- **One day before adding anti-SPARC antibody or rSPARC**, **trypsinize and plate 4 x 10^5 of cells in 12-well plates**.

[Day 2]: Culture cells in different conditions

- **In 12-well plates, culture 4 x 10^5 of cells in different conditions** (See table and picture below).

► **We need in total: 16 x 10^5 cells**

- **For well 1 to 6 : when cells are at 70-80% confluent transfer cells into differenciation medium (2% HS-DMEM):**
- Put differenciation medium in 37ºC water bath for 30 min.
- Prepare Pasteur pipettes.
- Aspirate the growth medium by using a pasture pipette.
- Rinse the cells with cold PBS (2 ml).
- Add 2 ml of differenciation medium in each well with different conditions (See table).
- Incubate cells at 37°C and 5% CO_2.

	Well 1	Well 2	Well 3	Well 4
Differenciation medium (ml)	2	2	2	2
PBS (µl)	80	40	40	-
Anti-SPARC antibody (µl) **at 0.5 mg/ml**	-	40	-	40
rSPARC (µl) **at 100 µg/ml**	-	-	40	40
Final concentration of anti-SPARC antibody (µg/ml)	-	10	-	10
Final concentration of rSPARC (µg/ml)	-	-	2	20

For 1 experiment: **20 µg × 3 changes of medium= 60 µg** of anti-SPARC antibody.

For 3 repetitions: **60 µg × 3 repetitions = 180 µg** of anti-SPARC antibody.

For 1 experiment: **4 µg × 3 changes of medium = 12 µg** of rSPARC.

For 3 repetitions: **12 µg × 3 = 36 µg** of rSPARC.

[Day 4]: Change differenciation medium

- Put differenciation medium in 37ºC water bath for 30 min.
- Prepare Pasteur pipettes.
- Aspirate the differenciation medium.
- Add 2 ml of new differenciation medium with other reagents in each well. (As mentioned in the table and picture above).
- Incubate cells at 37°C and 5% CO_2.
- **Culture cells for 4 days.** (*Ki Lim et al., 2000*) to 6 days.

[Day 6]: Change differenciation medium

- Change differenciation medium.

[Day 7]: Collect cells/ Protein extraction

Collect proteins from adherent cells :

- **Put centrifuge at 4ºC.**
- **Wash the cell 2X with 1 ml PBS/well (6-well plate) on ice.**
- **Add 0.5 ml of RIPA buffer sequentially to each well of the same experimental condition and scrape (detach) cells on ice by using cell lifter (VWR-Canlab Cat# 29442-200).**
- **Defreeze protease inhibitors cocktail** (Sigma, Cat# R0278 store at – 20 ºC) **at RT.**
- **Add 1 µl of Protease inhibitors cocktail directely in the wells.**
- **Add again 0.5 ml of RIPA buffer.Thus, total 1 ml of RIPA buffer per condition.**
- **Transfer the RIPA buffer containing cells into 2 ml tubes (Step II.7.2 well nº, date).**
- **Sonicate 3X for 1 sec (Fisher sonic Dismembrator model 100) with 10% amplitude** (Room R4739.1), **wash with DW** between different experimental conditions.
- **Centrifuge, 14,000 rpm, 15 min, 4 ºC.**
- **Transfer the supernatant into 2 ml tube (Exp, condition, name, date), Store sample at 80 ºC.**
- **Aliquot 20 µl of protein extracts to quantify. (See Western protocol Step 2.2)**
- **Make average of the concentrations of each sample.**
- **Total protein in 200 ml sample (mg) = The average protein concentrations (mg/ml) * 500 (dilution) / 5 (in 200 ml).**
- **10 mg protein (ml) = 200 * 10 / Total protein in 200 ml sample (mg).**

ECM remodeling: See Western protocol Step 3

Western conditions as follow:

Name	Protein (µg)	SDS gel (%)	Transfer (V)	Transfer (h)	Blocking (h)	1st AB (Dillution)	1st AB (h)	2nd AB (h)	Note
COL1A1	30	7	90	5	1	(1/200)	ON	1	-
COL1A2	5	7	90	5	1	(1/200)	ON	1	-
Fibronectin	5	7	90	5	1	(1/200)	ON	1	-
Myogenin	5	12	75	2	1	(1/200)	ON	1	2nd is an anti-mouse monoclonal antibody
Mitochondria (OXPHOS)	30	12	75	3	ON	(1/200)	2	2	Transfer buffer is 1X CAPS

ON: Over night.

Mitochondrial function:

Changed western conditions as follow:

- **Use 10% SDS-PAGE gel**
- **Load 30 mg of protein per well.**
- **Run gel at 120 V for approximately 10 min** until the dye front reaches to the bottom of the stoking gel. Then, **increase voltage to 150 V for approximately 60 min** until the dye front reaches to the bottom of the running gel.
- **After electrophoresis is finished the gel should be soaked in CAPS transfer buffer for 30 min before assembling the transfer sandwich.**

Using the CAPS buffer transfer system is highly recommended.

Or

► To prepare 1 liter of a 10X CAPS buffer for stock solution:

- Place 900 ml Milli-Q water in a 1 liter beaker; insert stir bar
- Dissolve 22.13 gm CAPS (Sigma C2632, RT) in water
- Titrate dropwise with 10 M NaOH to final pH 11.0
- Transfer to a 1000 ml graduated cylinder
- Add Milli-Q water to 1000 ml
- Store at 4C

►To prepare 1 liter of working solution:

- Measure 800 ml of Milli-Q water into a 1000 ml graduated cylinder

- Add 100 ml of 10X stock CAPS buffer
- Add 100 ml 100% HPLC grade methanol
- Mix well
- Store at RT

–

- **Transfer at <u>75 V for 3 h at 4ºC using CAPS transfer buffer.</u>**
- Romove the membrane and soack it in CAPS transfer buffer
- Under chemical hood, place the membrane in TrueBlot enhancer solution and soak for 2 min, then wash with PBST.
- RedAlert staining.
- **Blocking: Place the membrane in 10 ml of 5% TrueBlot Blocker in TrueBlot Assay Buffer (enough to cover the membrane) and incubate overnight at 4°C on a rocking platform.**
- **Remove the blocking buffer and wash the membrane for 10 min in PBST.**
- **Use the primary mouse immunoblotting antibody (40 uL) in 10 mL of TrueBlot Blocker at concentration of 6 µg/ml (The antibody cocktail 1.5 mg/ml should be diluted 250x).**
- **Incubate the blot with primary antibody for at least 2 h at RT on rocking platform.**
- **Wash the blot at least 3 times in PBST, each wash for a minimum of 10 min each.**
- **Prepare the secondary antibody Mouse IgG TrueBlot® at a 1:1,000 dilution in the Blocking.**
- **Incubate the blot with the Trueblot secondary antibody for 2 h at room RT on a rocking platform.**
- **Wash the membrane 3X in PBST solution for 10 min.**
- **The blot must be rinsed in PBS to remove any Tween-20, which can be inhibitory to the detection method.**

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Step 19.

NOTES
rimca@live.fr mouloudia87 09 Sep 2017

. Effect of induction / inhibition of SPARC on ECM and mitochondrial function during myoblasts proliferation.

ANNOTATIONS
rimca@live.fr mouloudia87 10 Sep 2017

<u>1Reagents preparation</u>

- **10% FBS-DMEM** (Step I.4)
- 1X PBS (Step I.4)
- 05% trypsin-EDTA solution (Step I.4)
- **Anti-SPARC antibody:** (R&D system Cat#**MAB942) (0.5 mg/ml PBS)**

- **Stock solution of recombinant SPARC protein (rSPARC):** (R&D system Cat#**942-SP-050) (100 µg/ml PBS)**
- **Protease inhibitors cocktail** (Sigma, Cat# R0278 store at – 20 ºC)
- **RIPA buffer** (Sigma, Cat# R0278 store at 4 ºC)
- **Protein Assay Kit II** (Bio-Rad, Cat# 500-0002)
- **COL1A1** (Santa Cruz Biotechnology, Cat# sc-8784-R store at 4 ºC)
- **COL1A2** (Santa Cruz Biotechnology, Cat# sc-28654 store at 4 ºC)
- **Fibronectin** (Santa Cruz Biotechnology, Cat# sc-9068 store at 4 ºC)
- **Myogenin** (Santa Cruz Biotechnology, Cat# sc-12732 store at 4 ºC)
- **Reagents for usual western**
- **MitoProfile Total OXPHOS** (Abcam, Cat# ab110413 store at 4 ºC)
- **Mouse TrueBlot Western Blot Kit**(Cedarlane, Cat# 88-8887-31 store at 4 ºC)
- **5% TrueBlot Blocker** (Step II.7.1)
- **TrueBlot Super Block** (Step II.7.1)
- **CAPS transfer buffer** (Step II.7.1)

8.2 Procedure

[Day 1]: Seed cells

- **One day before adding anti-SPARC antibody or rSPARC, trypsinize cells and plate 4 x 10^5 of cells in 12-well plates**.

[Day 2]: Culture cells in different conditions

- **In 12-well plates, culture 4 x 10^5 of cells in different conditions** (See table and picture below).

► **We need in total: 16 x 10^5 cells**

- Put growth medium in 37ºC water bath for 30 min.
- Prepare Pasteur pipettes.
- Aspirate the old growth medium by using a pasture pipette.
- Rinse the cells with cold PBS (2 ml).
- Add 2 ml of new growth medium in each well with different conditions (See table).
- Incubate cells at 37°C and 5% CO_2.

	Well 1	Well 2	Well 3	Well 4
Differenciation medium (ml)	2	2	2	2
PBS (µl)	320	160	160	-

Anti-SPARC antibody (µl) at 0.5 mg/ml	-	160	-	160
rSPARC (µl) at 100 µg/ml	-	-	160	160
Final concentration of anti-SPARC antibody (µg/ml)	-	40	-	40
Final concentration of rSPARC (µg/ml)	-	-	8	8

[Day 4]: Collect cells/ Protein extraction

Collect proteins from adherent cells :

- **Put centrifuge at 4ºC.**
- **Wash the cell 2X with 1 ml PBS/well (6-well plate) on ice.**
- **Add 0.5 ml of RIPA buffer sequentially to each well of the same experimental condition and scrape (detach) cells on ice by using cell lifter (VWR-Canlab Cat# 29442-200).**
- **Defreeze protease inhibitors cocktail** (Sigma, Cat# R0278 store at – 20 ºC) **at RT.**
- **Add 1 µl of Protease inhibitors cocktail directely in the wells.**
- **Add again 0.5 ml of RIPA buffer.Thus, total 1 ml of RIPA buffer per condition.**
- **Transfer the RIPA buffer containing cells into 2 ml tubes (Step II.9.2 well nº, date).**
- **Sonicate 3X for 1 sec (Fisher sonic Dismembrator model 100) with 10% amplitude** (Room R4739.1), **wash with DW** between different experimental conditions.
- **Centrifuge, 14,000 rpm, 15 min, 4 ºC.**
- **Transfer the supernatant into 2 ml tube (Exp, condition, name, date), Store sample at -80 ºC.**
- **Aliquot 20 µl of protein extracts to quantify.**

ECM remodeling:

- COL1A1 and Fn levels will be measured by western blot at **48 h** after the proliferation with or without inhibition/addition of SPARC (See Step II.7.2).

Mitochondrial function:

- The expression of mitochondria OXPHOS proteins will be measured **48 h** after the proliferation with or without inhibition/addition of SPARC (See Step II.7.2).

Effect of induction / inhibition of SPARC on ECM and mitochondrial protein in the myotubes using 24-well plate

Step 20.

Effect of the induction/inhibition of SPARC on ECM modulation and mitochondrial function in the myotubes: Set up the adequate concentration of rSPARC and anti-SPARC antibody.

Step 21.

ANNOTATIONS

rimca@live.fr mouloudia87 10 Sep 2017

► **Set up the number of cells, kind of plates and days for the differentiation:**

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9.1 Reagents preparation:

- **10% FBS-DMEM** (Step I.4)
- **2% HS-DMEM** (II.3)
- 1X PBS (Step I.4)
- 05% trypsin-EDTA solution (Step I.4)
- **Protease inhibitors cocktail** (Sigma, Cat# R0278 store at – 20 ºC)
- **RIPA buffer** (Sigma, Cat# R0278 store at 4 ºC)
- **Protein Assay Kit II** (Bio-Rad, Cat# 500-0002)
- **COL1A1** (Santa Cruz Biotechnology, Cat# sc-8784-R store at 4 ºC)
- **Fibronectin** (Santa Cruz Biotechnology, Cat# sc-9068 store at 4 ºC)
- **SDH** (Santa Cruz Biotechnology, Cat# sc-377302 or sc-390381store at 4 ºC)
- **Reagents for usual western**

9.2 Procedure :

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[Day 1]: Seed cells

- **Trypsinize** and **plate different number of cells** (in growth medium: 10% FBS-DMEM) as shown in tables below **in 2X: 6-well, 12- well and 24-well plates**.
- **Use an other 6-well plate**

6-well plate:

Well	Number of cells
1	8×10^4
2	9×10^4
3	$\mathbf{10^5}$
4	2×10^5
5	3×10^5

12-well plate:

Well	Number of cells
1	4×10^4
2	5×10^4
3	**6×10^4**
4	7×10^4
5	8×10^4
6	10^5

24-well plate:

Well	Number of cells
1	10^4
2	**2×10^4**
3	3×10^4
4	4×10^4
5	5×10^4

Other 6-well plate:

Well	Number of cells
1	3×10^4
2	**3×10^4**

[Day 3]: Culture cells in differentiation medium

- **When cells are at 70-80% confluent transfer cells into differenciation medium (2% HS-DMEM):**
- Put differenciation medium in 37ºC water bath for 30 min.
- Prepare Pasteur pipettes.
- Aspirate the growth medium by using a pasture pipette.

- Rinse the cells with cold PBS (2 ml).
- Add 0.5, 1 and 2 ml of differenciation medium in 24-well, 12-well and 6-well plates, successively.
- Incubate cells at 37°C and 5% CO_2.

NB: Keep cells from the 3rd 6-well plate (contains 2 wells only) in GM and change GM for the first well only.

[Day 4]: Change differenciation medium for the first 24-well, 12-well and 6-well plate

Change growth medium to differentiation medium for 6-well plate (with 2 wells only)

- Put differenciation medium in 37ºC water bath for 30 min.
- Prepare Pasteur pipettes.
- Aspirate the differenciation medium.
- Add 3 ml of new differenciation medium with other reagents in each well.
- Incubate cells at 37°C and 5% CO_2.

[Day 5]: Change differenciation medium for all the plates and for the second well of 6-well plate only

[Day 6]: Change differenciation medium for the first 24-well, 12-well and 6-well plate and for the 6-well plate with 2 wells.

[Day 7]: Change differenciation medium for all the plates and for 2nd well of 6-well plate

[Day 8]: Change differenciation medium for the first 24-well, 12-well and 6-well plate and for the 6-well plate with 2 wells.

[Day 9]: Change differenciation medium for all the plates and for 2nd well of 6-well plate

[Day 10]: Change differenciation medium for the first 24-well, 12-well and 6-well plate and for the 6-well plate with 2 wells.

[Day 10]: Protein extraction.

Remarks:

6-well plate:

Well	Number of cells	% Confluency
1	8×10^4	60
2	9×10^4	70
3	**10^5**	**80**
4	$2x\ 10^5$	90
5	3×10^5	100

12-well plate:

Well	Number of cells	% Confluency
1	4×10^4	60
2	5×10^4	70
3	**6×10^4**	**80**
4	7×10^4	90
5	8×10^4	90
6	10^5	100

24-well plate:

Well	Number of cells	% Confluency
1	10^4	40
2	**2×10^4**	**60**
3	3×10^4	70
4	4×10^4	80
5	5×10^4	90

Other 6-well plate:

Well	Number of cells	% Confluency
1	3×10^4	80
2	**3×10^4**	**80**

- Partial detachment starts on the **6th day** of the differentiation.
- Detachement is **less** in wells that DM is changed **every day**.
- It's better to **add SPARC** on the **5th day** of the differentiation.
- The adequate number of cells for each plate is mentioned **in red** (see tables above).

Step 22.
Effect of the induction/inhibition of SPARC on ECM modulation andmitochondrial function in the myotubes: Set up the adequate concentration of rSPARC and anti-SPARC antibody.

ANNOTATIONS
rimca@live.fr mouloudia87 10 Sep 2017

► **Set up the adequate concentration of rSPARC and anti-SPARC antibody:**

10.1 Reagents preparation:

- **10% FBS-DMEM** (Step I.4)
- **2% HS-DMEM** (II.3)
- 1X PBS (Step I.4)
- 05% trypsin-EDTA solution (Step I.4)
- **Anti-SPARC antibody:** (R&D system Cat#**MAB942) (0.5 mg/ml PBS)**
- **Stock solution of recombinant SPARC protein (rSPARC):** (R&D system Cat#**942-SP-050) (100 µg/ml PBS)**
- **Protease inhibitors cocktail** (Sigma, Cat# R0278 store at – 20 ºC)
- **RIPA buffer** (Sigma, Cat# R0278 store at 4 ºC)
- **Protein Assay Kit II** (Bio-Rad, Cat# 500-0002)
- **COL1A1** (Santa Cruz Biotechnology, Cat# sc-8784-R store at 4 ºC)
- **Fibronectin** (Santa Cruz Biotechnology, Cat# sc-9068 store at 4 ºC)
- **SDH** (Santa Cruz Biotechnology, Cat# sc-377302 or sc-390381store at 4 ºC)
- **COX 1** (Santa Cruz Biotechnology, Cat# sc-48143)
- **AMPK** (Santa Cruz Biotechnology, Cat#25792 sc-at 4 ºC)
- **Thr172** (Santa Cruz Biotechnology, Cat# sc-33524 store at 4 ºC)
- **Reagents for usual western**

10.1 Procedure :

[Day 1]: Seed cells

- **Trypsinize cells and plate 2 x 10^4 cells/well** (in growth medium: 10% FBS-DMEM) **in 3X 24-well plates**.

[Day 3]: Culture cells in differentiation medium

- **When cells are at 70-80% confluent transfer cells into differenciation medium (2% HS-DMEM):**
- Put differenciation medium in 37ºC water bath for 30 min.
- Prepare Pasteur pipettes.
- Aspirate the growth medium by using a pasture pipette.
- Rinse the cells with cold PBS (0.5 ml)
- Add 0.5 ml of differenciation medium in each well.
- Incubate cells at 37°C and 5% CO_2.

[Day 4]: Change differenciation medium

- Put differenciation medium in 37ºC water bath for 30 min.
- Prepare Pasteur pipettes.
- Aspirate the differenciation medium.
- Add 0.5 ml of new differenciation medium with other reagents in each well.
- Incubate cells at 37°C and 5% CO_2.

[Day 5]: Change differenciation medium

[Day 6]: Change differenciation medium

[Day 7]: Change differenciation medium

[Day 8]: Culture cells in different conditions

- **Change differenciation medium.**
- **Culture cells in different conditions.** (see table below)

	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6
Differenciation medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5
PBS (µl)	80	70 **(10)**	60 **(20)**	60 **(20)**	40	80
Anti-SPARC antibody (µl) at 0.5g/ml	-	10	20	-	-	-

rSPARC (µl) at 100 µg/ml (µg/ml)	-	-	-	20	40	80
Final concentration of anti-SPARC antibody	-	10	20	-	-	-
Final concentration of rSPARC (µg/ml)	-	-	-	4	8	16

NB: The volume of PBS that I took for my experiment is in red, the real volume must be the one in black.

[Day 9]: Collect cells/ Protein extraction for plate nº 1

Collect proteins from adherent cells :

- **Put centrifuge at 4ºC.**
- **Wash the cell 2X with 0.5 ml PBS/well (24-well plate) on ice.**
- **Add 0.25 ml of RIPA buffer sequentially to each well of the same experimental condition and scrape (detach) cells on ice by using cell lifter (VWR-Canlab Cat# 29442-200).**
- **Defreeze protease inhibitors cocktail** (Sigma, Cat# R0278 store at – 20 ºC) **at RT.**
- **Add 1 µl of protease inhibitors cocktail directely in the wells.**
- **Add again 0.25 ml of RIPA buffer.Thus, total 0.5 ml of RIPA buffer per condition.**
- **Transfer the RIPA buffer containing cells into 2 ml tubes (Step II.11.2 well nº, date).**
- **Sonicate 3X for 1 sec (Fisher sonic Dismembrator model 100) with 10% amplitude** (Room R4739.1), **wash with DW** between different experimental conditions.
- **Centrifuge, 14,000 rpm, 15 min, 4 ºC.**
- **Transfer the supernatant into 2 ml tube (Exp, condition, name, date), Store sample at -80 ºC.**
- **Dilute cell culture lysate** 2X**:** 10 µl of sample + 10 µl of DW in 1.7 ml tubes**.**

***Protein quantification should make with duplicate. Do not forget to make a pooled sample (western control sample) for protein quantification.**

- **Aliquot 20 µl of protein extracts to quantify with duplicate.**

- **Make average of the concentrations of each sample.**
- **Total protein in 200 ml sample (mg) = The average protein concentrations (mg/ml) * 500 (dilution) / 5 (in 200 ml).**
- **10 mg protein (ml) = 200 * 10 / Total protein in 200 ml sample (mg).**

[Day 10]: Collect cells/ Protein extraction for plate nº 2 and change medium for plate nº 3

- **Change differenciation medium.**
- **Culture cells in different conditions.** (see table below)

	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6
Differenciation medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5
PBS (µl)	80	70 **(10)**	60 **(20)**	60 **(20)**	40	80
Anti-SPARC antibody (µl) at 0.5g/ml	-	10	20	-	-	-
rSPARC (µl) at 100 µg/ml (µg/ml)	-	-	-	20	40	80
Final concentration of anti-SPARC antibody (µg/ml)	-	10	20	-	-	-
Final concentration of rSPARC (µg/ml)	-	-	-	4	8	16

[Day 11]: Collect cells/ Protein extraction for plate nº 3

ECM remodeling & Mitochondrial function& AMPK expression: See Western protocol Step 3

Western conditions as follow: To complete after doing WB.

Name	Protein (ug)	SDS gel (%)	Transfer (V)	Transfer (h)	Blocking (h)	1st AB (Dillution)	1st AB (h)	Note
COL1A1	30	7	90	5	1	(1/200)	ON	
Fibronectin	5	7	90	5	1	(1/200)	ON	
SDHB	10	12	75	2	1	(1/200)	ON	
COX 1	30	10	90	3	1	(1/200)	ON	The 2nd antidbody is anti-goat.
AMPK	20	10			1	(1/200)	ON	
Thr172	20	10			1	(1/200)	ON	

NB: Final concentration of **rSPARC is 4 µg/ml**, **anti-SPARC antibody**, we need to **set up** its concentration.