UNIVERSITA' DEGLI STUDI DI ROMA TOR VERGATA

MACROAREA DI SCIENZE MATEMATICHE, FISICHE E NATURALI

Revealing the molecular mechanism underlying inhibition of FAPs adipogenesis in co-culture with myotubes

Advisor:
Professor Gianni Cesareni

Candidate:
Arantxa Ferreiro

Co-advisor:
Milica Marinkovic, Ph.D.

INDEX

1. Abstract

Muscle regeneration is a complex and highly coordinated process mediated by various resident mononucleated cell populations. Satellite cells, skeletal muscle stem cells, play a pivotal role during regeneration being the main source of new myoblasts. However, their activation, proliferation and differentiation relies on environment cues shaped by cell populations such as macrophages, pericytes, and fibro-adipogenic progenitors (FAPs). FAPs are non-myogenic mesenchymal progenitor cells that ensure a proper environment for successful regeneration releasing factors that influence positively the differentiation of satellite cells. However, in pathological conditions such as muscular dystrophies or during aging, fibro/adipogenic progenitors contribute to fat and fibrous tissue depositions. In my thesis work I investigated the complex cross talk between these two cell populations during muscle regeneration. I isolated cells from C57/Bl6 mouse skeletal muscles and validated FAPs ability to differentiate into adipocytes and fibroblasts. In addition, I studied the interaction between satellite cells and FAPs in co-culture conditions. In *in vitro* experiments I have observed an improvement in the maturation of myotubes derived from satellite cells, when co-cultured with FAPs. Furthermore, I have also observed that direct contact of these two cell populations inhibits FAP adipogenic differentiation, inhibition that was not observed in the trans-well system. Even though this interaction has been observed earlier, it has not been thoroughly characterized yet. Since the Notch signalling pathway is mediated by direct cell contact and it is responsible for maintaining satellite cells in the quiescent state, thus preventing their differentiation, I investigated whether this pathway also modulates FAP adipogenesis.

My results demonstrate that adipogenic differentiation of FAPs is inhibited by myotubes and that this inhibition is not mediated by a paracrine way via small molecules, rather direct cell-cell contact mediated by Notch signalling pathway is essential for successful inhibition.

2. Introduction

2.1 Skeletal muscle structure

Skeletal muscle is a form of striated muscle comprising around 40% of body weight and it contributes to multiple body functions, such as voluntary contraction, maintaining posture and generating movement. Constant adjustments of skeletal muscle are needed for body balance or maintenance of homeostasis by generating heat [1]. During embryogenesis, the muscle cell precursors called myoblasts divide repeatedly by mitosis, line up and fuse forming multinucleated muscle cells called myofibers [2,3]. Each myofiber is surrounded by a layer of connective tissue called endomysium or basal lamina. The bundles of myofibers are bound together by perimysium (connective tissue) into fascicles. Multiple fasciculi, in turn, form a muscle, surrounded by the epimysium, a layer of connective tissue, and anchored to tendons and bones. A greater degree of complexity is achieved by interaction of muscle tissue with a dense network of nerve fibres and blood vessels that supply the tissue with the necessary oxygen and nutrients [2] (Figure 1). Myofibrils represent the smallest unit of a skeletal muscle, composed of

myofilaments, a complex of actin and myosin protein filaments, organized in force-generating sarcomeres.

In response to nerve stimulus calcium is released which drives the movement of actin and myosin filaments. The force generated by the actin-myosin cross-bridges is transmitted within the fibre and movement is produced [4].

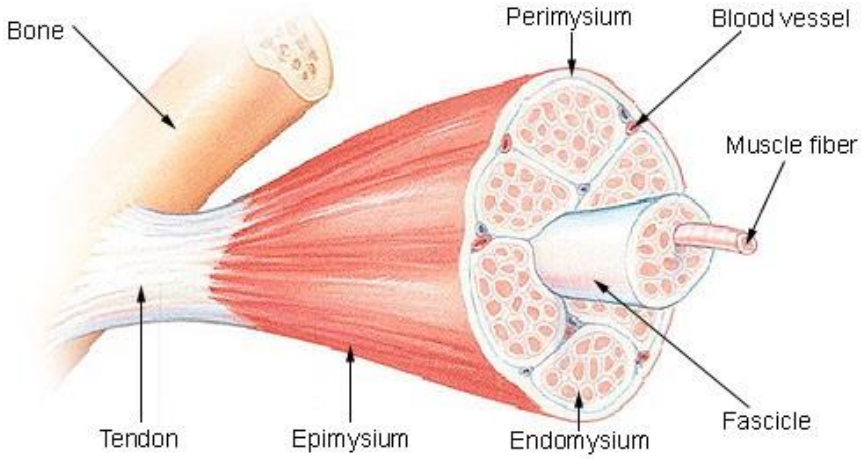

Figure 1. Structural organization of skeletal muscle.
(https://embryology.med.unsw.edu.au/embryology/index.php/Skeletal_Muscle_Histology)

2.2 Skeletal Muscle homeostasis and regeneration

Skeletal muscle tissue has a remarkable ability to regenerate. This property is essential to maintain its integrity and functionality, since it is exposed to constant micro injuries and stress, due to its primary function in locomotion, and to intense metabolic activities [5]. Muscle regeneration is a highly coordinated process occurring in four phases: degeneration, inflammation, regeneration and remodelling-repair. The muscle injury causes the disruption of myofiber integrity, which then induces the inflammatory response. Different inflammatory cell populations are recruited to the site of the damage, among which macrophages play a crucial role by providing necessary molecular cues that drive successful regeneration [6]. A remarkable characteristic of the regenerative phase is the massive proliferation of muscle stem cells known as satellite cells, followed by their differentiation into committed myoblasts. In normal conditions, satellite cells remain in a quiescent state but can quickly enter the cell cycle when activated in response to injury. Satellite cells can undergo both symmetric and asymmetric division; meaning that some satellite cells return to quiescent state to maintain the pool of stem cell population while some undergo myogenic differentiation to restore damaged fibers [7]. This last step can be achieved either repairing existing damaged myofibers or fusing to form new myotubes [8].

Satellite cells are located at the periphery of skeletal muscle myofibers and represent the main source of myoblasts during skeletal muscle regeneration. The proper functions of satellite cells greatly rely on their interaction with the environment, the so called stem cells niche, formed by different progenitor cell populations, vascular and neural network, that provide extracellular matrix components and diffusible molecules. Therefore, the

activity of satellite cells can be regulated by cell-cell interactions with myofibers or by autocrine and paracrine signals released by the components of the niche. For example, it was suggested that Wnt signalling promotes myogenic differentiation and that Notch signalling stimulates both the quiescence and the proliferation and expansion of satellite cells [9]. In particular, it has been demonstrated that myofibers regulate the quiescent state of satellite cells by cell-cell contact via the Notch signalling pathway [10].

2.4. Fibro-adipogenic progenitors and their dual role in skeletal muscle

Fibro-adipogenic progenitors (FAPs) are non-myogenic mesenchymal progenitor cells, identified as $CD31^-$, $CD45^-$, α-7 $integrin^-$, CD34+, Sca1+ population that can rapidly enter the cell cycle upon muscle damage [11]. FAPs have both adipogenic and fibrogenic potential, meaning that they can differentiate into adipocytes and fibroblasts by choosing alternative lineages. In response to severe damage FAPs expand and release factors that ensure a proper environment for successful regeneration and therefore influencing positively the differentiation of satellite cells. However, fibro/adipogenic progenitors in pathological conditions such as muscular dystrophies or during aging, play a different role and contribute to fat and fibrous tissue depositions [12]. Interestingly, it has been demonstrated *in vitro* that myotubes inhibit the adipogenic differentiation of FAPs, but, the molecular mechanism has not been characterized [14] (Figure 2). Due to their dual role in skeletal muscle, the identification of molecular mechanisms that control fibro-adipogenic progenitors behaviour are of great interest, since it could provide novel therapeutic targets that could restrain fat and fibrotic tissue deposition in order to ameliorate disease pathophysiology such as in muscular dystrophies.

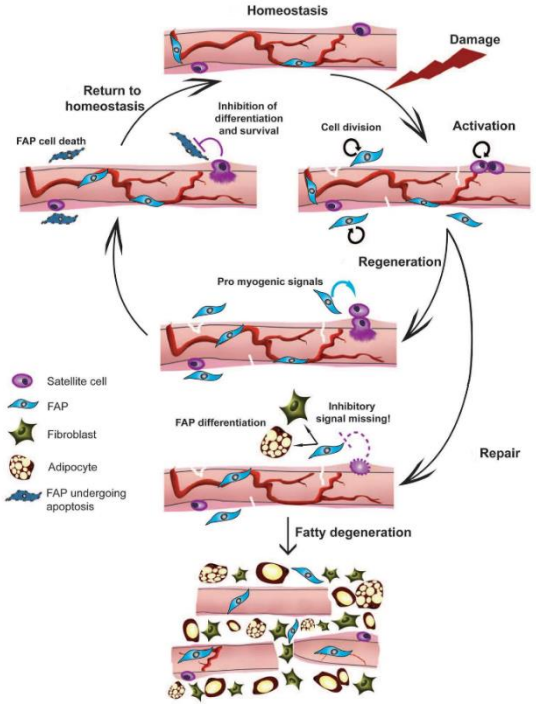

Figure 2. Schematic representation of the roles of fibro-adipogenic progenitors.

2.5. The Notch signalling pathway

Notch signalling is a major regulatory pathway of cell proliferation, differentiation and cell fate determination. It relies on cell-to-cell communications balancing neighbouring cell state. There are four Notch receptors in mammals (Notch-1, -2, -3 and -4), all trans-membrane proteins and 7 known ligands: five canonical ligands (DLL1, DLL3, DLL4, JAGGED1 and JAGGED2) and two non-canonical ligands (DLK1 and DLK2) [15]. The pathway is initiated by the binding of ligands such as Delta and Jagged family to Notch receptors resulting in receptor enzymatic cleavage [15]. Transmembrane receptors undergo two cleavages at two different sites: one is mediated by ADAM proteases and the other by the γ-secretase complex. The newly cleaved Notch intracellular domain (NICD) is a transcriptional coactivator that translocates to the nucleus and binds CCAAT-binding protein (CBF-1). In the absence of NICD, CBF-1 is a repressor, but NICD is able to displace this complex and recruit co-activators in order to regulate gene expression [15]. The Notch activation cascade induces the activation of genes such as Hes (Hairy/Enhancer of split), Hes-related repressor protein, NF-κB or PPAR [15]. Studies have demonstrated that γ-secretase inhibitors such as N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butylester (DAPT) can efficiently block Notch signalling by preventing the enzymatic cleavage of the four Notch receptors, due to the inhibition of γ-secretase complex activity.

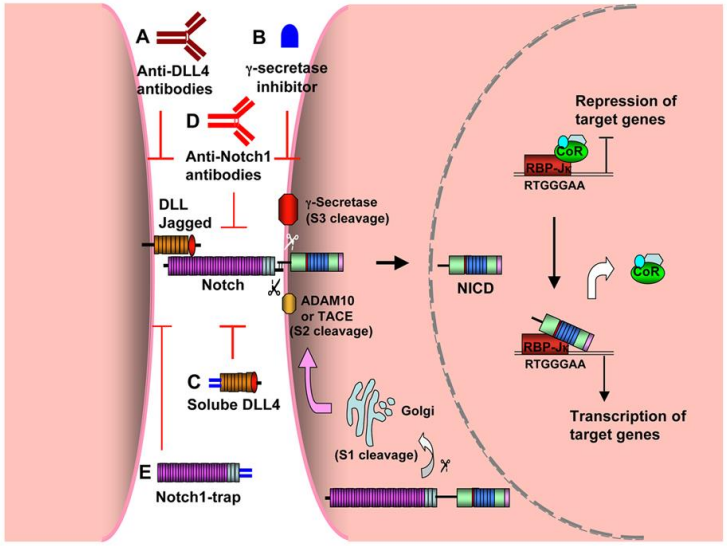

Figure 3. The Notch pathway.

In skeletal muscle during development, Notch activation stimulates proliferation of satellite cells. On the other hand, Notch signalling plays a key role in satellite cell quiescent state maintenance during adult homeostasis. Upon injury, Notch signalling is down regulated, satellite cells exit the G_0 phase and enter the cell cycle. After division, the majority of cells progress to terminal differentiation to form myofibers, but a fraction is able to self-renew and return to G_0 state. Thus, Notch signalling dramatically reduces in proliferating myoblasts but it again increases 4-5 days after injury, which correlates with the decline of proliferating cells and increases until quiescent satellite cell pool is restored. All these observations show that a decrease in Notch signalling is needed to expand the myogenic population during the regeneration process (Figure 4).

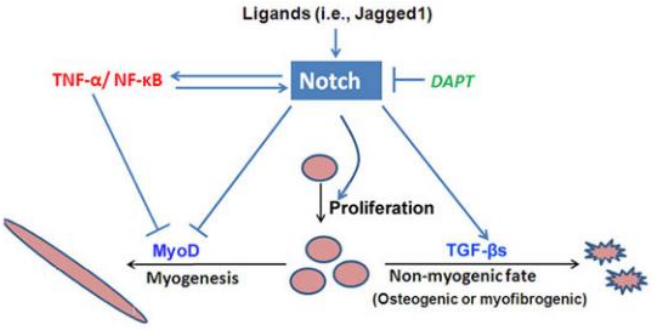

Figure 4. Effect of *in vivo* inhibition of Notch on the histopathology of skeletal muscle of dKO mice (Xiaodong et al, 2015)[16].

3. Aim of work

Skeletal muscle shows an extraordinary regenerative potential in response to severe injury. Muscle regeneration is a highly coordinated process mediated by different cell populations in the muscle. Satellite cells are quiescent stem cells in physiological condition, with the ability to enter in the cell cycle, proliferate and differentiate into myoblasts under pathological condition. However, their activity is highly affected by extracellular signals produced by different cell populations residing in the stem cell niche. Recently, a bipotent cell population has been identified as a key regulator in regeneration: fibro-adipogenic progenitors (FAPs). These cells have the potential to differentiate into adipocytes as well as into fibroblasts. FAPs have been proven to provide an optimal environment releasing pro-myogenic factors that positively affect myogenic differentiation of satellite cells. Nevertheless, under chronic damage FAPs are responsible for adipose and fibrotic deposition in muscle.

Due to the importance of satellite cell and FAP cross talk in muscle regeneration, in my project I aimed at characterising their interaction. I focused on understanding the inhibition of adipogenic differentiation of FAPs mediated by satellite cell derived myotubes. In particular, given the importance of Notch signalling in skeletal muscle I investigated a potential involvement of this pathway in satellite cells and FAPs crosstalk.

Considering that adipose and fibrotic deposition are common events in pathological conditions such as muscular dystrophies, obesity, type II diabetes or ageing, revealing the molecular mechanism that controls FAPs differentiation could lead to the discovery of novel therapeutic targets in order to improve such pathological conditions.

4. Materials and methods

4.1. Isolation of FAPs and satellite cells from mice

Materials

- Microsurgery equipment:
- 70 % Ethanol,
- Sterile Petri dishes (35x10, 60x15, 100x20 mm),
- Hank's Balanced Salt Solution ($HBSS^+$) containing Ca^{2+}/Mg^{2+} and Penicillin/Streptomycin,
- Bovine Serum Albumin (BSA),
- Disposable scalpel blades,
- 15 and 50 ml Falcon tubes,
- Sterile pipettes (5, 10 and 25 ml)
- Dispase II (stock 1.07 U/mg),
- DNase I (stock 10 mg/ml),
- Collagenase A (stock 100 µg/µl),
- Bürker counting chamber,
- Sterile cell strainers (100, 70, 40 and 30 µm),
- 0.22 µm strainers,
- Red blood cell lysis buffer (stock 10X)
- 10 and 50 ml syringes,
- Magnetic beads buffer: PBS 1X, BSA 0.5%, EDTA 2 mM;
- Anti-CD31, anti-CD45, anti-α7-integrin and anti-Sca1 antibodies (conjugated to magnetic beads).

Method

4.1.1. Cell isolation from mouse skeletal muscle

C57/Bl6 mice were sacrificed by cervical dislocation and washed in 70% ethanol. To obtain skeletal muscle, both limbs were gently dissected and placed into a petri dish with Hank's balanced salts solution ($HBSS^+$) containing Ca^{2+} and Mg^{2+} ions, 1% Penicillin/Streptomycin and 0.2% bovine serum albumin (BSA). Muscle was then stripped away from the bone, removing as much non-muscle tissue as possible, and was minced extensively until obtaining a homogenate. Minced muscles were transferred into a 50 ml falcon tube and centrifuged for 5 minutes at 1800 rpm at 4ºC. Supernatant was removed and the pellet was weighted for the estimation of enzyme mix needed. The enzyme mix contained 4 ml of Dispase II 2.4 U/ml, 4 µl of DNase I 10 µg/ml and 80 µl of Collagenase A 2 µg/ml per one gram of muscle. For muscle digestion, the pellet was resuspended in the enzyme mix (filtered before use through a 0,22 µm cell strainer) and incubated at 37ºC for one hour in agitation, vortexing it after 30 minutes. The digestion process was stopped by adding 30 ml of $HBSS^+$ solution. A 100 µm cell strainer was placed into a new falcon tube and activated with $HBSS^+$. The digested cell suspension was passed through the filter and washed. Cells were collected by centrifugation at 1800 rpm for 5 minutes at 4ºC and the supernatant was eliminated. The pellet was resuspended in 30 ml of $HBSS^+$ buffer and the process was repeated using a 70 µm filter. After centrifugation, 1 ml of red blood cell lysis buffer was added (RBC 1X in ddH2O, filtered through a 0.22 µm cell strainer) and cells were kept in ice for 2 min 30 sec. The reaction was then neutralized with $HBSS^+$ up to 30 ml and was filtered one last time through a 40 µm cell strainer. The solution was centrifuged at 1800 rpm for 5 minutes at 4ºC. The supernatant was removed and the pellet was resuspended in the appropriate volume of the magnetic beads buffer (500 µl for up to 10^8 cells). Cells were

filtered through a 30 µm cell strainer, previously washed with 500 µl of the magnetic beads. Resulting cells were counted using the Bürker chamber and centrifuged at 1800 rpm for 10 minutes.

4.1.2. Cell separation using MACS

For cell isolation we exploited the magnetic-activated cell sorting (MACS) technique, based on antibodies conjugated to magnetic beads. The cell preparation obtained after muscle digestion was suspended in 90 µl of magnetic beads buffer for 10^7 total cells and were incubated for 15 minutes at 4ºC with 5 µl of antibodies against CD31 and CD45 conjugated to magnetic beads. Then 2 ml of magnetic beads buffer were added and cells were centrifuged at 1800 rpm for 10 minutes at 4ºC. Supernatant was eliminated and the pellet was resuspended in 500 µl of magnetic bead buffer. The column was activated by rinsing 500 µl of magnetic beads and the suspension was passed through the column. As satellite cells and FAPs are $CD31^-$ and $CD45^-$, the cells which passed through without attaching to the conjugated antibodies were collected in a 15 ml falcon tube. Cells were counted and centrifuged at 1800 rpm for 10 minutes at 4ºC. The pellet was resuspended in 80 µl of magnetic beads buffer for up to 10^6 cells and the previously described process was repeated incubating this time with of anti-α7-integrin antibody (20 µl for up to 10^6 cells). Both α7-integrin positive and negative cells were collected. On one hand, to isolate satellite cells as $CD31^-$, $CD45^-$ and $\alpha7\text{-integrin}^+$ cell population, the remaining suspension was flushed out in a new falcon tube and cells were plated in desired conditions. On the other hand, α7-integrin negative cells were counted and centrifuged at 1800 rpm for 10 minutes. The pellet was resuspended in 90 µl of magnetic beads buffer and was incubated with 10 µl of anti-Sca1 antibody conjugated to magnetic beads. The separation was performed following the previously described procedure. FAPs were selected as $CD31^-/CD45^-/\ \alpha7\text{-integrin}^-/\ Sca1^+$ and were plated in desired conditions.

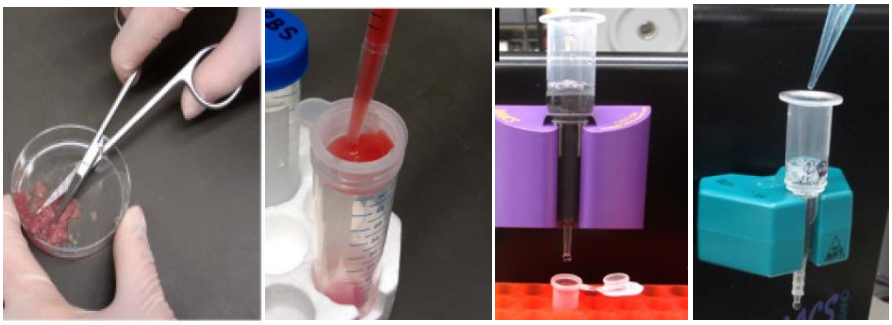

Figure 5. Cell isolation from mouse skeletal muscle. Skeletal muscle is minced until homogeneous, filtered and cells are isolated using MACS technique.

4.2. Cell cultures

Materials

- Dulbecco's modified Eagle medium (DMEM)
- Fetal bovine serum (FBS)
- Horse Serum (HS)
- Penicillin/Streptomycin (P/S), 100 U/ml-100µg/ml
- Sodium Pyruvate
- HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
- 96-mutiwell plates, 24-multiwell plates and transwell
- Sterile tips for Gilson pipettes (P1000, P200, P20, P2)
- Dexamethasone (DEXA)
- Methylisobutylxanthine (IBMX)
- Insulin (stock 10 mg/ml)

Method

Isolated fibro-adipogenic progenitors were cultured in growth medium consisting of Dulbecco modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 1% Penicillin-Streptomycin, 10 mM HEPES and 1 mM sodium pyruvate. Satellite cells were cultured in DMEM supplemented with 20% FBS and 10% horse serum (HS) and 2.5 ng/ml FGF.

For adipogenic differentiation, growth medium was replaced by adipogenic induction medium (AIM) consisting on DMEM supplemented with 20% FBS, 0.5 mM methylisobutylxanthine (IBMX), 0.4 mM dexamethasone and 1 µg/ml insulin.

Cells were maintained in AIM medium for 48 hours which was then changed by Adipogenic Maintenance Medium (AM) consisting of growth medium (DMEM+20%FBS) supplemented with 1 µg/ml insulin for 4 additional days. For inhibition of the Notch pathway, cells were treated with 10 µM DAPT inhibitor dissolved in DMSO, every 48h. Cells were then either fixed for immunofluorescence assay or protein extraction was performed for electrophoresis and blotting.

In an effort to study the interaction between satellite cells and FAPs co-culture experiments were designed. In these experiments FAPs were plated either in direct contact with satellite cells or in transwell cultures which were then placed in wells containing satellite cells (indirect co-culture). For direct co-culture experiments cells were seeded in 1:1 ratio. On the other hand, for indirect co-culture 1 µm porous transwell were used for 24-multiwell plates.

4.3. Western blot

Materials

- Phosphate-Buffered Saline (PBS)
- 1.5 ml eppendorf
- RIPA Lysis buffer
- Sodium fluoride (NaF); stock 500 mM

- Orthovanadate (stock 100 mM)
- PMSF (phenylmethylsulfonyl fluoride; stock 100X)
- Mammalian inhibitor cocktail (200X)
- Phosphatase inhibitor cocktail I (100X)
- Phosphatase inhibitor cocktail II (100X)
- Ice
- Gilson pipettes (P2, P20, P200, P1000)
- Protein Assay Dye Reagent (stock 5X)
- Spectrophotometer tubes
- Laemmil loading buffer 3X
- Thermoblock
- Resolving gel solution (30% acrylamide, 1.5 M Tris pH=8.8, 10% SDS, 10% ammonium persulfate, TEMED and ddH2O)
- Stacking gel solution (ddH_2O, 30% acrylamide, 0.5 M Tris pH=6.8, 10% SDS, 10% ammonium persulfate and TEMED)
- Isopropanol
- Casting glasses, casting stands, combs and running tank
- Running buffer (100 ml Tris-Glycine+ 5 ml 10%SDS+dH_2O)
- Molecular weight marker, PageRuler™ Prestained Protein Ladder
- Nitrocellulose membrane, Trans-Blot Turbo Transfer Packs
- Ponceau Red staining
- Blocking solution (5% non-fat milk in Tris Buffer Saline (TBS) Tween20)
- TBS 1X+Tween 0.1% solution

Method

4.3.1. Protein extraction from cell culture

To prepare samples, cells need to be lysed to release the protein of interest, disrupting the cell membrane and solubilising intracellular proteins. First, medium was removed and cells were washed with PBS 1X. The cells were then collected and incubated in RIPA lysis buffer (150 mM sodium chloride, 50 mM Tris-HCl pH 7.5, 0.1% sodium deoxycholate, 1 mM EDTA and 1% NP-40 detergent) supplemented with 1 mM sodium fluoride (NaF), 1 mM orthovanadate, PMSF 1X (phenylmethane sulfonyl fluoride),

mammalian inhibitor cocktail 1X and phosphatase inhibitor cocktails I and II 1X. Cell suspensions were transferred into a 1.5 ml eppendorf and were kept in ice for 30 minutes, followed by centrifugation at 13000 rpm for 30 minutes at 4ºC. Supernatants were collected and transferred into a new eppendorf tube.

4.3.2. Determination of protein concentration by the Bradford colorimetric assay

To determine protein concentration, a Bradford reagent solution (Bio-Rad Protein Assay reagent) was prepared by diluting it 1:5 in ddH20. This method is based on the absorbance shift of Coomassie Brilliant Blue G-250 after binding to proteins. In each spectrophotometer tube 1 µl of protein extract was added in 1 ml of Bradford Assay 1X and the absorbance of each sample was read with spectrophotometer at 595 nm. To prepare the blank, 1 µl of lysis buffer was used. In order to determine the concentration of proteins the obtained results were divided by 0.066, a value obtained from Lambert-Beer law: $A=\varepsilon LC$ (A- measured absorbance, ε - the slope of the standard curve, L - the length of the cuvette, and C - the concentration being determined).

In order to denature proteins the Laemmil loading buffer 3X was added and the samples were heated in a thermoblock at 95ºC for 10 minutes. Laemmil loading buffer contains 6% sodium dodecyl sulphate (SDS), an anionic detergent that binds and denatures proteins giving them a negative charge proportional to their molecular weight, 15% β-mercapto-ethanol, which breaks disulphide bonds; 30% glycerol, that increases the density of the sample so it will fall on the bottom of the well; 0.3% bromophenol blue, that colours the samples, 150mM Tris-HCl (pH 6.8) and ddH2O. Samples were stored at -20ºC.

4.3.3. SDA-PAGE gel electrophoresis

Using the SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) gel electrophoresis proteins are separated according to their molecular weight. In this procedure, denaturing agents are used to destroy protein secondary and tertiary structures disrupting non-covalent bonds. SDS molecules (sodium dodecyl sulphate) are negatively charged and by binding to proteins, the detergent provides all proteins with a similar net negative charge, thus a uniform charge to mass ratio. In this way, upon application of an electric field, proteins migrate independently of their original charge, and mobility only depends on molecular weight. The separation of molecules is influenced by the size of the pores within the gel, where smaller polipeptides travel faster through the pores. The size of gel pores depends on the concentration of the acrylamide. The higher the concentration the slower the protein migration rate (Table 1).

PROTEIN SIZE	GEL ACRYLAMIDE PERCENTAGE
4–40 KDA	20%
12–45 KDA	15%
10–70 KDA	12.5%
15–100 KDA	10%
25–200 KDA	8%

Table 1: Guide for choosing an appropriate gel percentage based on protein size, obtained from abcam protocols.

The gel is composed of two parts: stacking gel, which ensures sample alignment and resolving gel, used for separation of polipeptides. First the resolving gel, containing 30% acrylamide, 1.5 M Tris pH=8.8, 10% SDS, 10% ammonium persulfate, TEMED and ddH2O was prepared and poured between two casting glass frames previously set on the casting stands and left until solidification. To level the top of the separating gel, isopropanol was used. After solidification, isopropanol was removed. The stacking gel (ddH_2O, 30% acrylamide, 0.5 M Tris pH=6.8, 10% SDS, 10% ammonium persulfate and TEMED) was poured on top and a gel comb was carefully inserted in order to form the wells for sample loading. After polymerization was completed the glasses were taken out of the casting frame and were set in the running tank. Combs were gently removed and 500 ml of running buffer (100 ml Tris-Glycine+ 5 ml 10%SDS+dH_2O) was added.

Samples were heated at 95ºC for 5 minutes before use, spinned and 30 µg of proteins were loaded. A molecular weight marker, PageRuler™ Prestained Protein Ladder, was also loaded to enable the determination of protein size. In order to run the gel an electric field was applied with a desired voltage, inducing the migration of the negatively charged proteins towards the positive electrode, separating them according to their molecular weight.

4.3.4. Western Blotting

Following electrophoresis, proteins were transferred to a nitrocellulose membrane using the commercial kit Trans-Blot Turbo Transfer Packs (Biorad). The equipment was assembled placing the gel between the two layers, facing the proteins towards the nitrocellulose membrane. The transfer was carried out for 12 minutes at 1.3 A and 25 V. After the transfer protein visualization was achieved using Ponceau Red staining. Due to the ability of Ponceau (negative stain) to bind positively charged amino groups, this staining allows to check if proteins have transferred to the membrane successfully. Ponceau solution was recovered and the membrane was extensively washed with water. The membrane was then incubated with the blocking solution (5% non-fat milk in Tris Buffer Saline (TBS) Tween20) for 1 hour at room temperature or over-night at 4ºC. This is performed to prevent unspecific binding. After three washes with PBS 1X+Tween 0.1% 10 minutes of rotation each, the membrane was incubated with the primary antibody for 2h at room temperature or at 4ºC over-night. Primary antibodies that were used are anti-MyHC (1:1000), anti-aSMA (1:1000) and anti-Perilipin (1:1000). For normalization vinculin and actin were used. This was followed by three washes with TBS 1X+Tween 0.1%. Incubation with the secondary antibody (directed at a specie-specific portion of the primary antibody) was performed for 1h at room temperature in agitation and it was then washes three times as previously described. Secondary antibodies that were used are anti-rabbit and anti-mouse (1:2500).

The secondary antibody is conjugated to the enzyme horseradish peroxidase (HRP). When the membrane is incubated with the Clarity Western ECL substrate solution containing peroxide solution and luminol/enhancer solution (1:1), the peroxidase is able to catalyse the oxidation of luminol producing chemiluminescent signal which can be visualized with a digital imaging instrument Luminescence Analyzer System (LAS), which converts the chemi-luminescent signal into digital image.

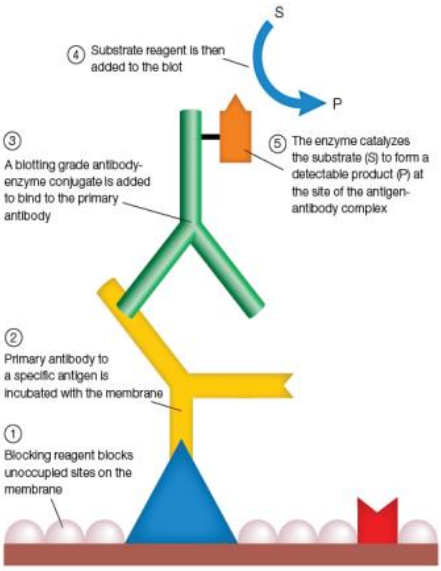

Figure 6. Representation of the Western Blotting technique. The antigen is recognised by a primary antibody which is then recognised by a secondary antibody conjugated to HRP enzyme.

4.4. Immunofluorescence assay

Materials

- Phosphate-Buffered Saline (PBS)
- Paraformaldehyde (PFA) 2%
- Triton X-100
- Fetal bovine serum (FBS)
- DAPI (4,6-diamidino-2-phenylindole)
- Oil-red-O solution (Sigma Aldrich)

Method

The culture medium was removed carefully and cells were washed once with PBS. To “freeze” cell metabolic activities they were fixed using 2% paraformaldehyde, which causes covalent crosslinks between macromolecules such as proteins, stabilising them and preserving the cell structure, for 12 minutes at room temperature and were washed with PBS 1X. In order to make intracellular structures accessible to the antibody, cells were permeabilized for 5 minutes using PBS 1X+Triton 0,1% and were then washed using the same solution. For minimizing unspecific binding of the primary antibody, cells were first incubated with the blocking solution containing PBS 1X+Triton 0,1%+Fetal

bovine serum (FBS) 10% for one hour at room temperature. Cells were incubated for 1 hour at room temperature with primary antibodies diluted in blocking solution. The antibodies used were anti-Smooth muscle actin, SMA (mouse, 1:300, Sigma) or anti-Myosin heavy chain, MyHC, (mouse,1:200, DSHB). Four washes were performed with PBS 1X+Triton 0,1% for 5 minutes each. All the following steps were performed in the dark. Cells were then incubated with the anti-mouse secondary antibody conjugated to the green fluorophore Alexa Fluor488 diluted in blocking solution (1:250, Life technologies) for 30 minutes at room temperature. After incubation three washes were performed with PBS 1X+Triton 0,1% for 5 minutes, and then twice with PBS. To stain the nuclei, cells were incubated with DAPI (4,6-diamidino-2-phenylindole; 1:5000 diluted in PBS 1X) for 5 minutes at room temperature. Finally, cells were washed twice with PBS and cells were conserved at 4ºC.

The Oil-red-O solution (Sigma Aldrich) was used for detection of lipid droplets in adipocytes. The stock solution (0.5% filtered solution of Oil red-O in isopropanol) was dissolved in ddH20 in 3:2 ratios and filtered. The cells were incubated for 5 min at RT, followed by two washings with PBS and DAPI staining.

5. Results

5.1 Isolation of satellite cells and FAPs using MACS

Our purification method for isolation of satellite cells and FAPs includes enzymatic dissociation of skeletal muscle isolated from hind limbs of C57/Bl6 mice, followed by magnetic-activated cell sorting (MACS) technology.

Mice are sacrificed by cervical dislocation, hind limbs are isolated and dissociated mechanically until homogeneous. This is followed by an enzymatic digestion using a solution containing 2 µg/ml Collagenase A, 2.4 U/ml Dispase II, and 10 µg/ml DNase I for one hour with agitation. Dispase II is an enzyme that disrupts the tissue extracellular matrix by degrading connective tissue proteins such as fibronectin and collagen. Collagenase A is used to break peptide bonds in the collagen. Finally, DNase I catalyses the hydrolytic cleavage of phosphodiester linkages in DNA released in the isolation and it is used to reduce sample viscosity.

After digestion, a series of filtrations were performed through 100 µm, 70 µm, 40 µm and 30 µm cell strainers and a red blood cells lysis buffer (RBC Lysis Buffer) was used to discard red blood cells from the suspension. Due to the ammonium chloride from the lysis buffer, the osmotic pressure inside the cells changes and membranes of red blood cells are broken. Then, several purification steps were performed using established antibodies and MACS technology.

Cell isolation can be achieved using antigen-antibody affinity methods due to the fact that different cell populations express specific cell surface markers. MACS technology allows purification of different cell populations according to the specific markers by using antibodies that are conjugated to magnetic beads. For this process, after incubating the cell suspension with the specific antibody, a column is placed between magnets and the cells are transferred into the column. The magnetic field captures the cells attached to microbeads and retains them in the column; whereas the cells that don't express the antigen flow through. Affinity purified cells are released after removal of the column from

the magnet. Thus, by this approach, both cell populations that express the markers and those that do not can be isolated and collected.

The first separation step was performed using anti-CD31 and anti-CD45 antibodies to label and eliminate endothelial cells and hematopoietic cells from the suspension. Cells that do not express CD31 and CD45 antigens (Lin^-) were collected and a second isolation step was performed using α7-integrin as a positive marker for satellite cells. The cell fraction that did not express α7-integrin marker was used in the last step of purification using anti-Sca-1 antibody. Finally, FAPs were selected as Sca-1 positives.

Before the isolation process by MACS and after each step of purification cells were counted. The average number of total cells obtained from one mouse (counted before the isolation step) was approximately $3.5x10^6$, around 30% of which were characterized as Lin^-. $Lin^-/α7\text{-integrin}^+$ cells identified as satellite cells and $Lin^-/α7\text{-integrin}^-/Sca\text{-}1^+$ cells identified as FAPs represented each about a 5% of the total cells population (Figure 7).

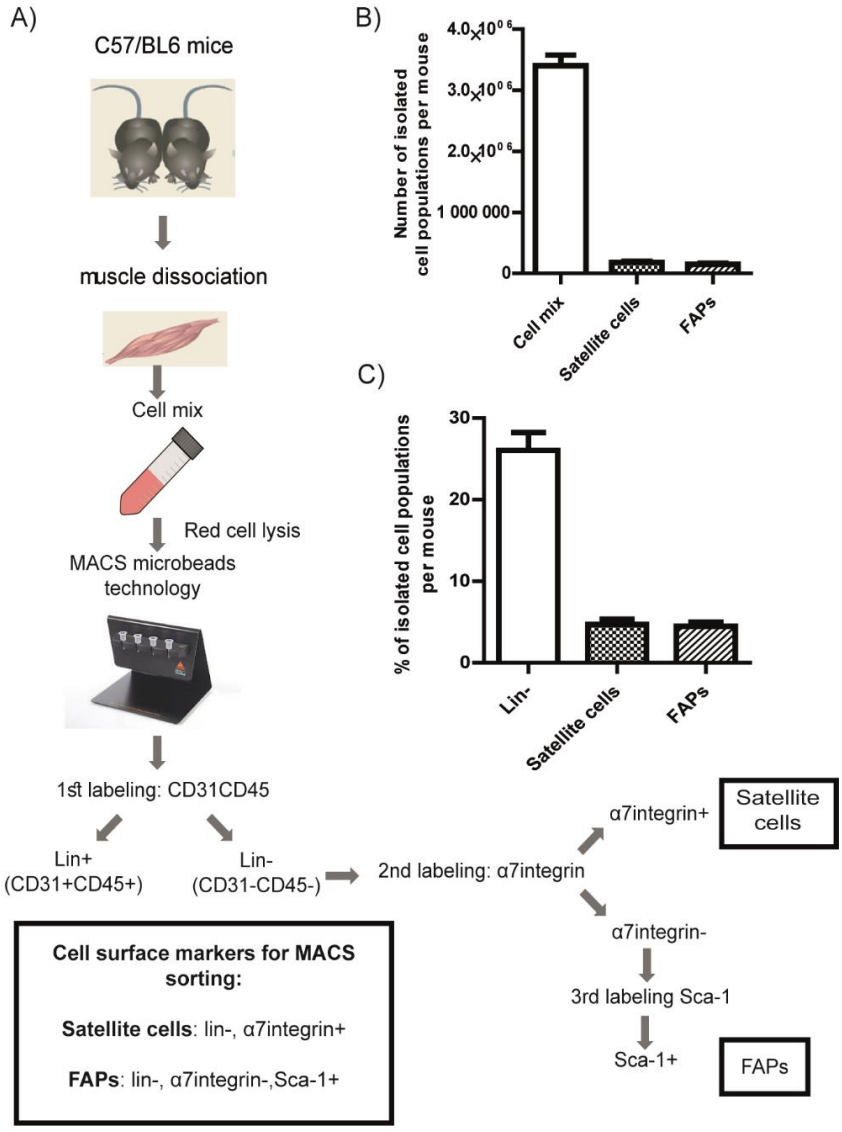

Figure 7. MACS microbeads isolation strategy. Schematic representation of cell isolation using MACS microbeads technology (A), average number (B) and percentage (C) of satellite cells and FAPs per mouse

5.2. Validation of FAPs differentiation potential

Isolated fibro-adipogenic progenitors were plated in 96-well plates at a concentration of 3000 cells per well in growth medium (DMEM+20% FBS) and kept in the incubator at 37ºC and in humidified environment with CO_2 5%. In order to assess the differentiation potential of fibro-adipogenic progenitors, cells were specifically induced to either adipogenic or fibrogenic differentiation. The adipogenic induction medium (AIM) consisted of growth medium supplemented with dexamethasone (2250X), IBMX (1000X) and insulin 1 µg/ml. Dexamethasone is an anti-inflammatory steroid that stimulates adipogenic differentiation in a concentration dependent manner. IBMX in combination with dexamethasone regulates PPARγ, a master regulator of adipogenesis, promoting adipogenic differentiation. IBMX is a phosphodiesterase inhibitor that increases intracellular levels of cAMP and PKA, required for adipogenic gene expression. Finally, insulin is widely used to induce the differentiation of preadipocytes as at high concentrations it mimics insulin-like growth factor-1 (IGF1), triggering the activation of transcriptional factors regulating adipogenic differentiation. Conversely, for fibrogenic differentiation transforming growth factor-β (TGFβ) was used at a concentration of 10 ng/ml. TGFβ is a cytokine that directs the transformation of fibroblasts and accelerates the synthesis of ECM proteins [17].

Fibro-adipogenic progenitors that were cultivated only in growth medium differentiated in adipocytes as well as in fibroblast. Adipogenic differentiation was confirmed by Oil-red-O (ORO) staining, which specifically marks triglycerides and lipid droplets. Fibrogenic differentiation, instead, was detected by immunofluorescence staining of α-smooth muscle actin (αSMA), expressed in fibroblasts. Nuclei were stained using DAPI, a fluorescent stain that recognizes A-T rich regions in DNA (Figure 8).

Upon adipogenic differentiation induction we observed a considerable increase of lipid droplets in comparison with cells cultivated in growth medium, suggesting that in conditions that favour adipocytes formation FAPs preferentially choose the adipogenic lineage. In contrast, cells that are grown in TGFβ containing medium showed a higher percentage of αSMA positive cells, and absence of adipocytes.

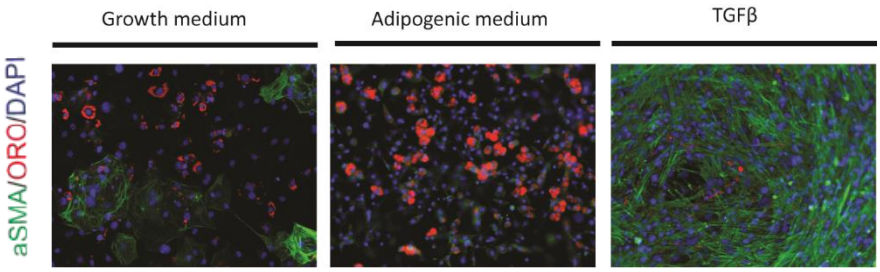

Figure 8. Differentiation properties of FAPs. Cells were isolated and plated in growth medium and in conditions favouring adipogenic and fibrogenic differentiation. After fixing adipocytes were stained with Oil-red-O and fibroblasts with antibodies against aSMA. Nuclei were counterstained with DAPI.

5.3. Adipogenic differentiation of FAPs is inhibited *in vitro* by the direct contact with myotubes

In order to study the cross talk between satellite cells and FAPs *in vitro*, FAPs and satellite cells were isolated and seeded alone or in co-cultures in 1:1 ratio in adipogenic medium. The co-cultures were performed in two different ways: in direct and indirect conditions. For direct co-cultures cells were plated on the same surface allowing a cell-to-cell interaction between satellite cells and FAPs. For indirect co-cultures, the transwell inserts (TW), with 1 µm pore size, containing FAPs were transferred to the wells containing satellite cells. Cell migration does not occur with pores smaller than 3.0 µm, thus using transwells with smaller pores, cell-to-cell contact is inhibited and communication is only allowed via soluble molecules that can diffuse through the pores. In this experiment, the adipogenic induction was performed as described above.

Differentiated cells were evaluated by immunofluorescence staining. Myosin heavy chain (MyHC) is a major contractile protein and was used to detect myogenic differentiation. Instead, adipogenic differentiation was detected by Oil-red-O (ORO) staining.

The immunofluorescence staining shows that isolated satellite cells, even when cultivated in adipocyte induction medium, have a strong potential to undergo myogenic differentiation as myotubes were detected by the presence of MyHC. This strong myogenic differentiation potential was also observed in co-culture. Isolated FAPs, as already shown, differentiated into adipocytes under conditions favouring adipogenic differentiation.

Interestingly, after co-culturing FAPs with satellite cells we did not observe the formation of adipocytes even after adipogenic induction, suggesting that myotubes inhibit the adipogenic differentiation of FAPs. However, this inhibition was not observed in the indirect co-culture, as both myotubes (detected by MyHC) and adipocytes (stained by ORO) were observed, leading to the conclusion that direct contact between satellite cells as FAPs was necessary for the inhibition to occur (Figure 9).

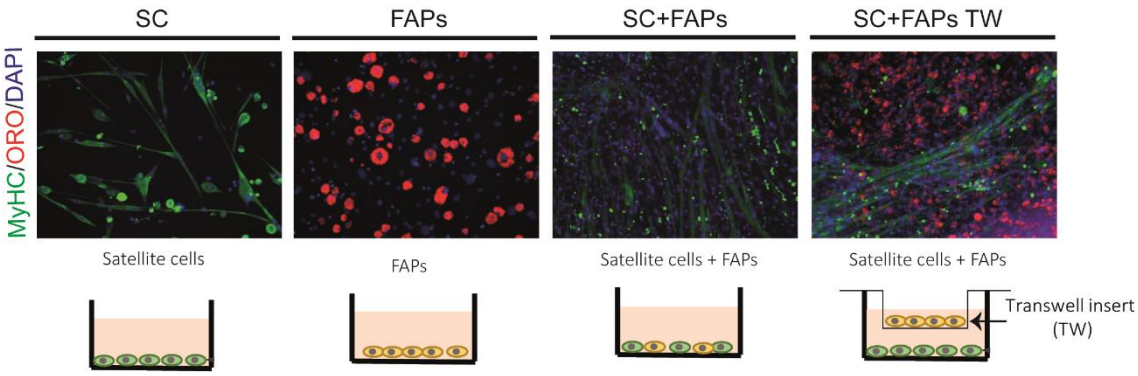

Figure 9. Satellite cells derived myotubes inhibit FAPs adipogenic differentiation by direct cell-cell contact. Satellite cells and FAPs were isolated and plated alone or in direct and indirect co-cultures (1:1) in adipogenic medium for 8 days. Myotubes were detected with immunofluorescence staining for MyHC, adipocytes with Oil-red-O stain and nuclei were counterstained with DAPI.

5.4. Myotube inhibition of FAPs adipogenesis is Notch dependent

In order to understand the mechanisms by which adipogenic differentiation is repressed in co-cultures and given the importance of Notch signalling in cell-to-cell contact, we further studied if the activation of Notch signalling was responsible for regulating the differentiation of FAPs. For this experiment, isolated satellite cells and FAPs were seeded either alone or in co-culture and adipogenic differentiation was induced. In separate experiments cells were also treated with 5 μM DAPT. This compound can efficiently block Notch signalling by preventing the enzymatic cleavage of Notch receptors, due to the inhibition of the γ-secretase complex activity.

Immunofluorescence assays were performed to study the differentiation potential after Notch inhibition. Satellite cells did not show any changes in differentiation, as myotubes were observed in both control and treated cells. On the other hand, a significant increase of lipid droplets was observed in treated FAPs, suggesting that Notch signalling represses the adipogenic differentiation of fibro-adipogenic progenitors. In untreated co-cultures we confirmed inhibition of the adipogenic differentiation of FAPs mediated by myotubes. However, when Notch signalling was inhibited, we observed a significant decrease in adipogenic inhibition, as lipid droplets were detected by ORO staining.

The results observed in co-culture experiments were further confirmed by Western Blot assay. Cells were seeded at the same conditions and protein extractions were performed in order to study protein expression. Western blot analysis confirmed that the inhibition of Notch signalling pathway promotes FAPs differentiation into adipocytes in direct co-culture, since perilipin was only detected in co-cultures treated with DAPT. Perilipin localizes at the surface of lipid droplets to regulate triglyceride storage [18]; thus, it can be used as a late marker of adipogenic differentiation. Moreover, we observed hardly any difference in the expression of MyHC in both control and treated groups, confirming that the inactivation of Notch signalling did not interfere with myogenic differentiation. In addition to these observations, we also investigated the expression of aSMA in order to monitor fibrogenic differentiation. As expected, the co-culture treated with DAPT showed no expression of this protein, indicating that FAPs took the adipogenic fate (Figure 10). Overall these results suggest that myotubes inhibit FAP differentiation by activating Notch signalling, and that a disruption of Notch signalling can lead to excessive adipocyte deposition.

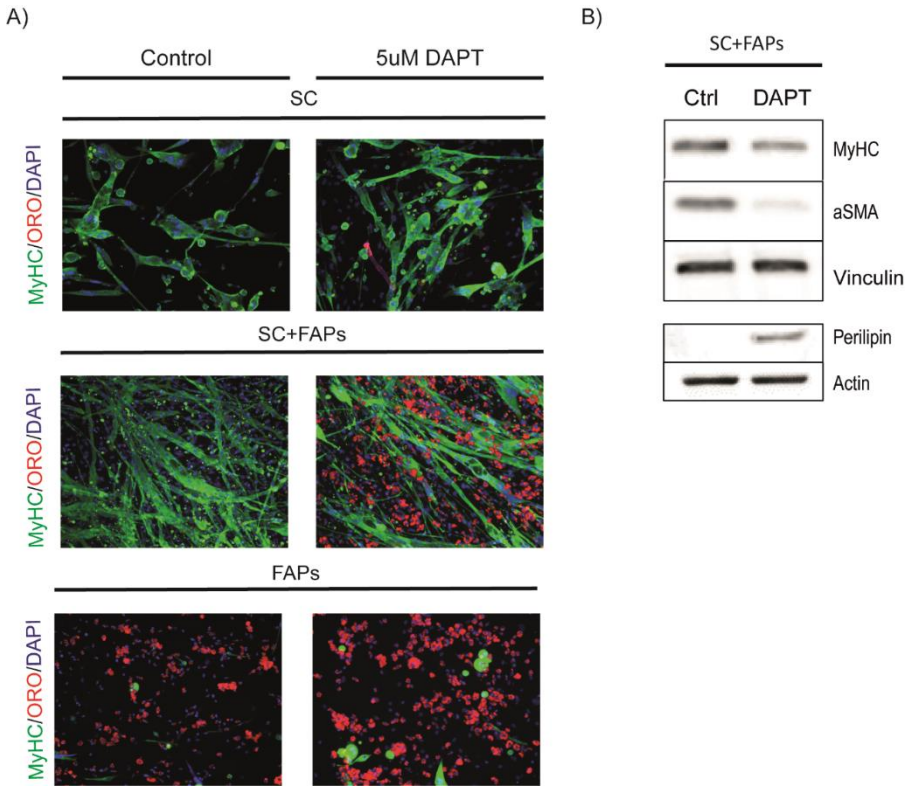

Figure 10. DAPT promotes FAPs differentiation into adipocytes in co-culture with myotubes. Direct co-cultures of satellite cells and FAPs treated with 5μM DAPT every 48h in adipogenic medium were stained for MyHC, Oil-red-O and DAPI (A). Western blot analysis of MyHC, aSMA and Perilipin expression in control and treated co-cultures (B).

6. Discussion

In physiological conditions, skeletal muscle is a relatively stable tissue undergoing limited cellular turnover. However, in response to a trauma, micro-injuries or stress, it displays a remarkable ability to regenerate, which is essential to maintain its integrity and functionality [2]. The regeneration process is highly coordinated by different cell populations that contribute to the maintenance of skeletal muscle such as satellite cells (SCs), fibro-adipogenic progenitor (FAPs) and inflammatory cell populations [5]. Among them the myogenic potential resides mainly in satellite cells, the undifferentiated muscle progenitor cells. Satellite cells remain in a quiescent state in physiological conditions, but can quickly enter the cell cycle when activated in response to injury and differentiate into myoblasts that eventually fuse to form myofibers [7]. However, their activity is affected by extracellular signals produced by different cell populations residing in the stem cell niche. Fibro-adipogenic progenitors (FAPs) are a bipotent non-myogenic mesenchymal progenitor cell population able to differentiate into both adipocytes and fibroblasts due to alternative lineage choice [12]. They are important in regeneration because they influence positively the differentiation of satellite cells. However, in pathological conditions they are responsible for fat and fibrous tissue depositions. Interestingly, it has been reported that satellite cells derived myotubes can inhibit FAPs adipogenic differentiation *in vitro* [19]. Since FAPs are responsible for adipose and fibrotic deposition in skeletal muscle, identification of potential molecular mechanisms by which the

undifferentiated state is maintained could be helpful to provide novel therapeutic targets to mitigate pathological conditions.

Given the importance of satellite cells and FAPs in regeneration and degeneration processes, in this project we studied the molecular cross talk between these two populations, with the aim to offer a rational explanation of the observed adipogenic inhibition of FAPs occurring when FAPs are co-cultivated with satellite cells derived myotubes.

Satellite cells and FAPs were isolated from C57/Bl6 mice hind limbs and were maintained in culture in different conditions. Before setting up co-culture experiments we tested the bipotent differentiation properties of FAPs. We assessed the dual differentiation potential in conditions that favoured either adipogenic or fibrogenic differentiation. FAPs, when cultivated in growth medium, differentiated spontaneously into either fibroblasts or adipocytes confirming their divalent differentiation potential. When cells were subjected to the environment favouring adipogenic differentiation we could observe a considerable increase of adipocytes compared to cells in growth medium. In contrast, cells that were induced for fibrogenic differentiation by TGFβ showed a substantial increase in the percentage of αSMA positive cells. Through this approach we validated that FAPs have the potential to differentiate into two different cell types. We further showed that we can selectively induce them to differentiate into adipocytes or fibroblasts by providing specific stimuli.

As FAPs are reported to play a key role in regeneration positively affecting satellite cells, their interaction was further studied in co-cultures. In order to ensure FAPs differentiation into adipocytes, all experiments were done under adipogenic conditions. In our studies we considered both direct co-culture conditions, seeding both types of cells on the same surface; and indirect co-culture conditions, physically separating the cells by using transwell inserts. In these conditions cell-to-cell contact is prevented and communication can only occur by soluble molecules able to diffuse through pores that prevent cell migration. Immunofluorescence staining showed that satellite cells either isolated or in co-culture have a strong potential to undergo myogenic differentiation. Additionally, adipogenic induction did not change the myogenic properties of satellite cells as myotubes were detected by the presence of MyHC. Moreover, we could observe amelioration of myogenic differentiation in co-culture with FAPs compared with satellite cells plated in isolation, confirming their positive effect of FAPs on myogenesis. FAPs alone, as expected, differentiated into adipocytes under conditions favouring adipogenic differentiation. Interestingly in direct co-culture with satellite cells we did not observe any adipocytes, even though cells were maintained in adipogenic medium. However, in indirect co-cultures lipid droplets were detected by ORO staining, meaning that inhibition does not occur. These data indicate that adipogenic differentiation of FAPs is inhibited by myotubes. Furthermore this inhibition is not mediated by a paracrine process via small molecules, rather a direct contact is essential for successful inhibition.

We further aimed to identify the molecular mechanism that underlies observed inhibition of FAPs adipogenesis in direct contact with myotubes. Notch signalling is a major regulatory pathway of cell proliferation, differentiation and cell fate determination [13]. This pathway is activated by cell-cell contact, upon biding of the receptor to a ligand expressed on the membrane of neighbouring cells. In skeletal muscle the delta-like-

ligand 1 (Dll1) is expressed on the cell membrane of satellite cells and myofibers [20]. Moreover, it is established that Notch signalling governs the satellite cell quiescent state during skeletal muscle homeostasis [13]. However, little is known about its effect on fibro-adipogenic progenitors. Since Notch is a known regulator of stem cells quiescence and given that Notch signalling is transmitted through cell-to-cell contact, we decided to investigate a possible role of Notch signalling in controlling FAPs adipogenesis in co-cultures. In order to evaluate if the inhibitory effect was mediated by Notch signalling, purified cells and co-cultures were treated with the γ-secretase inhibitor (DAPT). The treatment did not affect satellite cells differentiation since no difference in myotubes formation was observed. On the other hand, FAPs alone showed a greater fraction of ORO stained cells upon DAPT treatment. These data suggest that FAPs differentiation is regulated by Notch signalling, and that the inhibition of such pathway leads to an increase in adipogenic differentiation. Further, in co-cultures without DAPT treatment myotubes showed, as previously observed, the potential to inhibit adipogenic differentiation of FAPs. Upon Notch pathway inhibition, however, adipocytes differentiate efficiently as observed by ORO staining. These data indicate that the inhibition of FAP adipogenesis by myotubes is mediated by the Notch signalling pathway. Western Blot data from co-cultures further confirmed the data obtained with immunofluorescence assays. While the expression on myosin heavy chain barely changed under DAPT treatment, the expression of perilipin, a late marker of adipogenic differentiation was only observed when Notch pathway was inhibited. Moreover, in treated co-culture we observed a decrease in the expression of aSMA indicating that FAPs were completely committed to the adipogenic lineage.

Overall, these results support the conclusion that Notch signalling plays an important role in modulating the adipogenic differentiation of fibro-adipogenic progenitors and that *in vitro* this pathway is responsible for mediating the myotube inhibitory effect on FAPs. Given the importance of FAPs in both physiological or pathological skeletal muscle conditions, the identification of a new molecular mechanism that is able to suppress their differentiation is of great interest. Our results could provide potential new therapeutic targets for amelioration of disease pathophysiology such as in muscular dystrophies.

7. Bibliography

1. Saladin, K.S. & Miller, L. 1998, Anatomy & physiology, WCB/McGraw-Hill New York (NY).
2. MacIntosh, B.R., Gardiner, P.F. & McComas, A.J. 2006, Skeletal muscle: form and function, Human Kinetics.
3. Zhao, P. & Hoffman, E.P. 2004, "Embryonic myogenesis pathways in muscle regeneration", Developmental Dynamics, vol. 229, no. 2, pp. 380-392.
4. Frontera, W.R. & Ochala, J. 2015, "Skeletal muscle: a brief review of structure and function", Calcified tissue international, vol. 96, no. 3, pp. 183-195.
5. Farup, J., Madaro, L., Puri, P. & Mikkelsen, U.R. 2015, "Interactions between muscle stem cells, mesenchymal-derived cells and immune cells in muscle homeostasis, regeneration and disease", Cell death & disease, vol. 6, no. 7, pp. e1830.

6. Carosio, S., Berardinelli, M.G., Aucello, M. & Musarò, A. 2011, "Impact of ageing on muscle cell regeneration", Ageing research reviews, vol. 10, no. 1, pp. 35-42.
7. Yin, H., Price, F. & Rudnicki, M.A. 2013, "Satellite cells and the muscle stem cell niche", Physiological Reviews, vol. 93, no. 1, pp. 23-67.
8. Kuang, S., Kuroda, K., Le Grand, F. & Rudnicki, M.A. 2007, "Asymmetric self-renewal and commitment of satellite stem cells in muscle", Cell, vol. 129, no. 5, pp. 999-1010.
9. Bentzinger, C., von Maltzahn, J. & Rudnicki, M.A. 2010, "Extrinsic regulation of satellite cell specification", Stem cell research & therapy, vol. 1, no. 3, pp. 27.
10. Natarajan, A., Lemos, D.R. & Rossi, F.M. 2010 "*Fibro/adipogenic progenitors: a double-edged sword in skeletal muscle regeneration*", Cell Cycle, 9:11, 2045-2046.
11. Joe, A.W., Yi, L., Natarajan, A., Le Grand, F., So, L., Wang, J., Rudnicki, M.A. & Rossi, F.M. 2010, "Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis", Nature cell biology, vol. 12, no. 2, pp. 153-163.
12. Uezumi, A., Fukada, S., Yamamoto, N., Takeda, S. & Tsuchida, K. 2010, "Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle", Nature cell biology, vol. 12, no. 2, pp. 143-152.
13. Song, B.Q., Chi, Y., Li, X., Du, W.J., Han, Z.B., Tian, J.J., Li, J.J., Chen, F., Wu, H.H., Han, L.X., Lu, S.H., Zheng, Y.Z. & Han, Z.C. 2015, "Inhibition of Notch Signaling Promotes the Adipogenic Differentiation of Mesenchymal Stem Cells Through Autophagy Activation and PTEN-PI3K/AKT/mTOR Pathway", Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology, vol. 36, no. 5, pp. 1991-2002.
14. Mourikis, P. & Tajbakhsh, S. 2014, "Distinct contextual roles for Notch signalling in skeletal muscle stem cells", BMC developmental biology, vol. 14, no. 1, pp. 2.
15. Huang, Y., Yang, X., Wu, Y., Jing, W., Cai, X., Tang, W., Liu, L., Liu, Y., Grottkau, B. & Lin, Y. 2010, "γ-secretase inhibitor induces adipogenesis of adipose-derived stem cells by regulation of Notch and PPAR-γ", Cell proliferation, vol. 43, no. 2, pp. 147-156.
16. Xiaodong Mu, Ying Tang, Aiping Lu, Koji Takayama, Arvydas Usas, Bing Wang, Kurt Weiss, Johnny Huard, 2015, "The role of Notch signaling in muscle progenitor cell depletion and the rapid onset of histopathology in muscular dystrophy", Hum.Mol.Genet, 24(10): 2923–2937.
17. Clara Sciorati, Emilio Clementi, Angelo A. Manfredi, Patrizia Rovere-Querini, 2015, "Fat deposition and accumulation in the damaged and inflamed skeletal muscle: cellular and molecular players" Cell. Mol. Life Sci.
18. Christina C. Westhoff, Janice Mrozinski, Ina Riedel, Hans W. Heid, Roland Moll, 2017, "Perilipin 1 is a highly specific marker for adipocytic differentiation in sarcomas with intermediate sensitivity" Journal of Cancer Research and Clinical Oncology, Volume 143, Issue 2, pp 225–232.
19. Chu W, Wei W, Yu S, Han H, Shi X, Sun W, Gao Y, Zhang L, Chen J, 2016, "C2C12 myotubes inhibit the proliferation and differentitation of 3T3-L1 preadipocytes by reducing the expression of glucocorticoid receptor gene" Biochem Biophys Res Commun.;472(1):68-74.
20. Conboy, I.M., and Rando, T.A. (2002). The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell 3, 397-409.