Optimizing Ex Vivo Culture of Satellite Cells

A Thesis

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BY

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Abstract

Satellite cells are responsible for regeneration of skeletal muscles. They have the ability to form new muscle fibers and to repopulate satellite cell pool. Transplantation of human satellite cells to muscle dystrophy patients is an aim. To achieve this aim, we must understand mechanisms governing satellite cell proliferation and differentiation. We must also have tools that allow us to control these mechanisms. In this thesis, I tried to understand *in vitro* behavior of satellite cells and to find factors that can control their differentiation pathways. I showed the effect of *in vitro* culture on transcription of various myogenic regulatory factors. I also showed the effect of various inhibitors on these factors. I found that treatment of freshly isolated satellite cells and of established myoblast cells with SR1 (Stemregenin 1, one of the compounds screened at the initial part of the thesis) results in inhibition of myogenic commitment factors, MyoD and Myogenin. SR1 also succeeded to block myoblast differentiation into myotubes. SR1 treated established myoblast cells succeeded to repopulate satellite cell pool of recipient muscles. SR1 is described to work through an action on AHR (aryl hydrocarbon receptor). So, I described the expression pattern of AHR in different stages of satellite cells.

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Introduction

Muscular dystrophies (MDs) are inherited disorders that cause muscle weakness affecting variable muscle groups and is having variable severity (1). There are nine major types of MD, with each type involving an eventual loss of strength, increasing disability, and possible deformity. These types include Duchenne MD (DMD), Becker MD, Congenital MD, Distal MD, Emery-Dreifuss MD, Facioscapulohumeral MD, Limb girdle MD, Myotonic MD and Occulopharyngeal MD. Of these types the most common is DMD (32). DMD is an X-linked recessive disease, affecting around 1 in 6,291 boys (33). The molecular defect in DMD is a severe deficiency of dystrophin (67), a subsarcolemmal protein resulting in instability of the sarcolemma during cycles of muscle contraction. Dystrophin deficiency causes myofiber necrosis due to sarcolemmal damage during contraction and relaxation cycles (34). Myofiber necrosis is followed by myofiber regeneration in repetitive cycles until the regeneration process is exhausted. This results in progressive muscle degeneration and eventual death of the patient which happens in late teens or early twenties typically from respiratory failure (1,2). Despite the high morbidity and mortality of the disease, there is no cure for any form of MD. Therapy ranges from treatment with corticosteroids which have severe side effects to range of motion exercises and mobility aids (46).

Recently, parallel to new breakthroughs in stem cell research, cell therapy has emerged as a hope for treatment of many diseases that have been considered for a long duration as incurable, including MD. The most settled example of cellular therapy was started here in Minnesota when the first successful haematopoietic stem cell transplantation was done by Dr. Robert A. Good in 1968 (35). This thesis can be considered as an attempt to continue the mission through enabling the first successful and efficient transplantation of ex vivo expanded skeletal muscle stem cells for treatment of patients with MD. In 1961, Cooper and Konigsberg demonstrated that the multinucleated skeletal myofibers, the contractile units of muscle, are formed by the fusion of mononucleate myoblasts (13). In the same year, Alexander Mauro from the Rockefeller institute described the existence of mononuclear cells residing beneath the basal lamina of skeletal muscle fibers during an electron microscopic study of skeletal muscle fibers of

the frog (3). These cells were given the name “satellite cells”. The function of satellite cells is that they are responsible for regeneration of the injured skeletal muscle by having the ability to form new contractile myofibers that can restore the function of the damaged muscle.

Satellite cells have the ability to divide, proliferate and to fuse to form skeletal muscle fibers (4), but what is interesting about satellite cells is their ability to replenish the satellite cell population of the injured muscle after this cycle is finished (11). So, transplanting satellite cells to DMD patients holds the ability to simultaneously deliver normal copies of genes to dystrophic muscle and to continue delivering it as normal cellular turnover occurs. These characteristics of SCs opened the door to think about transplanting genetically competent satellite cells to patients with muscular dystrophy (5,38). The cells are expected to proliferate and fuse to form fully functional skeletal muscle fibers that can restore the function of the diseased muscle and at the same time maintain a long lasting population of satellite cells that can maintain the normal homeostasis of the muscle over the long run in terms of maintaining regenerative capabilities in case of muscle injury. Freshly isolated satellite cells have been successfully transplanted into mouse models of MD (5). Transplanted satellite cells succeeded to form new muscle fibers that have been associated with improvement in the contractile force of recipient muscles (42). The muscle stem cell pool has also been replenished by donor freshly harvested satellite cells (11). These studies showed that SC transfer could restore dystrophin to recipient muscles.

However, though it seems interesting, the idea of using satellite cells as transplantation therapy for patients with MD faces some challenges. To be noted, there is no treatment, so far, for MD based on cells. The first challenge is the method of delivery of myogenic cells to the diseased muscles. Myopathies are generalized diseases; it is important to consider that the target tissue to be treated (skeletal muscles) represents 40-50% of body weight (36). Intramuscular delivery would require a very high number of cell injections, taking into consideration the surface area, the volume and the distribution of the body muscles. But, intramuscular injection of the cells is still the most commonly used method in muscle stem cell research at least to test for the engraftment ability of a

certain cell population in model organisms. The other route is intravascular delivery whether intravenous or intra-arterial. The intra-arterial route by going directly to the target muscle and covering it uniformly has resulted in much more favorable results in engraftment experiments on mice (37). But, the intra-arterial injection in human patients would be dangerous, posing many serious possible complications because of the high pressure within and the vital functions of the arteries in the body. Although easier and being accompanied by less local complications, the intravenous route was unsuccessful even after extensive muscle injury (38). In addition, the intravenously injected cells face the barrier of the capillary network of the lung which may be a source of many complications as well as loss of cells before they reach the muscles. Together, these points indicate the challenge of delivering the cells to the body of the MD patients.

Another serious challenge to satellite cell therapy of MD patients which is actually the one served by this thesis is that satellite cells are a rare cell population accounting for less than 1% of the total mononuclear cell population of a muscle (31). More over, isolation of satellite cells requires destruction of the donor muscle which adds to the limitation of the whole process. In order to achieve the goal of curing patients with MD through transplantation of satellite cells, we will need large numbers of satellite cells. Therefore, expanding them ex vivo is an indispensable goal on the way to transplantation therapy for muscular dystrophy. So, the question should be "can we grow satellite cells *in vitro*?" The answer is yes, we can do that. Isolated satellite cells from skeletal muscle fibers can grow *in vitro* very rapidly achieving huge numbers. But, the problem is that they are no longer satellite cells. They are now just proliferating myoblasts. Their stemness properties have been lost. The myogenic potential after transplantation of cultured satellite cells is markedly limited compared to freshly isolated satellite cells. Trying to transplant myoblasts resulting from growing satellite cells *in vitro* also results in marked reduction in their ability to self renew and to replenish satellite stem cell pool (9,10). All the previous studies observed a rapid loss of satellite cell phenotype upon *in vitro* cultivation.

So, the need to optimize conditions which can allow satellite cells to grow and at the same time conserve their stem cell properties is a critical goal for muscle stem cell research work. Pax7 gene is a universal marker of satellite cells (29). A transgenic mouse model generated by recombineering a Pax7 BAC to put the gene for the fluorescent protein ZsGreen into the first coding exon of Pax7 was generated by my laboratory (31). This Pax7-ZsGreen model allows the isolation of cells expressing Pax7 by fluorescence-activated cell sorting (FACS). When we attempt to grow cells isolated on the basis of the Pax7 ZsGreen, they proliferate very much but when transplanted they lose their ability to contribute to the satellite cell pool of the muscle. Meanwhile, they also lose their ZsGreen signal (Figure.1). It is interesting that they lose their ZsGreen signal in parallel with losing their transplantation efficiency. Based on this observation, I used the level of ZsGreen in the cultured cells as readout, growing cells *in vitro* for 4 days and analyzing for the level of ZsGreen by FACS on day 4. This was done in the context of testing different small molecules and cytokines that affect different signaling pathways of the cell. The experiment begins by isolation of satellite cells from transgenic Pax7-ZsGreen mice, then growing them in media containing the molecules, and analyzing by FACS, aiming to discover a molecule that can allow the cells to grow and proliferate while conserving their ZsGreen signal. Those cells are planned to be transplanted into MDX mice (X chromosome linked muscle dystrophy) and tested for their ability to differentiate into dystrophin+ myofibers and Pax7-ZsGreen+ satellite cells. This was the first stage of my project which was not associated with significantly positive results in terms of preservation of ZsGreen in ex vivo cultured satellite cells.

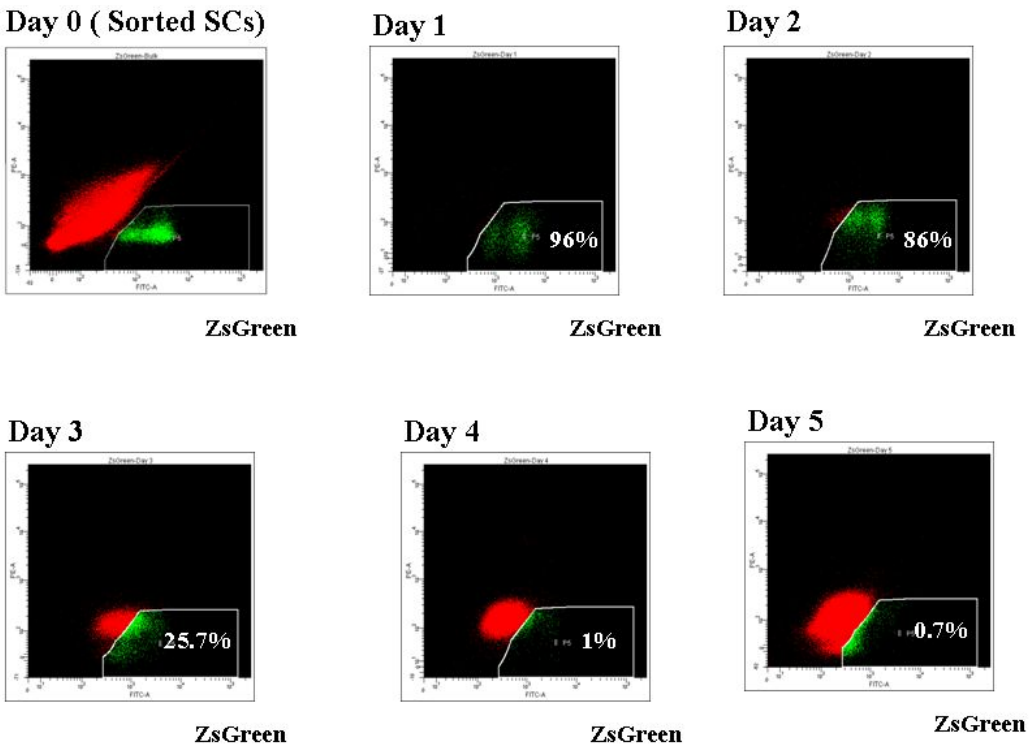


Figure (1). Decline in the level of ZsGreen fluorescence in cultured Pax7-ZsGreen satellite cells. The figure is showing FACS analysis for ZsGreen signal in day 0 (the day of ZsGreen cells from the total mononuclear cell population of the muscle), day 1,2,3,4 and 5 of stay in 20% FBS medium *in vitro*.

The idea of using ZsGreen as readout to monitor satellite cell differentiation status was partly based on the assumption that cells losing ZsGreen are also losing Pax7 expression. I hypothesized that losing Pax7 expression is cause of the decline in transplantation efficiency of cultured vs. fresh satellite cells that express Pax7. So, in the next part of my project, I tried to assess transcriptional profile of myogenic regulatory factors (MRFs), specifically Pax7. I used immunofluorescence for MRFs coupled with FACS analysis for ZsGreen. I found that Pax7 expression is preserved in cells that have lost ZsGreen expression. So, Losing Pax7 expression in proliferating myoblasts was not the cause of the decline in their engraftment ability according to my last hypothesis. I therefore moved forward to use another metric of assessment. Matrigel coating of the wells results in a high level of proliferation of satellite cells but it is also associated with rapid differentiation in the form of transformation of satellite cells into elongated

committed myocytes that are morphologically distinct from the rounded shape of satellite cells *in vitro*. I assessed molecules and chemicals for their ability to preserve the rounded shape of the cells on matrigel, an easy and practical way of screening molecules that can prevent satellite cell differentiation.

The transcriptional state of satellite cells and that of myoblasts plays a major role in explaining the differences in function of each cell type. So far, there is no single pure marker for quiescent satellite cells versus activated myoblasts. Comparing the expression profile of satellite cells and myoblasts shows that both cell populations express Pax7 the major factor that is supposed to be satellite cell specific and to be lost upon differentiation. This is true but for this to occur satellite cells can still make several rounds of proliferation as myoblasts during which they are do no efficiently engraft, although they still express Pax7. A more time selective factor is necessary for distinction between SCs and differentiated myoblasts. Time is an essential factor in satellite cell function, that is to say, their function and characteristics are not the same over time after being harvested. For my project, aiming at improvement of the transplantation efficiency of cultured myoblasts, the very early period of satellite cell culture shortly after being harvested from the body of the mouse is the most critical during which cell stemness properties and the ability to engraft is gradually lost. For assessment of this period, I thought that MyoD expression would be suitable. MyoD protein expression is one distinct factor that can discriminate between quiescent satellite cells that are known to be MyoD negative (48) and proliferating myoblasts that are MyoD positive, from as early as few hours following harvesting satellite cells (43). So, MyoD expression and the effect of different molecules and chemicals on it was a second parameter that I thought suitable for the project. MyoD is as an important factor of myogenic commitment (43). To be noted that MyoD m-RNA has been detected in a proportion of around 25% of freshly harvested satellite cells (11) but, due to post-transcriptional regulation processes, the translation of MyoD protein is prevented in quiescent cells. In my assessment, I am searching for MyoD protein by immunofluorescence.

Assessment of matrigel-induced morphological differentiation and MyoD expression was a clear and practical approach to judge the effect of a given compound on satellite

cell differentiation. Soon after the first few experiments using this approach, favorable results were obtained with the compound SR1 (stemregenin-1) SR1 has been previously described to be able to promote expansion of CD34 positive haematopoietic stem cells through blockage of the aryl hydrocarbon receptor (44). In my assay, SR1 succeeded to prevent matrigel induced differentiation of satellite cells and prevented MyoD protein expression in cultured satellite cells. However, these results were associated with a marked negative effect on the proliferative capacity of the cells. It will be discussed that I adopted a low level of serum in my assays of SR1 because serum inhibited the activity of SR1. So, the adopted low serum levels were having an additional negative effect on the level of cell proliferation. It has been previously described that serum constituents have a negative effect on the ligand stimulation of halogenated aromatic compounds to aryl hydrocarbon receptor (45). SR1 is a phenolic aromatic compound which suggests that it has the same properties. A period of trouble shooting followed in a trial to stimulate satellite cell proliferation in the presence of SR1 by different molecules that have been described to stimulate satellite cell proliferation. But, these trials were widely unsuccessful. So, a new approach was followed trying to see if the compound has an effect on established myoblasts that have already activated MyoD. These trials were successful and the compound proved to be active against cultured myoblasts that have been passaged several times and have been in culture for several weeks. SR1 succeeded to turn the treated myoblasts into MyoD-negative phenotype. SR1 treated cells were characterized by QRT-PCR to find different levels of expression of myogenic regulatory factors, and finally we transplanted satellite cells cultured for 5 days, either exposed to SR1 or not. These results will be discussed in detail in the results section.

SR1 is described as an antagonist of AHR (aryl hydrocarbon receptor) (44). Aryl hydrocarbon receptor a member of the basic helix loop helix and PAS domains (50) is a cytoplasmic receptor that is upon ligand binding forms a ligand receptor complex and dissociates from heat shock proteins (51). The ligand-receptor complex translocates to the nucleus where it interacts with aryl hydrocarbon receptor nuclear translocator known as ARNT. The tripartite complex then interacts with the XRE (xenobiotic response element) in responsive genes and activates transcription (51) (Figure .2). The receptor has been most commonly described to be stimulated by halogenated or polycyclic aromatic

hydrocarbons (HAH or PAH) of which the most famous is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and its related toxicity. Stimulation of aryl hydrocarbon receptor is associated with induction of P450 enzyme system (52) which has been used as the appropriate assay system for the receptor activity. Knock out experiments of AHR in mice have shown multiple defects in the new born mice particularly in liver development (53). Several defects also appeared upon follow up of the adult mice that lack the receptor (53). But, no defects in muscle development have been described in the new born knock out models. The defective regeneration of the injured muscle of the adult has not been investigated. I hypothesized that SR1 effect on satellite cells is due to an interaction with AHR. If interfering with AHR is having an effect on satellite cell proliferation and differentiation, then its expression is thought to have a distinctive pattern in satellite cells and differentiated myoblasts. Aryl hydrocarbon receptor was tracked by immunofluorescence in different stages of satellite cells, proliferating myoblasts and fully differentiated myotubes. I will try to describe the possible role that Aryl hydrocarbon receptor may serve in myogenesis and the assumed relationship with the already described myogenic regulatory factors trying to prove any possible association or superior control relationship of aryl hydrocarbon receptor over any of these genes.

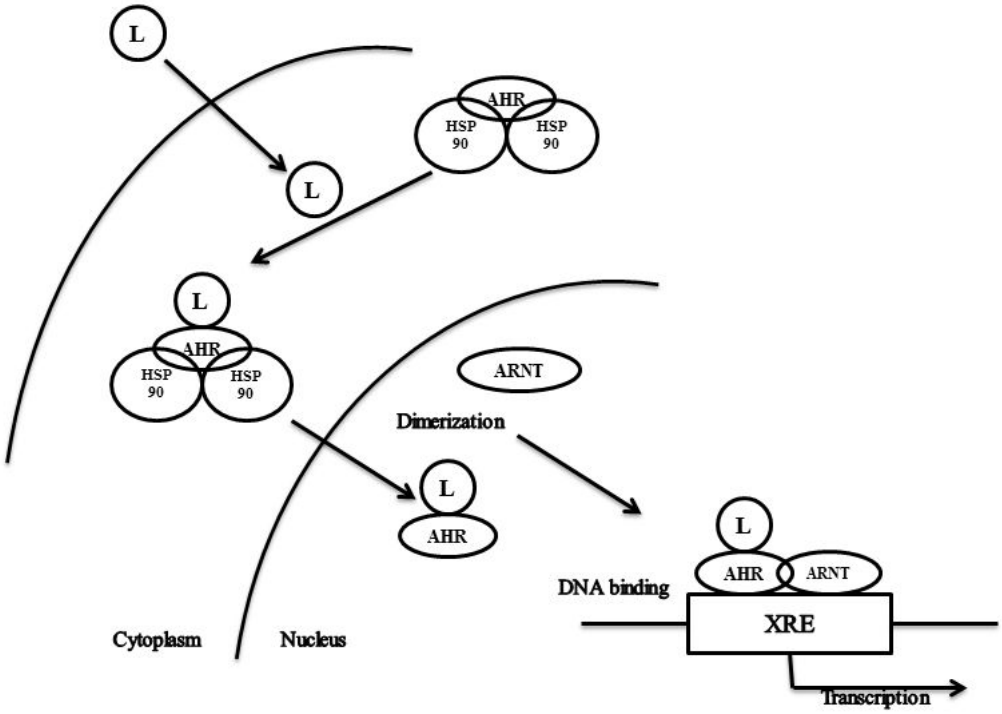


Figure (2). Mechanism of aryl hydrocarbon receptor induced stimulation of target gene expression. The figure is showing how binding of the ligand (L) leads to dissociation of heat shock proteins (HSP). This is followed by translocation of the receptor-ligand complex to the nucleus. In the nucleus, dimerization occurs with aryl hydrocarbon receptor nuclear translocator (ARNT). Then receptor-ligand-ARNT complex binds to the xenobiotic response element (XRE) of target genes to stimulate transcription.

The supplementation of culture media with different types of sera is an essential step for growing different cell types. Serum components are important for attachment after plating, as well as cell proliferation. Serum contains many growth factors and chemical substances that exert multiple actions on cells, but these growth factors and chemicals are widely undefined. This may not be very important when the aim is simply to grow cells. But when trying to assay activity of a certain compound on cells, it may be a problem if, as we have found, some component of serum interacts with the molecules you are

screening. These factors may be synergistically acting with the screened compound or they may have an antagonistic effect on it. In case you have a positive result, reproducing it may be a problem, especially in another lab, because not only are the constituents of serum are ill defined but different lots of serum may be different in composition. Being extracted from animal origin you do not know if this animal has been exposed to the same environment which will be reflected on the composition of the serum of that animal. This will probably have an effect on your experiment. So, depending on these fore mentioned causes and because I am trying to find a factor which may have a positive effect in terms of preventing or delaying satellite cell differentiation, finding a serum free medium (if possible) or low serum medium was one of my goals. So, I performed several experiments trying to find a serum free media condition or a low serum media which can still allow satellite cells to grow and proliferate. It was very hard for satellite cells to accommodate a serum free medium. Finally I adopted the lowest serum level that would allow the cells to proliferate. Together with using matrigel coating of my wells, 0.5% FBS serum succeeded to allow satellite cell proliferation in a b-FGF containing medium and at the same time clearly showed the effect of different molecules that can be very easily masked by the high serum level.

Materials and methods

Mice

Transgenic Pax7-ZsGreen/Bl6 mice were used to isolate stem cells of skeletal muscle by flow-cytometry (31).

Harvesting Satellite cells

All the hind limb muscles, all the fore limb muscles, the paravertebral muscles were harvested. Muscle fibers were dissected with razor and forceps. The chopped muscles were suspended in digestion solution I (0.2% collagenase II (Gibco 17101-015) in DMEM medium supplemented with 1% P/S.). The resulting suspension was incubated shaking for 75 minutes at 37°c. The muscle suspension was then washed by rinsing solution (10 ml F10 medium (Hyclone - SH3002501), 10% horse serum (Gibco, 26050-088), 1% hepes buffer solution (Gibco 15630-106), 1% P/S (Gibco-15140-122)) for a couple of times. Further mechanical digestion of the fibers was done through manipulation of the muscle suspension by snapped glass Pasteur pipette. More washing of the suspension was done by rising solution before it was suspended in digestion solution II (7 ml of rinsing solution, 0.5 ml DS1, 1.25 ml of 0.4% dispase (Gibco 17105-041) in rinsing solution) and was incubated shacking for another 30 minutes. After retrieval of the tube, last purification steps were done through filtration of the cells solution through a 40 Millipore cell strainers while more mechanical processing is done through 10 ml syringes. Lastly, Muscle cells were suspended in staining medium ready to be sorted by FACS machine (FACS analysis was performed on a FACSAria (BD Biosciences, San Diego, CA.).

Myoblast and satellite cell culture

Myogenic Medium (MM), Composed of Ham's nutrient mixture F10 medium (Hyclone - SH3002501) supplemented with 20% FBS, 100x P/S (Gibco-15140-122), 100x glutamax (Gibco-35050061), β-mercapto ethanol (Sigma-M3148) 4 µl per 500 ml media, hbFGF (peprotech 100-18B) 10 ng/ml. The 20% FBS concentration were used for assessment of the effect of different chemicals on ZsGreen expression on cultured satellite cells and for immunostaining of cultured satellite cells for the expression of myogenic regulatory

factors. The lowest tolerable serum content by satellite cells were assessed, as well as testing other types of serum free media (Mesenchymal stem cell medium/Gibco-A10334-01 , Stempro-34 medium/Gibco- 10639-011 , Essential-6 medium/Gibco- A1516401, Neurobasal medium/ Gibco- 21103-049) ,Neurobasal medium was supplemented by 2%N2 (Gibco- 17502-048) & 2%B27 (Gibco- 17504-044) supplements. All the serum free media were supplemented with P/S, glutamax, β-ME in the same concentrations. For differentiation assays, I used differentiation medium composed of 2% Horse serum in DMEM medium with 100X p/s, 100X ITS (Gibco, 51300-044). Duration of differentiation is 12 days unless otherwise indicated with media changed once after 3 to 4 days. 0.5% FBS serum concentration was used for experiment concerned with assessment of the effect of different chemical on MyoD expression. The plates were coated with 0.1% gelatin (Sigma G1890-100G) in distilled water, geltrix (Gibco A15696-01) or non-coated depending on the experiment type. Duration of the cell culture was variable depending on the experiment as indicated in the results section. For harvesting the cells on the day of the analysis, trypsin 0.25% (Gibco 15050-057) was used by incubating the cells for 3 minutes at 37ºc and neutralized with an equal amount of 20% FBS medium. For retrieving the cells centrifugation at 1200 rpm for 3 minutes was done. All the experiments were done in low oxygen incubator 5% oxygen, 5% CO2 at 37 °c. The used serum types: Fetal Bovine serum (FBS) (Hyclone), Horse serum (Gibco, 26050-088), Charcoal stripped serum (Life technologies, 12676011)

Immunofluorescence

For fixation, 20 minute incubation with 4% PFA at room temperature was used. Blocking and permealizing for 1 hour using 0.3% triton x in 3% BSA in PBS was done. I incubated my cells with primary antibody (suspended in the blocking solution) overnight at 4°c. I incubated with the secondary antibody for 45 minutes at room temperature. DAPI mounting was used to counterstain the nuclei for 20 minutes. The Primary antibodies used are MHC (mouse antibody MF 20S, 1:20), Myogenin (mouse antibody F5D-S, 1:50), MyoD (mouse antibody, BDpharmigen, 1:400), Pax7 (mouse antibody from hybridoma labs, 1:10 or :20), Myf5 (mouse antibody from santa cruz biotechnology, 1:250), Pax3(mouse monoclonal antibody, R&D systems, 1:200), AHR (mouse and

rabbit antibodies from thermiscientific 1:200), laminin (mouse antibody, from abcam 1:500).And the secondary antibodies are Appropriate secondary antibodies were used at a concentration of 1:750 or 1:500. Alexa Fluor® 555 Goat Anti-Mouse IgG (H+L), life technologies- A-21422, Alexa Fluor® 555 Goat Anti-Rabbit IgG (H+L), life technologies- A-21428. DAPI from Santa Cruz Biotechnology was used to counterstain the nuclei. The microscopy facility of the histology core of the AHC has been used for taking the images (Carl-zeis inverted microscope).

QRT-PCR

RNeasy kit (Qiagen- 74004) was used for RNA extraction. 350 µl of RLT buffer were used for cell lysis per well of a 6-well plate of grown myoblasts. One volume of 70% ethanol was then added before the 700 µl were then transferred to minispin column. Centrifugation was done to get the RNA of the lysed cells on the spin column membrane. This was followed by using RW1 buffer and RPE buffer for washing the membrane bound RNA. Membrane bound RNA was then eluted to a collection tube using RNAse free water. The needed amount of RNA to make c DNA was calculated using nanodrop. Verso-kit (Thermoscientific-AB-1453/A) was used for making cDNA. 11 µl of RNA diluted with RNase free water was added to 9 µl of verso enzyme kit master mix. The reaction solution was then allowed to start making c DNA by incubation for 60 minutes at 42º. The resulting 20 µl of c DNA was then diluted with water. Relative levels of RNA of different myogenic regulatory factors were detected through mixing the c DNA with probes designed to detect different factors. The relative levels were normalized against GAPDH enzyme RNA levels. The probes are from Applied biosystems of life technologies.

Muscle injury and transplantation (done by Dr. Robert Arpke, Post doctoral associate at Kyba lab). (figure 41).

4-monthold NSG-mdx4Cv mice were used. 2 days before transplantation both hindlimbs of mice were irradiated(using an X-RAD 320 Biological Irradiator, Precision X-Ray, Inc., Branford, CT, http://www.pxinc.com) to ablate any satellite cells of the recipient mouse. 24 hours before transplantation 15 µl cardiotoxin (10 lM, Calbiochem, Philadelphia, PA,

http://www.emdbiosciences.com) were injected into TA muscles (Tibialis anterior muscle) of the irradiated limbs. Cardiotoxin causes injury to the muscle stimulating the regenerative system of the recipient muscle and makes it ready to receive the transplanted cells. 3000 cells were transplanted per TA. 4 weeks later both TA muscles of every mouse were harvested and subject to testing. One TA were frozen, sectioned and stained for dystrophin and laminin to detect the percentage of dystrophin positive fibers. Mononuclear cells were harvested from the other TA and were subject to FACS analysis for ZsGreen positive cells. Two groups of animals were used each consisted of six animals.

Histology and immunofluorescence for analysis of fiber engraftment data of the transplantation experiment. (Sectioning of the frozen muscles was done by Dr. Robert Arpke, Post doctoral associate at Kyba lab).

The harvested muscles were immediately frozen by placing in OCT using liquid nitrogen-cooled 2-methylbutane (Sigma-Aldrich, St Louis, MO, http://www.sigmaaldrich.com). Muscle sections of the frozen muscles were made, fixed with acetone and stained for laminin (Sigma Aldrich) and dystrophin (Abcam). I blocked with 3% BSA in PBS for 1 hour. This was followed by incubation with primary antibodies for 1 hour at RT. Then the sections were incubated with the appropriate secondary antibodies for 45 minutes (as previously described) at RT, mounted with DAPI and imaged using carl-Zeis upright microscope of the AHC facility. And (a) The number of donor (dystrophin positive fibers) muscle fibers and total muscle fibers (laminin positive) were quantified in crosssections from the transplanted TA. (b)The counting process was done using the counting tool of Photoshop program.

FACS analysis of the TA muscles for satellite cell engraftment.

The mononuclear cell population of each TA muscle was harvested using the same protocol used for harvesting the bulk of muscles with some modifications for the change of the volumes. The harvested cells were then analyzed to detect the total myogenic population of the muscle by finding V-cam (antibody from BDbiosciences, concentration

0.5 µL/million cells) & alpha7 integrin (antibody from BDbiosciences, concentration 1 µL/million cells) double positive population. This population is further analyzed for ZsGreen to find the definite donor derived cells, then: (a)The number of ZsGreen cells (by FACS) got from each T.A. sample was recorded and plotted in the graph shown in the results section.(b)The total number of ZsGreen cells of cells treated versus untreated cells with SR1 was counted and shown in the results.(c)The total number of Vcam&alpha7 integrin double positive population (by FACS) was also shown in the results and the proportion of ZsGreen cells of them was also shown.

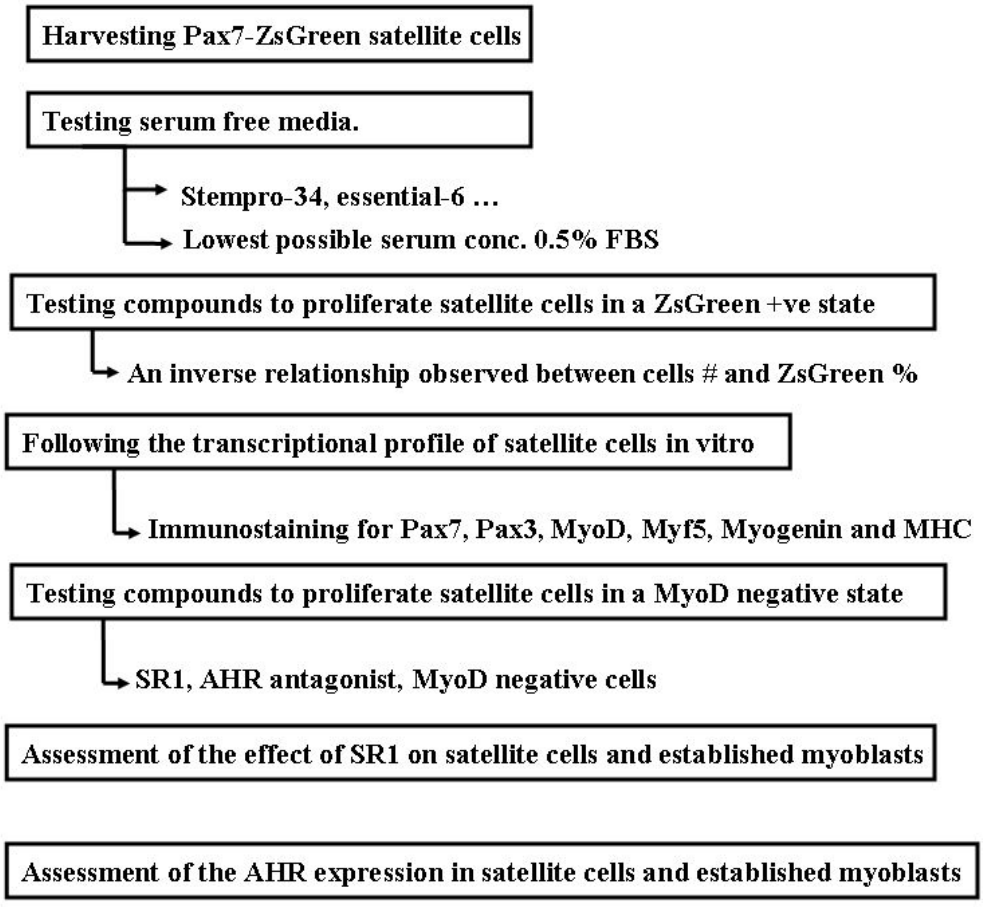
Compounds tested

Compound	Company
Stemregenin 1	Cellagen technology (C7710-1s).
Hh-Ag-1.5	Cellagen technology.
IWR1	Sigma Aldrich (I0161-5MG).
CHIR99021	Tocris Bioscience (4423).
IWP2	Cayman Chemical(13951).
A-83-01	Cellagen technology (C2831-2s).
SU5402	Cellagen technology (C7854-1s).
SAHA	Cayman Chemical (10009929).
BMP4	Peprotech.
PD098059	Promega (V1191).
LIF	Millipore

Table 1. Names of different compounds tested in the project and the companies of purchase.

Results

Here, I will show the milestones of the results section of the thesis. These are the main points that will be covered, the points that represented important stations in the flow of the work.



Harvesting Pax7-ZsGreen satellite cells

The total mononuclear cell population of the muscle is harvested from mice by mechanical and enzymatic digestion of muscles. Then, Pax7-ZsGreen fluorescent cells are isolated by FACS sorting based on the gates explained in figure (3). The sorted cells are then cultured with different compounds acting on different pathways of the cells. The gates used for isolation of the cells are the same gates used in the analysis on day 4. These gates are also the ones used in ZsGreen FACS analysis in all the remaining experiments shown in this thesis.

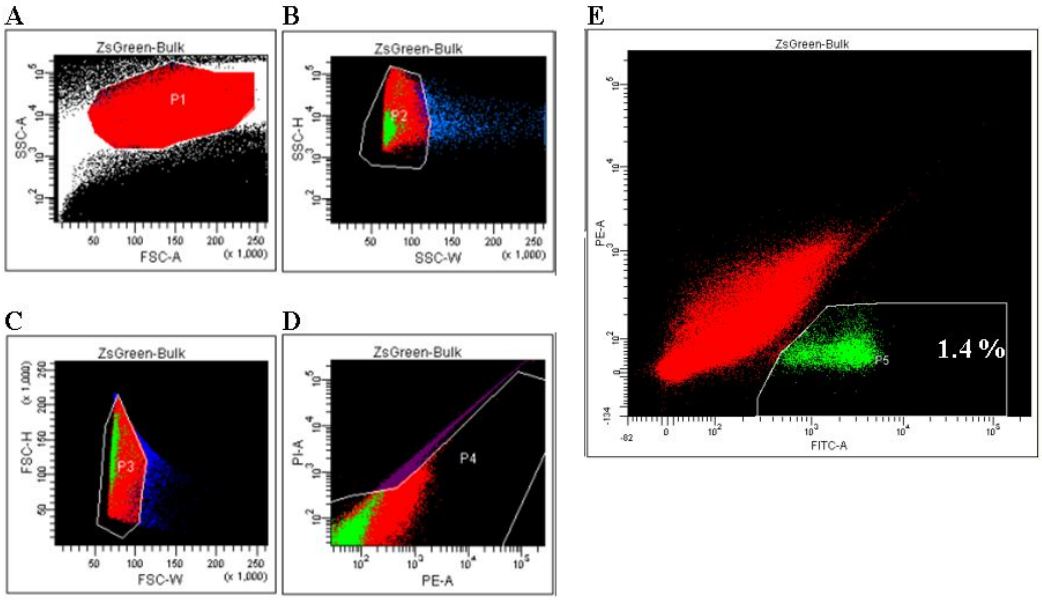


Figure (3). Fluorescent activated cell sorting of Pax7-ZsGreen satellite cells.

(A,B,C) Forward scatter and side scatter of the total cells.

(D) Fluorescence of live cells in the FSC/SSC gates gated using propidium iodide.

(E) Sorting of ZsGreen positive cells. Uncompensated green fluorescence is shown on the x axis.

Serum free media assay

I started this assay by testing a number of serum free media (Neurobasal medium, Essential-6 medium, Stempro-34 medium, Stempro-MSC, F-10 medium) (Figure 3,4). I tried to grow freshly isolated satellite cells using the mentioned media types but the cells did not grow. A big lag was present in the growth rate of the cells using serum free media in comparison with the control myogenic media (MM, 20% FBS f10 media plus hbFGF) (Figure 5). Essential-6 medium supplemented by hbFGF was accompanied by an acceptable growth rate but the result did not reproduce.

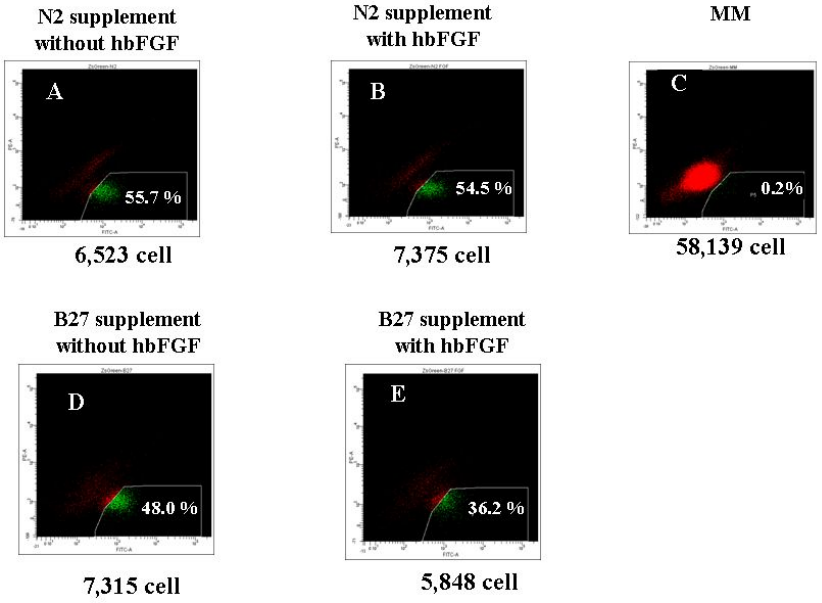


Figure (4). Assessment of Neurobasal medium. With the total number of cells obtained and the percentage of ZsGreen positive cells indicated in each corresponding figure. Control myogenic medium is shown in (C). (n=3, presented data are from one experiment)

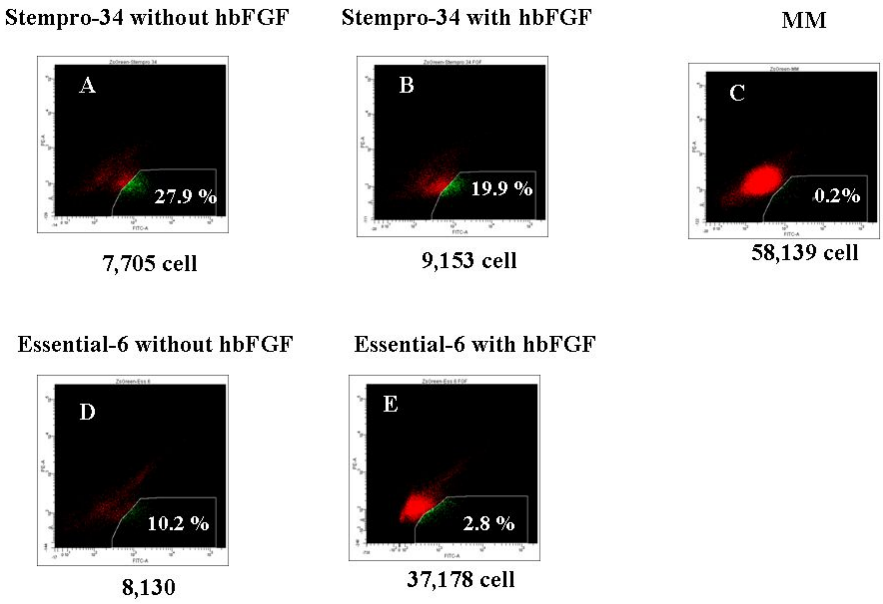


Figure (5). Assessment of stempro-34 and essential-6 medium with the total number of cells obtained and the percentage of ZsGreen positive cells indicated in each corresponding figure. Control is shown in (C). (n=3, presented data are from one experiment)

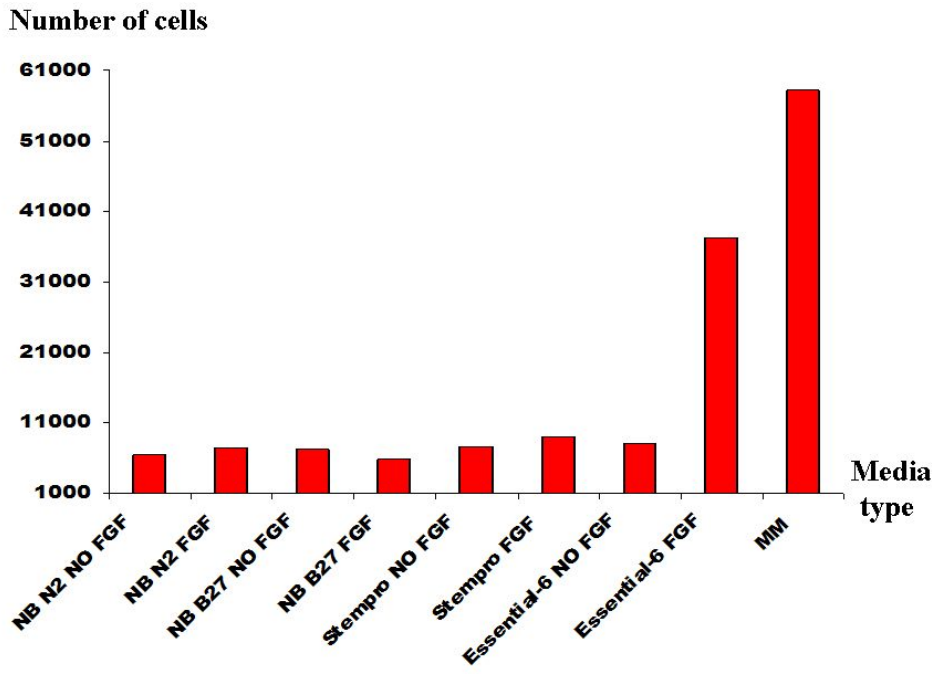


Figure (6). Number of cells after 4 days in different types of serum free media. NB (neurobasal medium), N2, B27 (supplements), NO FGF (without hbFGF), MM (myogenic medium, 20% FBS f10 medium plus hbFGF). (n=3, presented data are from one experiment)

Next I tried to stimulate cell proliferation. Different media types was supplemented by a number of cytokines that are supposed to stimulate their proliferation, namely, BMP4 in a concentration of 20 ng/ml, LIF in a concentration of 1000 unit/ml, IL-6 in a concentration of 10 ng/ml in addition to b-FGF (Figure 46). The cell proliferation increased under the effect of these factors to an acceptable level with Stempro-34 medium and Essential-6 medium although it was still lower than the control medium containing serum. But the problem is that these results could not be reproduced later on. The cells failed to achieve a good level of growth and proliferation in the mentioned media types even under the stimulatory effect of the cytokines.

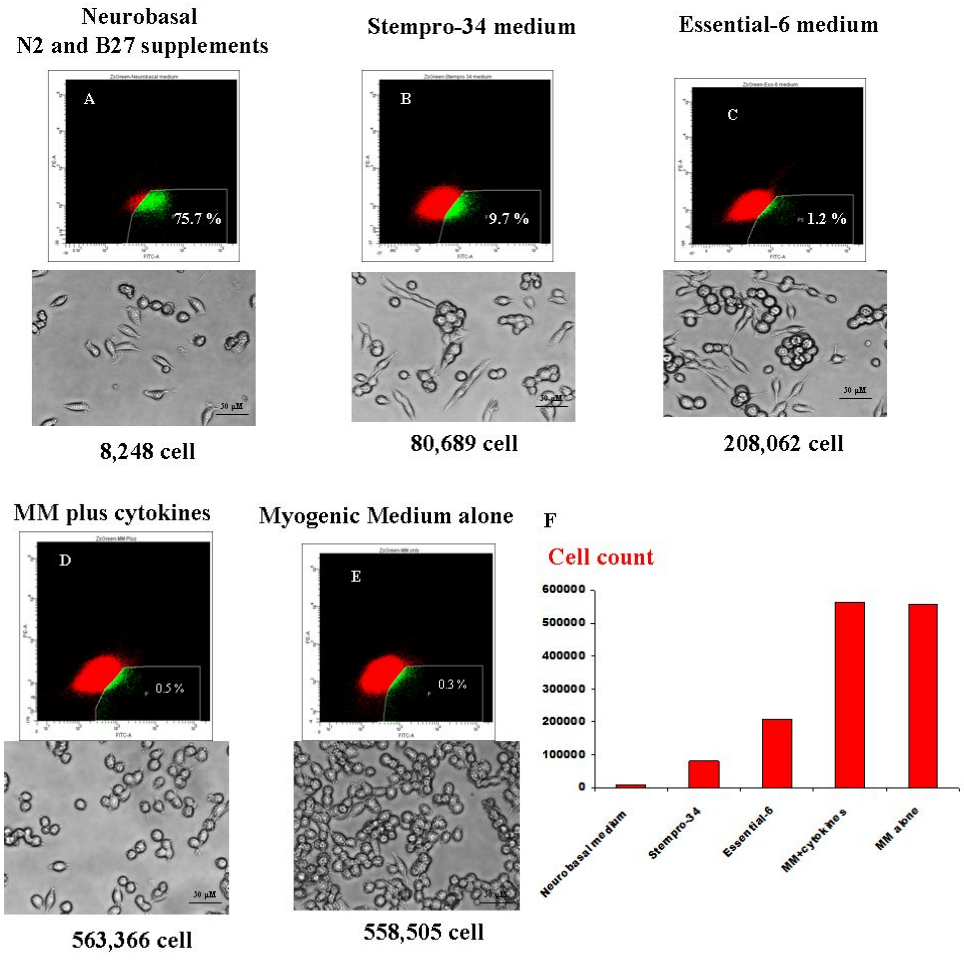


Figure (7). Assessment of serum free media with cytokines. The total number of the obtained cells, percentage of the ZsGreen positive cells and phase contrast images of the cells are shown in each corresponding figure scale bar 50 µm. (F) Graphical presentation of the number of cells with each media type.(n=1).

Satellite cell cultivation in the absence of serum was broadly accompanied by poor growth of the cells and low growth rates. So, next I attempted searching the lowest serum level that can allow satellite cells to grow. The cells succeeded to grow in a serum level of 2.5% FBS F10 medium (Figure 7). This serum concentration was supplemented with the fore mentioned growth factors (FGF, IL6, BMP4 and LIF). Of these factors, hbFGF was the most important while other factors appeared to be dispensable. The role of FGF as an important stimulant to satellite cell growth has been thoroughly investigated in previous literature since a long time (61). In my studies, I found that satellite cells can go without external supplementation of FGF to the media in the presence of high serum concentration on the short run (Figure 8 A&B). Satellite cells have a normal growth rate without FGF in the presence of high serum concentration. But, on the long run a marked drop in the rate of satellite cell growth *in vitro* without FGF (Figure 8 E). Without FGF, Satellite cells start to differentiate which means lack of satisfactory proliferation signals Figure (8 C&D). I think presence of hbFGF in the medium represents the artificial stimulus that forces the cells to proliferate *in vitro*. That is unlike the normal physiology of satellite cells in vivo in the form of a number of proliferation cycles followed by differentiation into new fibers.

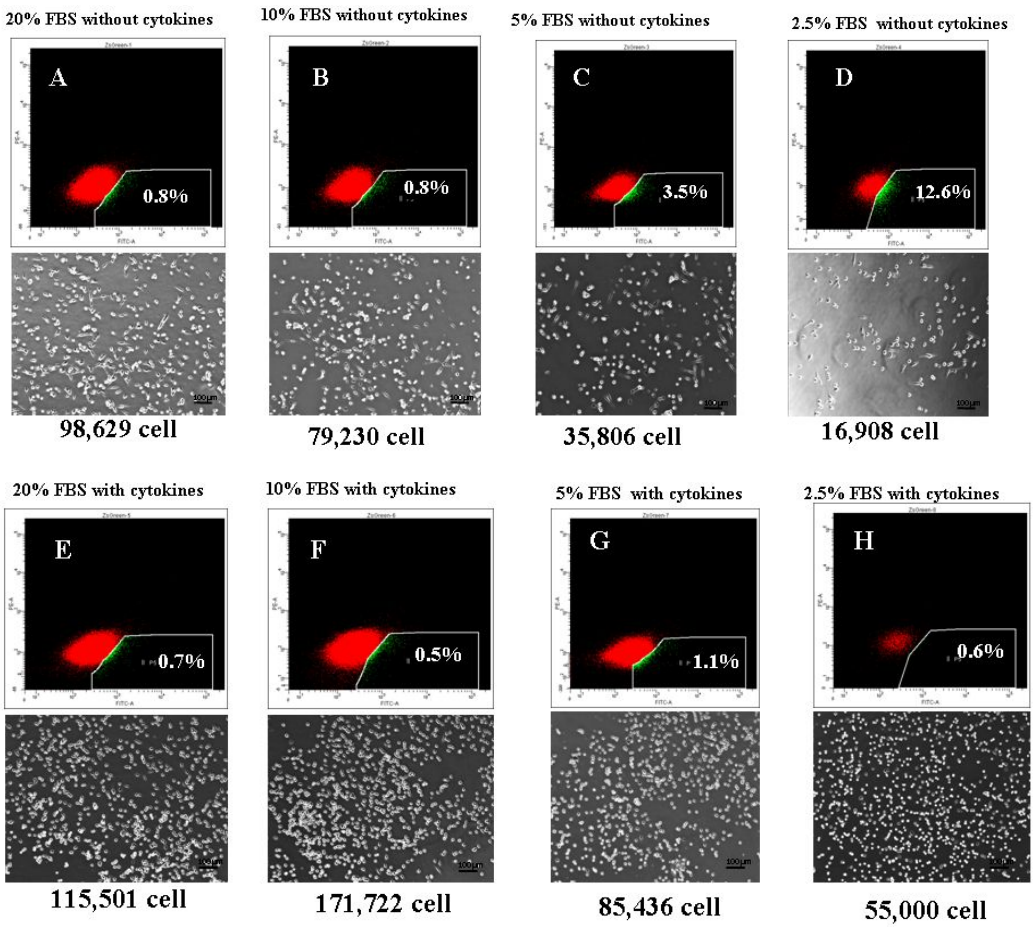


Figure (8) Assessment of the lowest applicable serum concentration needed to grow satellite cells. 20, 10, 5, 2.5% FBS in F10 medium, with and without 4 cytokines (BMP4 20 ng/ml, LIF 1000 U/ml, FGF 12.5 ng/ml, IL6 10 ng/ml). Scale bar 100 µM. (n=1).

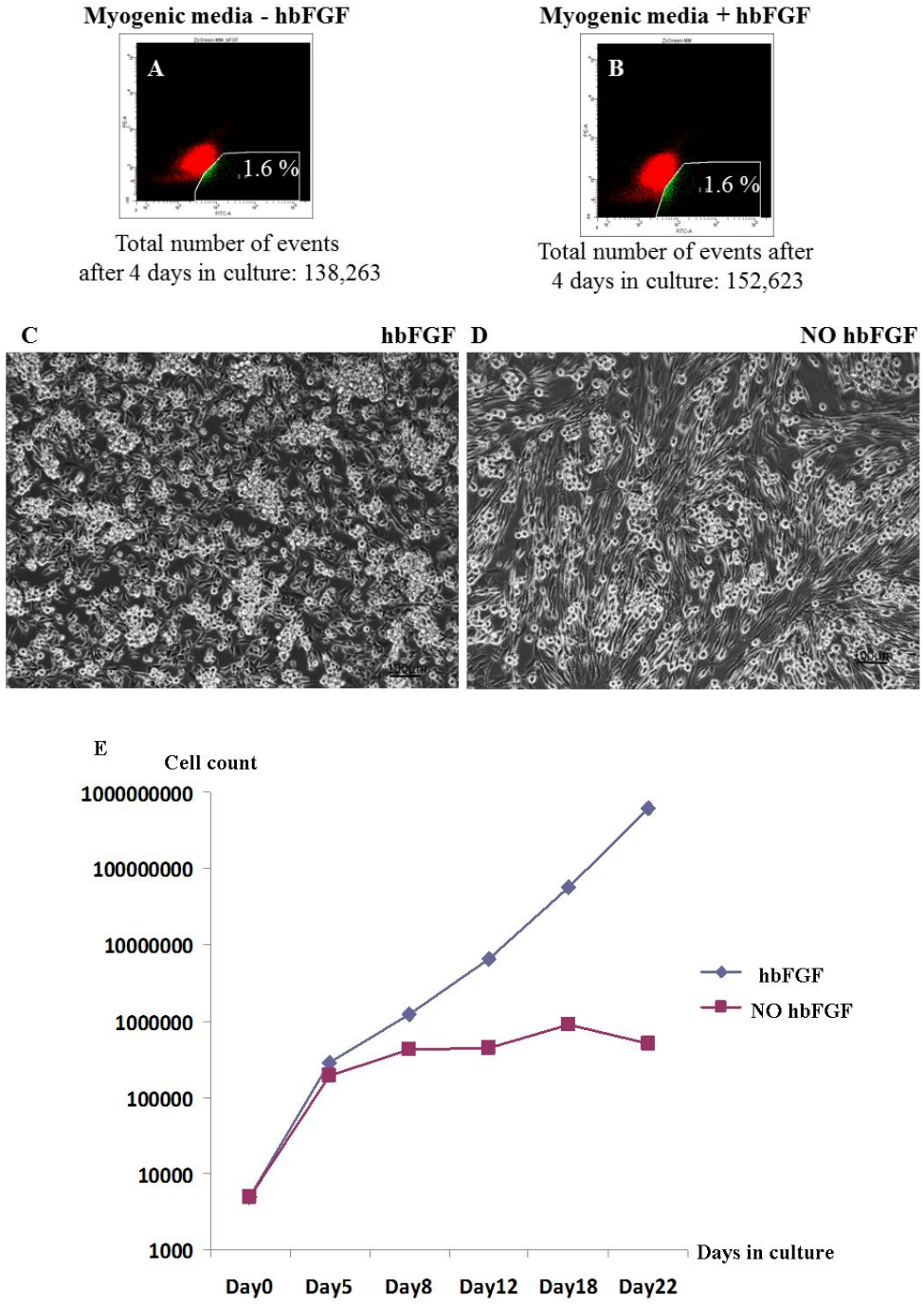


Figure (9). Effect of hbFGF on cultured satellite cells.

(A&) FACS analysis of satellite cells cultured with and with without hbFGF for 4 days. (C&D) Phase contrast images of satellite cells cultured with and without hbFGF for 11 days showing more differentiation in absence of FGF. Scale bar 100 µM. (E) Growth curve of the same cells shown in c&d after culture for 22 days showing drop in growth rate in absence of FGF coinciding with increasing differentiation. (n=1).

Matrigel coating of the wells has been described to support and stimulate satellite cell proliferation, though at the same time stimulating their differentiation (62). So, I tried to take advantage of this stimulatory effect. Growing the cells on matrigel coated wells, they survived and proliferated in a serum level down to 0.5% (Figure 9).

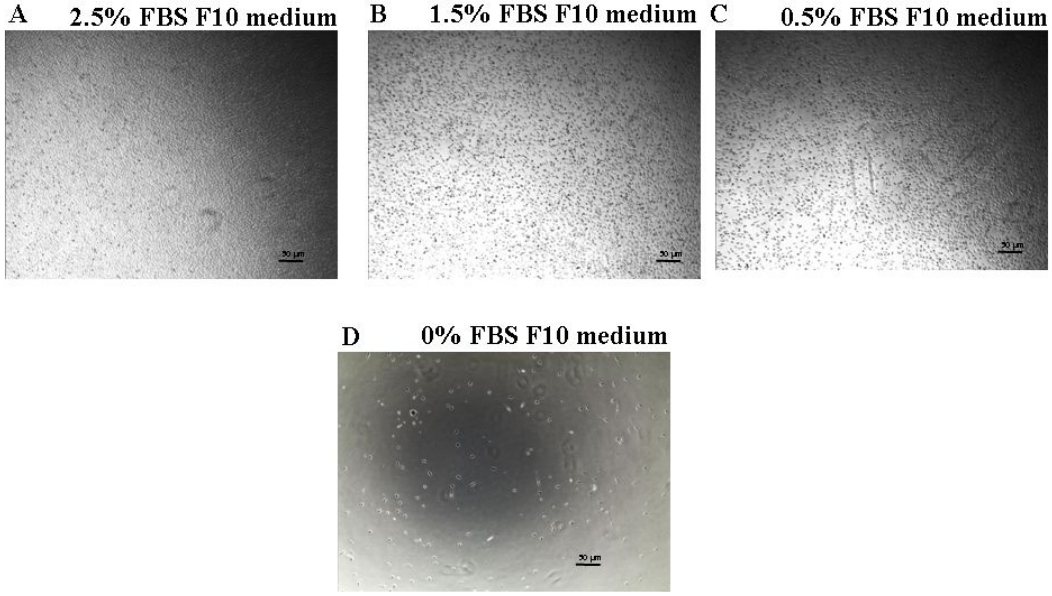
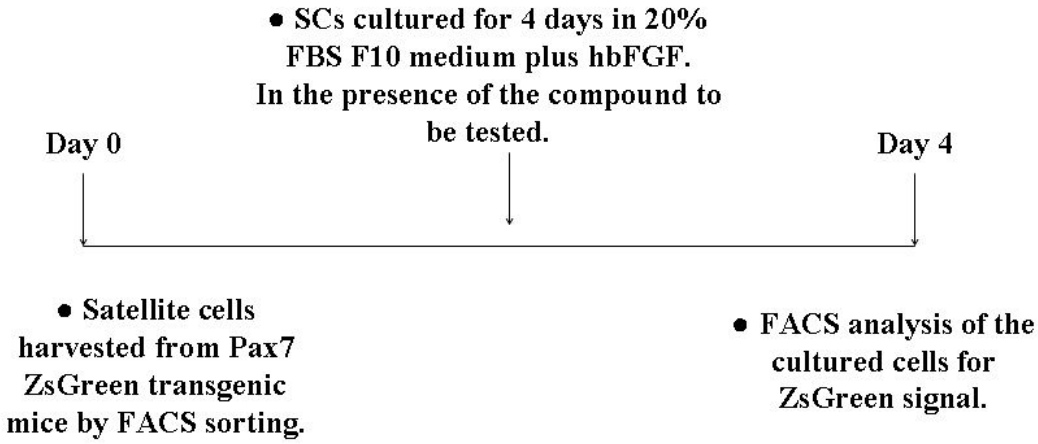


Figure (10). Testing the ability of Matrigel to support satellite cells proliferation in low serum media All conditions plated on Matrigel, with FGF, for 5 days. Scale bar 50 μM. (n=1).

So, in the next sections of this thesis the following media types and serum concentrations was used. For the second part concerned with using FACS ZsGreen in the assessment of satellite cell differentiation and also for the third part used concerned with immunostaining of cultured satellite cells for different myogenic regulatory types, the used media was myogenic medium (MM, composed of 20% FBS plus hbFGF. In the third part concerned with using MyoD expression as an assessment point, 0.5% FBS f10 medium with hbFGF was used on matrigel coated plates. All the assessment experiments of SR1 were done using a serum concentration of 0.5%. Staining experiments for AHR in different stages of satellite cell growth was done using a proliferating 20% FBS medium for proliferating cells and using a low serum medium of 2% horse serum for differentiating cells.

FACS analysis of 4-days cultured satellite cells with different compounds



The aim of this experiment is to find a compound that can allow transgenic Pax7-ZsGreen satellite cells to proliferate and at the same time preserve their ZsGreen signal. I am assuming two points (a)The cells having the ZsGreen signal means that they have Pax7 gene turned on and that losing ZsGreen means losing Pax7 expression. (b)Pax7 gene is the one responsible for stemness properties of satellite cells and that losing Pax7 (as evidenced by losing ZsGreen) is the cause of the inefficient transplantation of cultured satellite cells. That is because Pax7 gene expression is known to be down-regulated upon differentiation of satellite cells (43). So, FACS analysis ZsGreen positive cells are Pax7 positive cells that are assumed to be the most undifferentiated state of satellite cells capable of achieving successful transplantation accompanied by restoration of satellite cell pool in the recipient muscle.

I was unable to find a compound that can preserve *in vitro* cultured satellite cells in their ZsGreen positive state and at the same time allow them to proliferate. Preservation of ZsGreen signal of the cells occurred only when cell proliferation is inhibited. A reverse relationship exists between the number of cells and the percentage of ZsGreen (Figure 12). One example that shows this relationship is SAHA (a deacetylase inhibitor). As I increased the dose of SAHA satellite cell proliferation was inhibited. Inhibition of cell proliferation was associated with increased percentage of ZsGreen positive cells (figure 11).

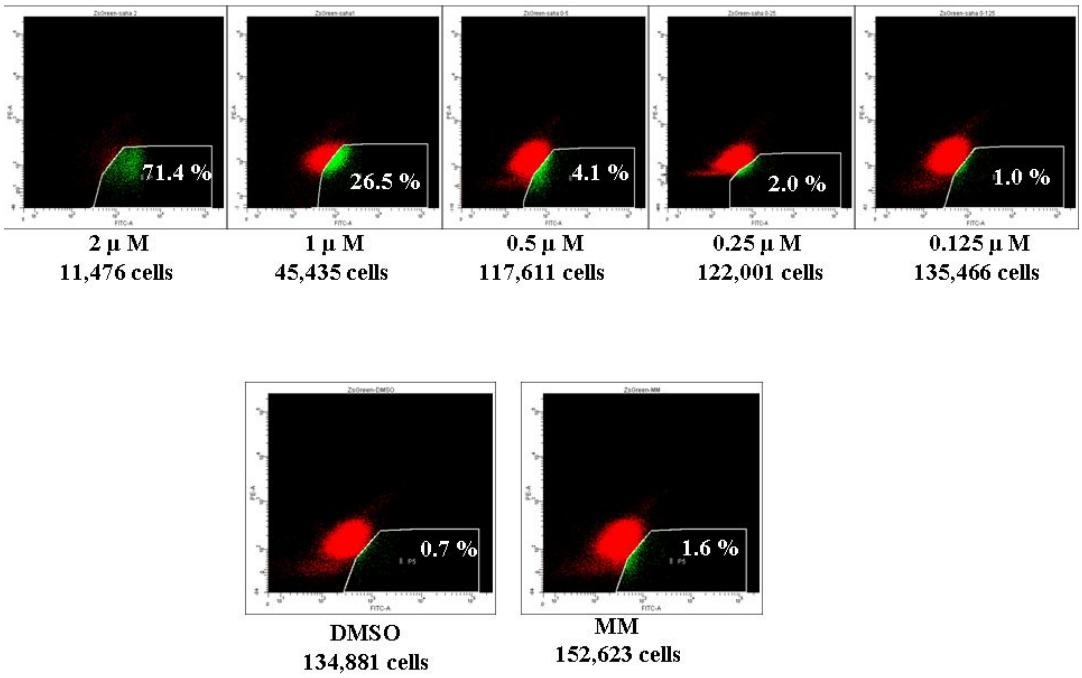


Figure (11). FACS analysis of satellite cells exposed to different doses of SAHA.

The percentage of ZsGreen positive satellite cells among live cell (refer to the used FACS analysis gates at figure 3)., the concentrations used and the total number of cells (Total number of events as shown in figure 3) are shown in each corresponding figure. Controls are shown at the bottom. (n=4, presented data are from one experiment)

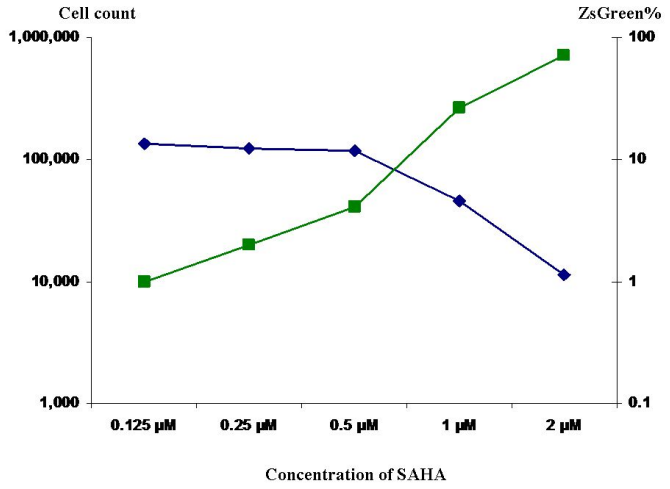
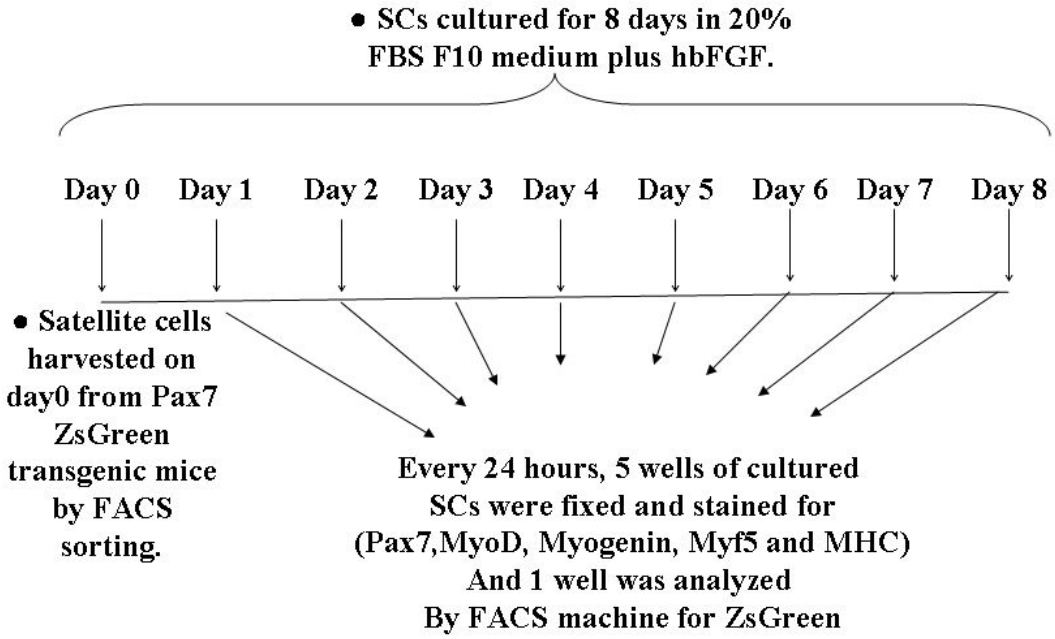


Figure (12). Graphical presentation of the relationship between the number of cells (blue), the percentage of ZsGreen positive cells (Green) and the used concentration of SAHA.

I also tested several other compounds in the same way, with similar results. Toxic doses that slowed proliferation allowed the cells to retain ZsGreen. But, this does not allow us to use any of them as a practical tool to proliferate satellite cells. So, we conclude that even if the percentage of ZsGreen cells seems to be high, still, this effect is not useful to achieve our goal of expanding ZsGreen positive cells. We should take into consideration that in each of these conditions (figure 11) the seeded number of cells at day 0 was more than 15000 cells. So, the net result is loss of ZsGreen positive cells.

Effect of *in vitro* culture on changing transcriptional profile of satellite cells



The aim of this experiment is to assess transcriptional profile of cultured satellite cells. Transcriptional changes of satellite cells have been previously described in literature, but different labs use different ways for harvesting the cells, different media types, growth factors and serum levels. All these variables would certainly affect cell behavior and necessitates individual characterization of the cells. In addition, I tried to specifically find the relationship between the level of ZsGreen signal and the transcriptional state of the cells i.e. what are the genes that have been turned on or off when my cells have lost their ZsGreen signal. The most important gene to be tracked in this experiment was Pax7 gene due to the dependence of ZsGreen signal on Pax7 promoter and my hypothesized relationship between losing ZsGreen signal *in vitro* and losing Pax7 expression.

	Immunostaining data					FACS analysis	
Day	Figure	Pax7	MyoD	Myogenin	MHC	ZsGreen	Number of cells
1	15	+++	+++	-	-	96%	2323
2	16	+++	+++	_	_	86%	3670
3	17	+++	+++	-	_	25.7%	13730
4	18	+++	+++	-	_	1%	69713
5	19	++	+++	+	+	0.7%	344544
6	20	++	+++	+	+	0.2%	648672
7	21	++	+++	+	+	0.5%	> 288000
8	22	++	+++	+	+	0.4%	> 700000
Established myoblasts.	23	++	++	+	+	Not analyzed	Not counted.

Table 2. Effect of *in vitro* cuture on Pax7-ZsGreen satellite cells. The table describes the data analysis of every individual day of the 8 day experiment (Number of plated cells is 5,000 cells on day 0).

Satellite cell activation starts immediately following starting the harvesting process. Around 25-30% of freshly isolated uncultured satellite cells have made MyoD protein within 4 to 5 hours after starting harvesting (figure 13). Harvesting satellite cells in the presence of cycloheximide (translation inhibitor) succeeded to block formation of MyoD protein and preserved the cells in a MyoD negative quiescent-like state (figure 13). 24 hours following the harvest, all the cells are MyoD positive (Figure 15). The majority of the cultured satellite cells remain Pax7 positive after one week in culture, although they become ZsGreen negative after only 4 days in culture. All the cells are Pax7/MyoD double positive for the first three days. Starting from day4 scattered MyoD negative and Pax7 negative cells start to appear. Yet, the majority of the cells remain positive for either factor over several passages. Early cultured Satellite cells are Myogenin/MHC double negative over the first 4 days in culture. Starting from day 5 scattered positive cells for Myogenin and MHC start to appear in the culture with the percentage of these cells maintained over several passages. Following several passages the level of Pax7 and MyoD expression declines in the cultured cells with a parallel rise in the level of Myogenin and MHC expression. A growth curve for the number of cells every day over

the first 6 days of a starting number of 5,000 cells was done (Figure.14). It shows that the greatest growth spike happened after 48 hours in culture. This was consistent with literature describing that harvested fiber associated satellite cells made their first division after 40 hours of their harvest and activation (45). This was also coinciding with loss of ZsGreen signal (Figure. 15)

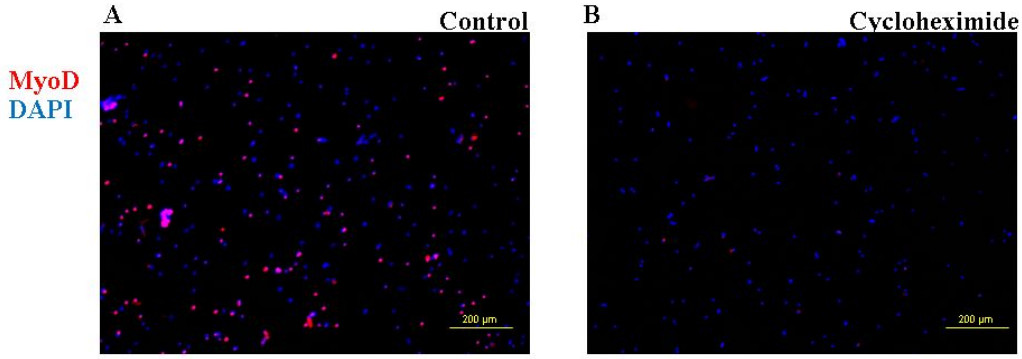


Figure (13). Immunostaining for MyoD protein in cytospin preparation of freshly isolated SCs harvested with and without cycloheximide. (A) Without cycloheximide (B) in the presence of 100 µgm cycloheximide. Scale bar 200 µm.

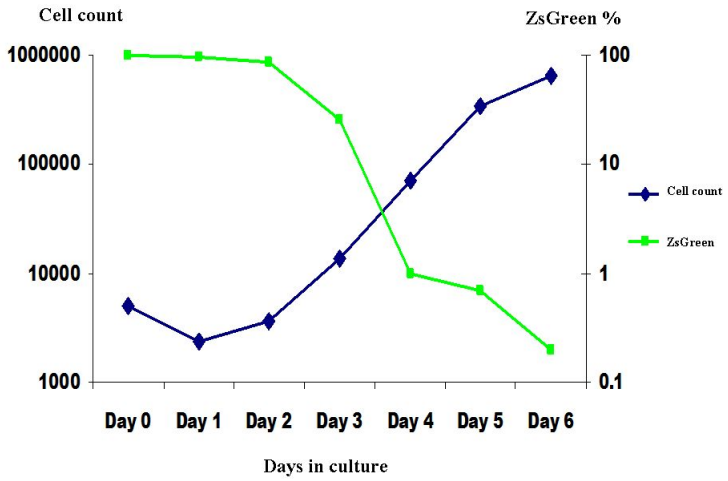


Figure (14). Relationship between satellite cell growth and ZsGreen. Graphical presentation showing the numbers of satellite cells by FACS analysis over 6 days in culture (Blue) and the change in the percentage of ZsGreen cells over the same days (green).

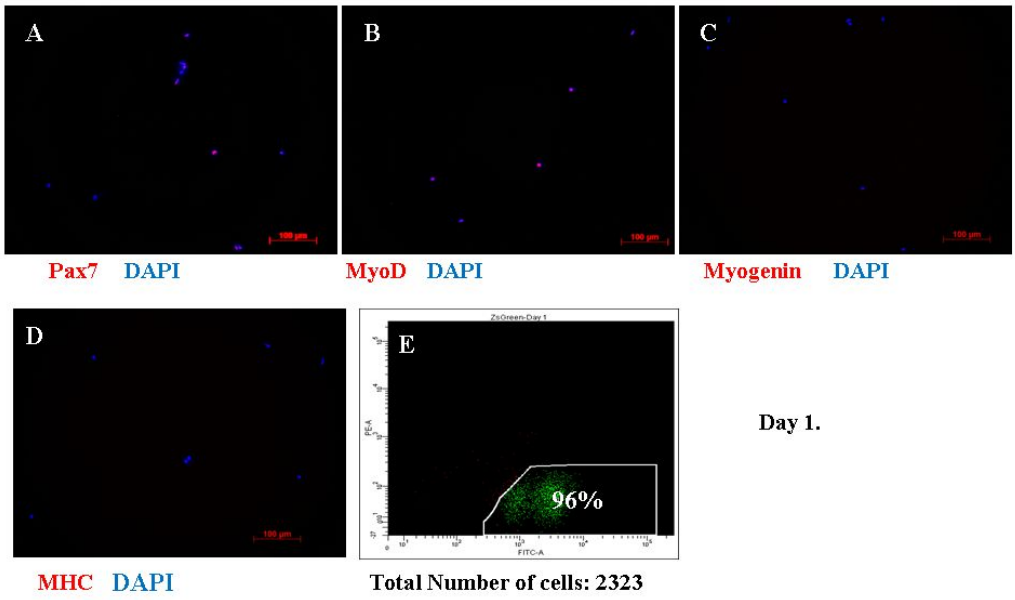


Figure (15). Day1-Immunofluorescence for (A) Pax7, (B) MyoD, (C) Myogenin and (D) MHC in red. DAPI (blue) was used for counterstaining the nuclei. Scale bar 100 µM. (E) FACS analysis for the percentage of ZsGreen positive satellite cells.

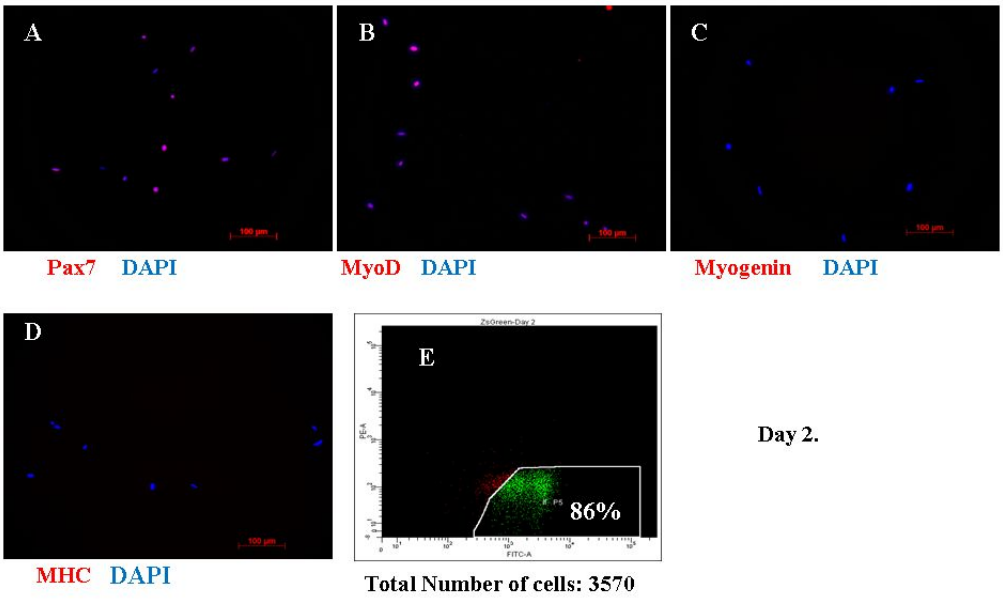


Figure (16). Day2-Immunofluorescence for (A) Pax7, (B) MyoD, (C) Myogenin and (D) MHC in red. DAPI (blue) was used for counterstaining the nuclei. Scale bar 100 µM. (E) FACS analysis for the percentage of ZsGreen positive satellite cells.

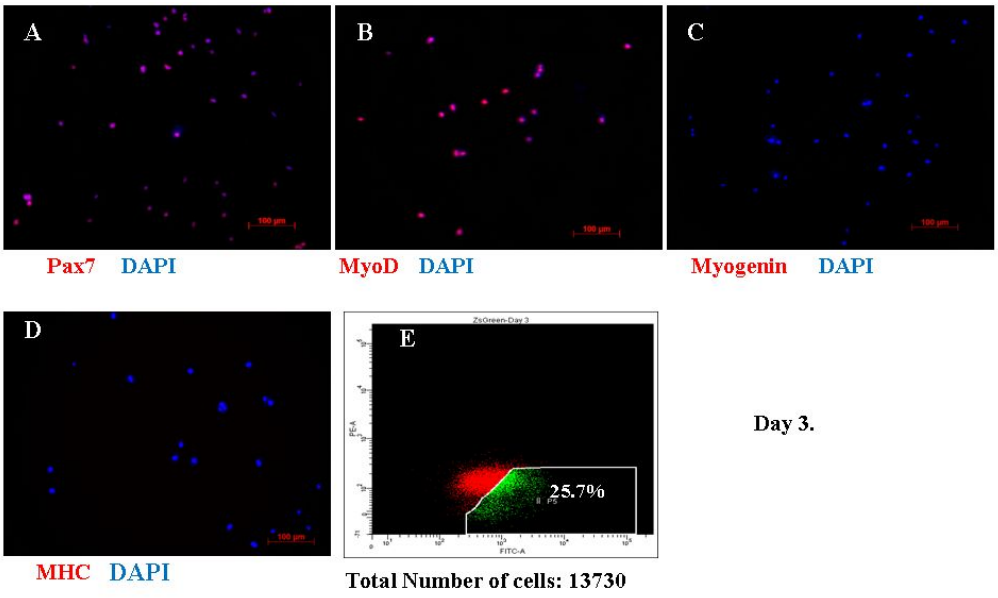


Figure (17). Day3-Immunofluorescence for (A) Pax7, (B) MyoD, (C) Myogenin and (D)MHC in red. DAPI (blue) was used for counterstaining the nuclei. Scale bar 100 µM. (E) FACS analysis for the percentage of ZsGreen positive satellite cells.

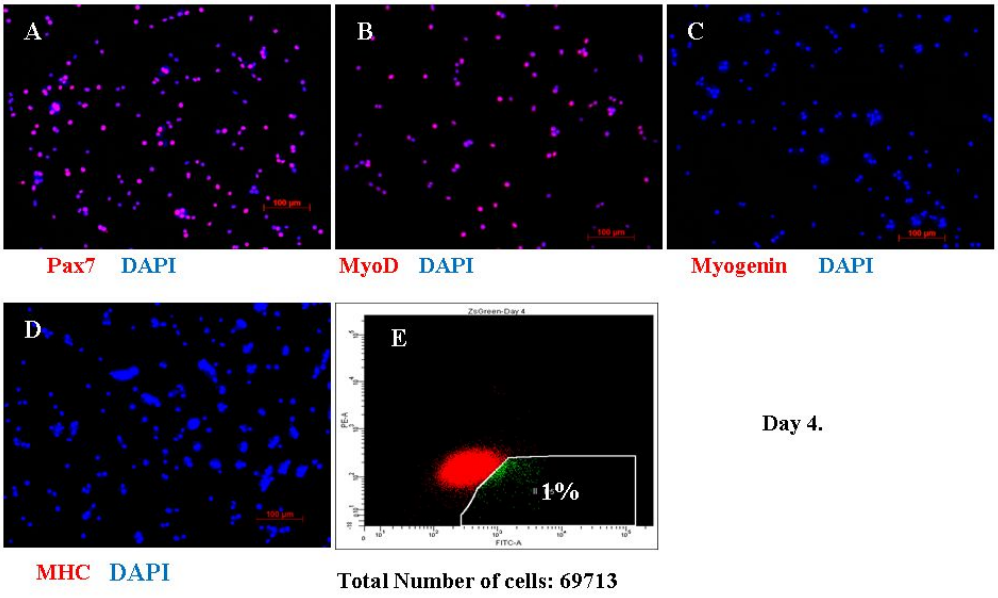


Figure (18). Day4-Immunofluorescence for (A) Pax7, (B) MyoD, (C) Myogenin and (D) MHC in red. DAPI (blue) was used for counterstaining the nuclei. Scale bar 100 µM. (E) FACS analysis for the percentage of ZsGreen positive satellite cells.

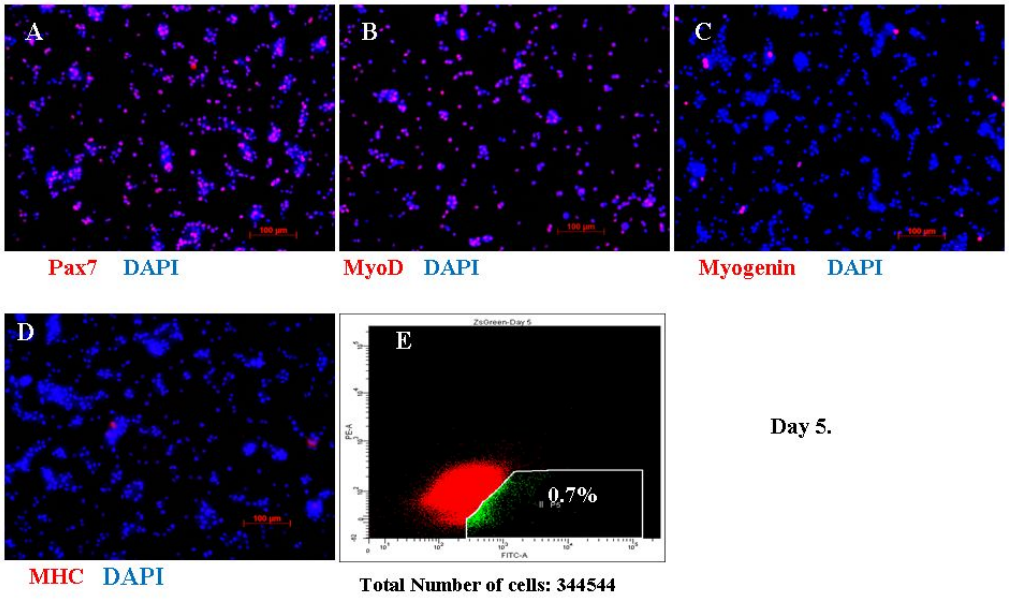


Figure (19). Day5-Immunofluorescence for (A) Pax7, (B) MyoD, (C) Myogenin and (D) MHC in red. DAPI (blue) was used for counterstaining the nuclei. Scale bar 100 µM. (E) FACS analysis for the percentage of ZsGreen positive satellite cells.

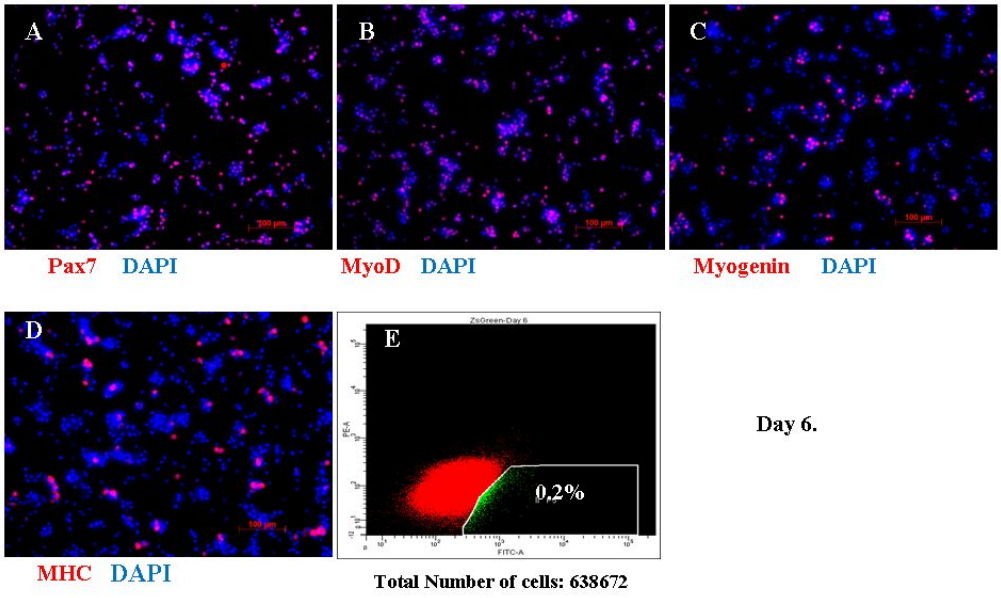


Figure (20) Day6-Immunofluorescence for (A) Pax7, (B) MyoD, (C) Myogenin and (D)MHC in red. DAPI (blue) was used for counterstaining the nuclei. Scale bar 100 µM. (E) FACS analysis for the percentage of ZsGreen positive satellite cells.

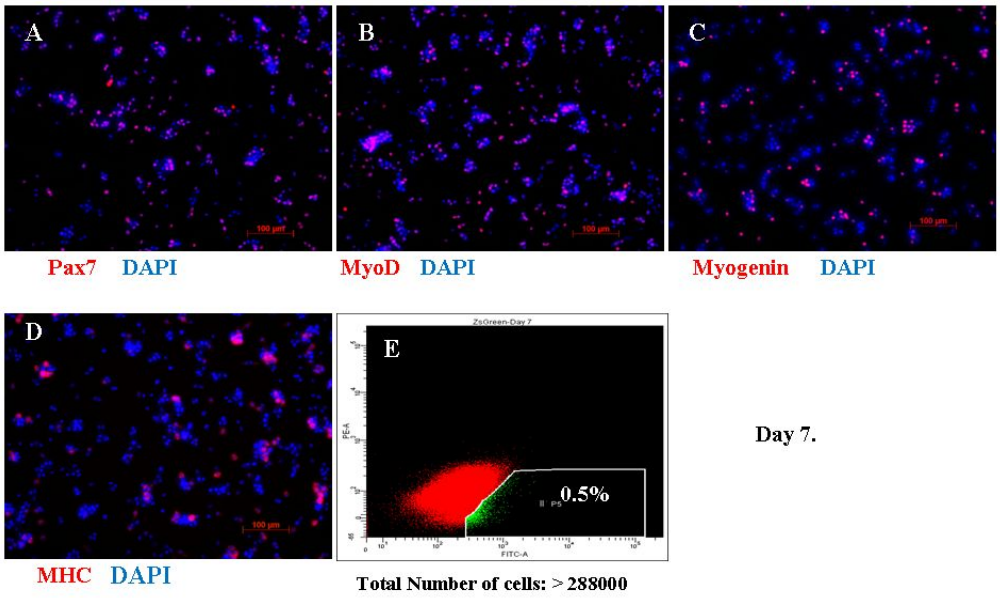


Figure (21) Day7-Immunofluorescence for (A) Pax7, (B) MyoD, (C) Myogenin and (D) MHC in red. DAPI (blue) was used for counterstaining the nuclei. Scale bar 100 µM. (E) FACS analysis for the percentage of ZsGreen positive satellite cells.

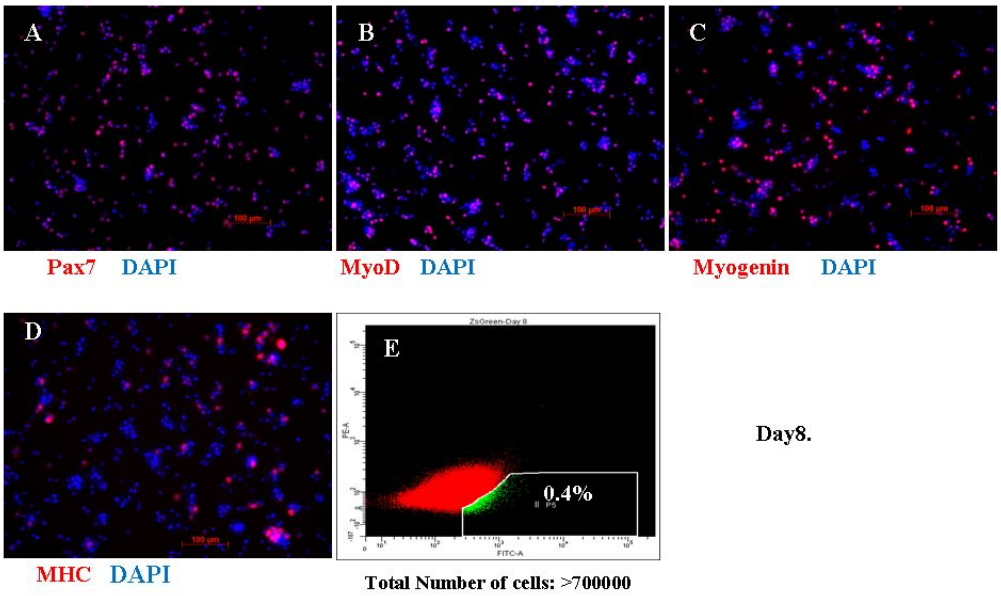


Figure (22) Day8-Immunofluorescence for (A) Pax7, (B) MyoD, (C) Myogenin and (D) MHC in red. DAPI (blue) was used for counterstaining the nuclei. Scale bar 100 µM. (E) FACS analysis for the percentage of ZsGreen positive satellite cells.

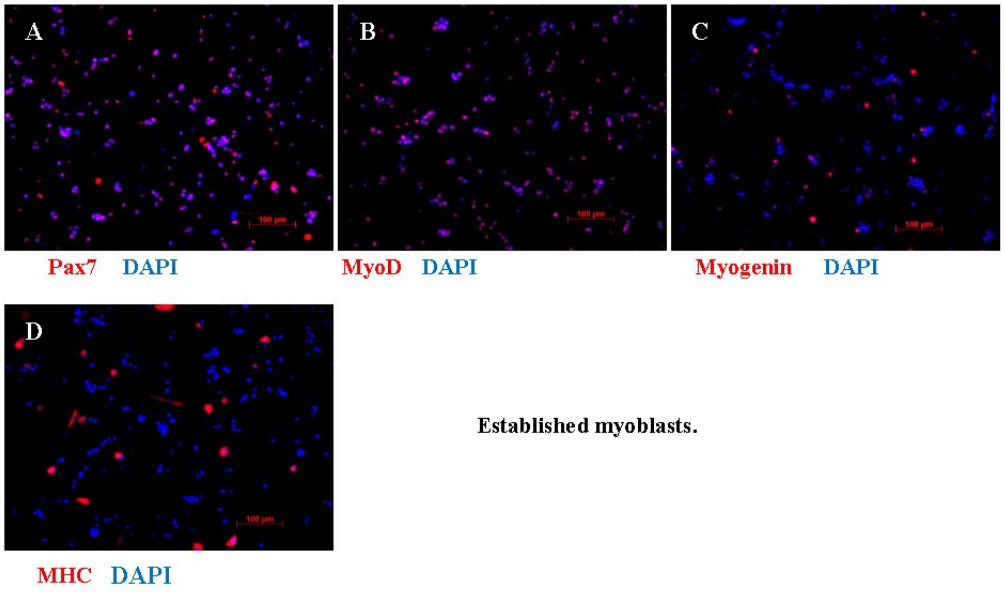


Figure (23). Established myoblasts Immunofluorescence for (A) Pax7, (B) MyoD, (C) Myogenin and (D)MHC in red. DAPI (blue) was used for counterstaining the nuclei. Scale bar 100 µM. (F) FACS analysis for the percentage of ZsGreen positive satellite cells.

The experiment was repeated for confirmation of Pax7 data. This time the cells were tracked for only 4 days. The cells were stained for Pax7, MyoD and Pax3 (Figures 24&25). Previous data describing Pax7 and MyoD expression in cultured satellite cells were confirmed. Immunofluorescence for Pax3 showed a similar pattern to that of Pax7 expression. All the cells are positive for Pax3 over the first three days in culture. Pax3 negative cells start to appear on day 4.

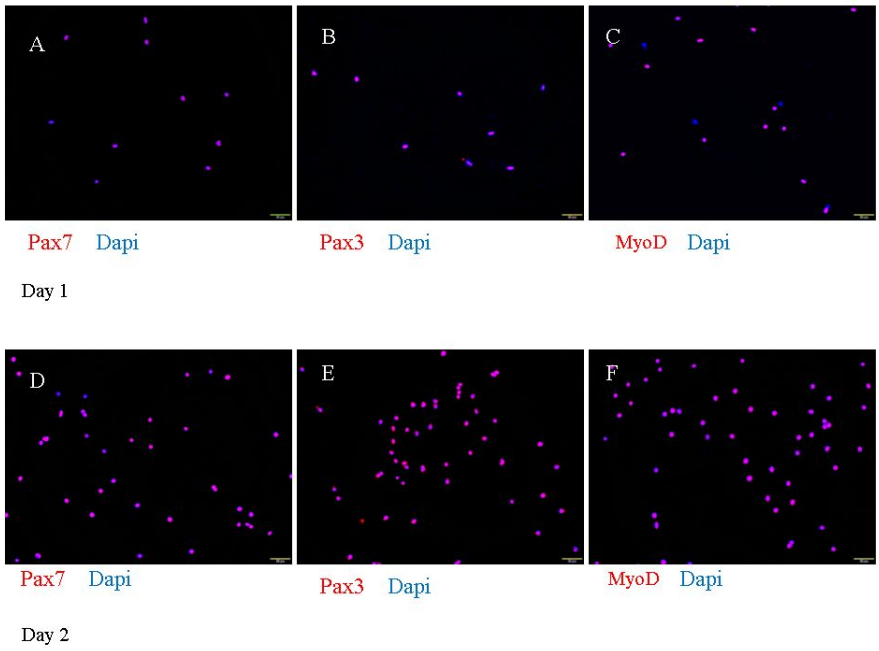


Figure (24). Immunofluorescence for (A,D) Pax7, (B,E) Pax3, and (C,F) MyoD in red. DAPI (blue) was used for counterstaining the nuclei. Scale bar 100 µM. The upper panel is for day-1 cells and the lower panel is for day-2 cells.

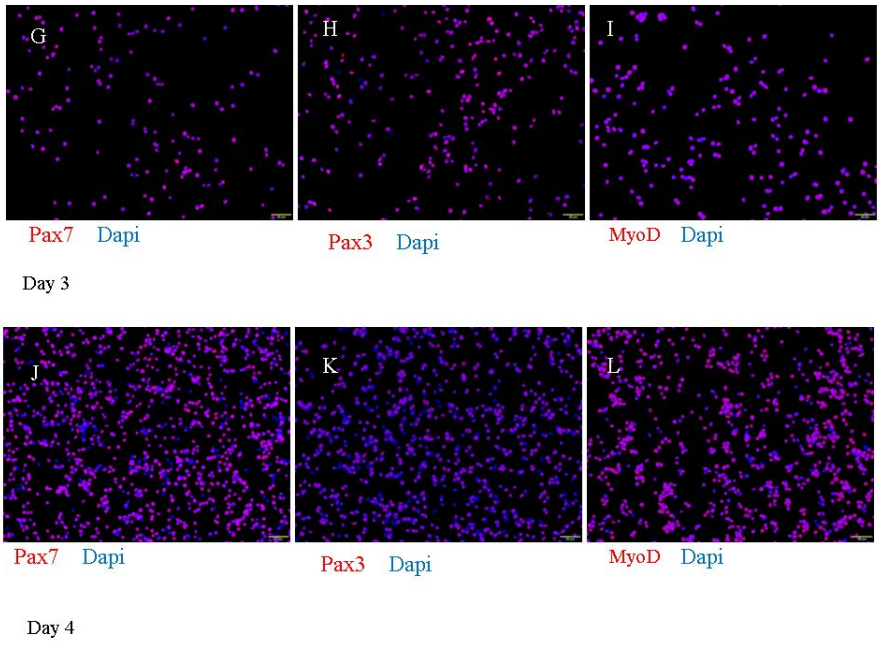
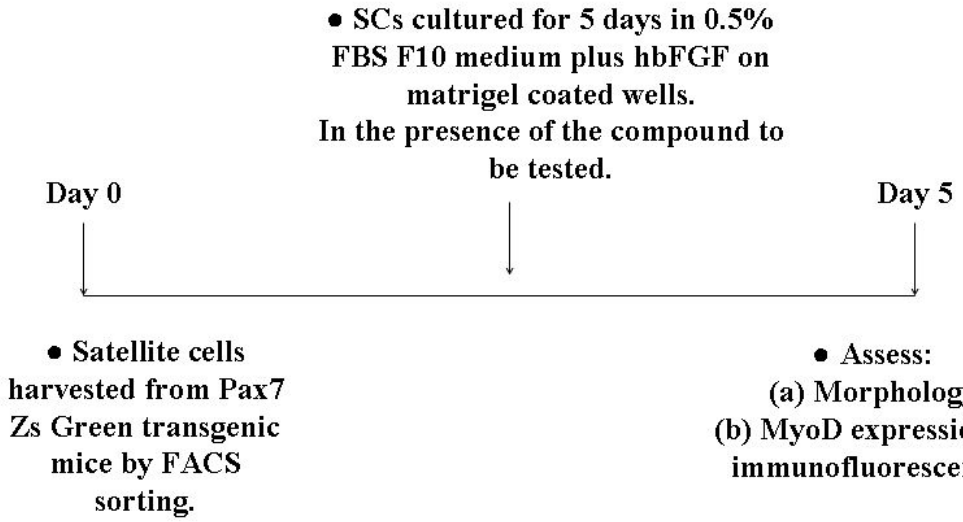


Figure (25). Immunofluorescence for (A,D) Pax7, (B,E) Pax3, and (C,F) MyoD in red. DAPI (blue) was used for counterstaining the nuclei. Scale bar 100 µM. The upper panel is for day-3 cells and the lower panel is for day-4 cells.

Based on the Pax7 data, the assessment points were changed for the following studies.

Effect of inhibitors on morphology and MyoD expression of cultured SCs



The aim of the experiment is to find a compound that can serve two functions (a) Prevent matrigel induced differentiation effect on satellite cells and preserve the rounded undifferentiated shape of the cells. (b) Inhibit MyoD expression in cultured satellite cells.

The following table shows the results.

Compound	Conc.	Figure.#	Mechanism.	Cell morphology	MyoD	Cell count.
SR1	1 µM	26	AHR blocker.	Rounded	Inhibitory	Inhibitory.
SDF1	500 ng/ml	26	Chemotaxis.	Elongated	No effect.	No effect.
SAHA	0.5 µM	26	Deacetylase inhibitor	Elongated	No effect.	Inhibitory.
CHIR9902	3 µM	27	GSK3β inhibitor	Elongated	No effect.	Inhibitory.
PD098059	3 µM	27	ERK1 pathway inhibitor.	Elongated	No effect.	No effect.
A83-01	1 µM	28	TGFß 1 inhibitor	Elongated	No effect.	No effect.
Hh Ag.1.5	2 µM	28	Shh agonist	Some rounded.	No effect.	Inhibitory.
IWR1	10 µm	29	Wnt inhibitor	Elongated myocytes.	No effect	No effect.
IWP2	5µm	29	Wnt inhibitor	Elongated myocytes.	Inhibitory.	Inhibitory.

Table 3. Effect of different compounds on cultured satellite cells.

The effect on proliferation and the morphological assessment was described on basis of cell shape in under the microscope as shown in the figures. MyoD expression was assessed based on indirect immunofluorescence staining of the cells.

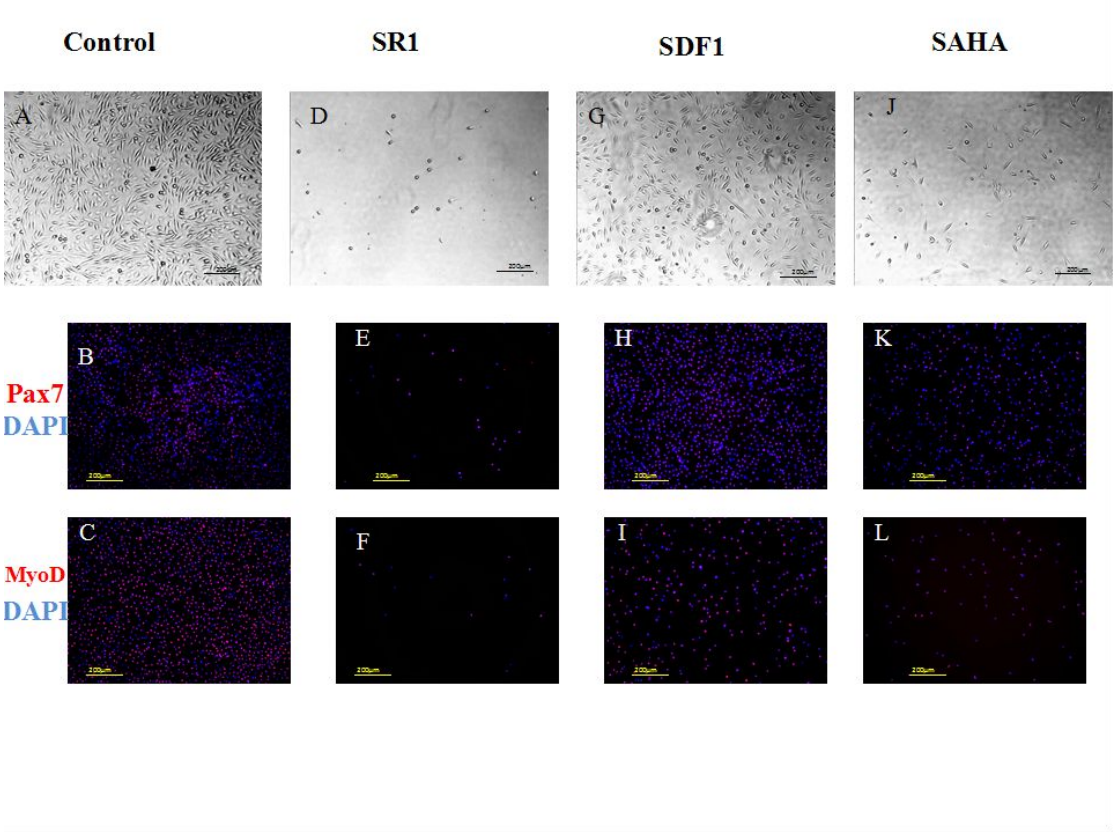


Figure (26). Effect of SR1, SDF1, and SAHA on cultured SCs. The upper panels are showing cell morphology through phase contrast images. The middle and lower panels are showing Pax7 and MyoD immunostaining (red) with DAPI counter staining of the nuclei. (A,B&C) control, (D,E&F) SR1, (G,H&I) SDF1, (J,K&L) SAHA. Scale bar 200µM.**(n=1).**

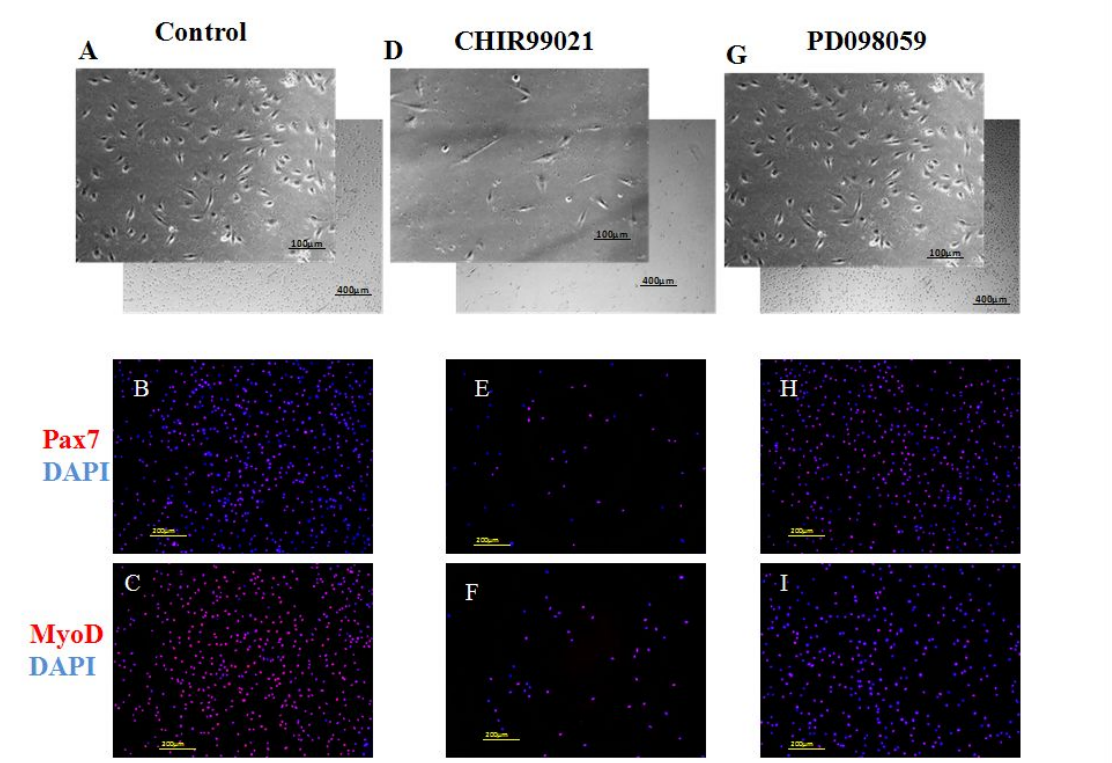


Figure (27). Effect of CHIR99021, PD098059 on cultured SCs. The upper panels are showing cell morphology through phase contrast images. The middle and lower panels are showing Pax7 and MyoD immunostaining (red) with DAPI counter staining of the nuclei. (A,B&C) control, (D,E&F) CHIR99021, (G,H&I) PD098059. Scale bar is 200µM for immunofluorescent images and 100, 400 µM for phase contrast images. (n=1).

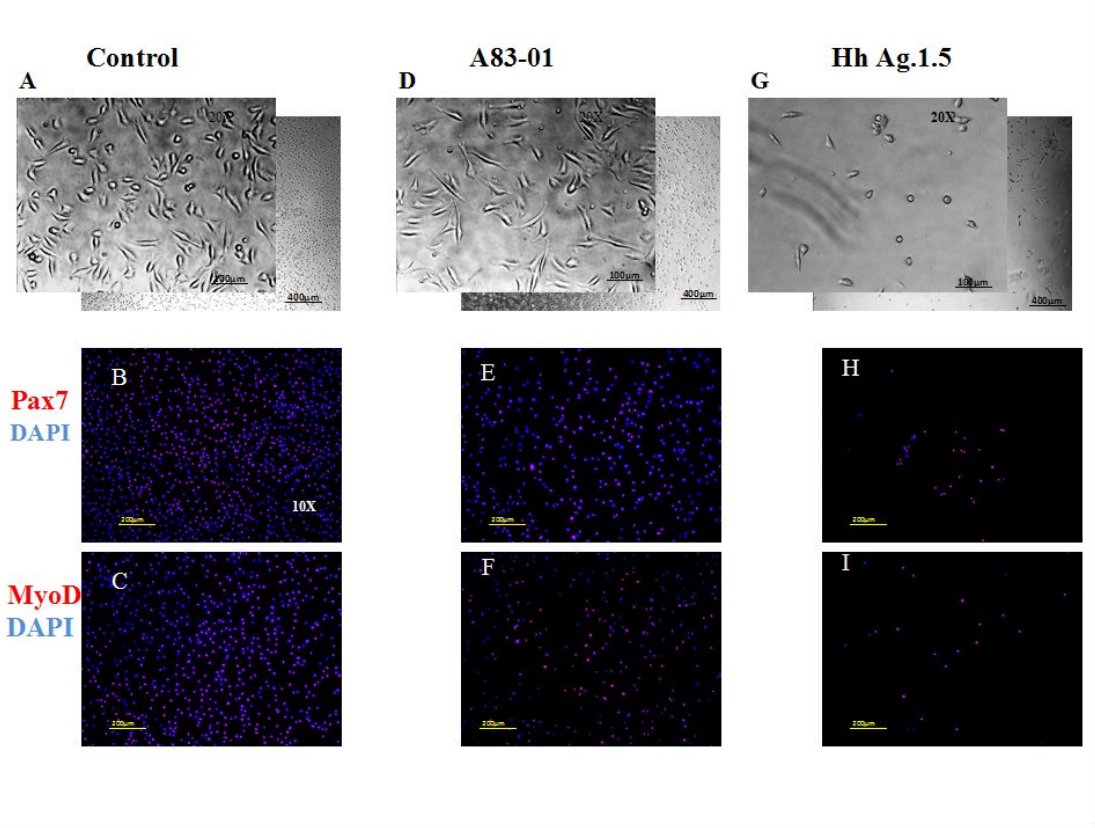


Figure (28). Effect of A83-01, Hh Ag.1.5 on cultured SCs. The upper panels are showing cell morphology through phase contrast images. The middle and lower panels are showing Pax7 and MyoD Immunostaining (red) with DAPI counter staining of the nuclei. (A,B&C) control, (D,E&F) A83-01, (G,H&I) Hh Ag.1.5. Scale bar is 200µM for immunofluorescent images and 100,400 µM for phase contrast images. (n=1).

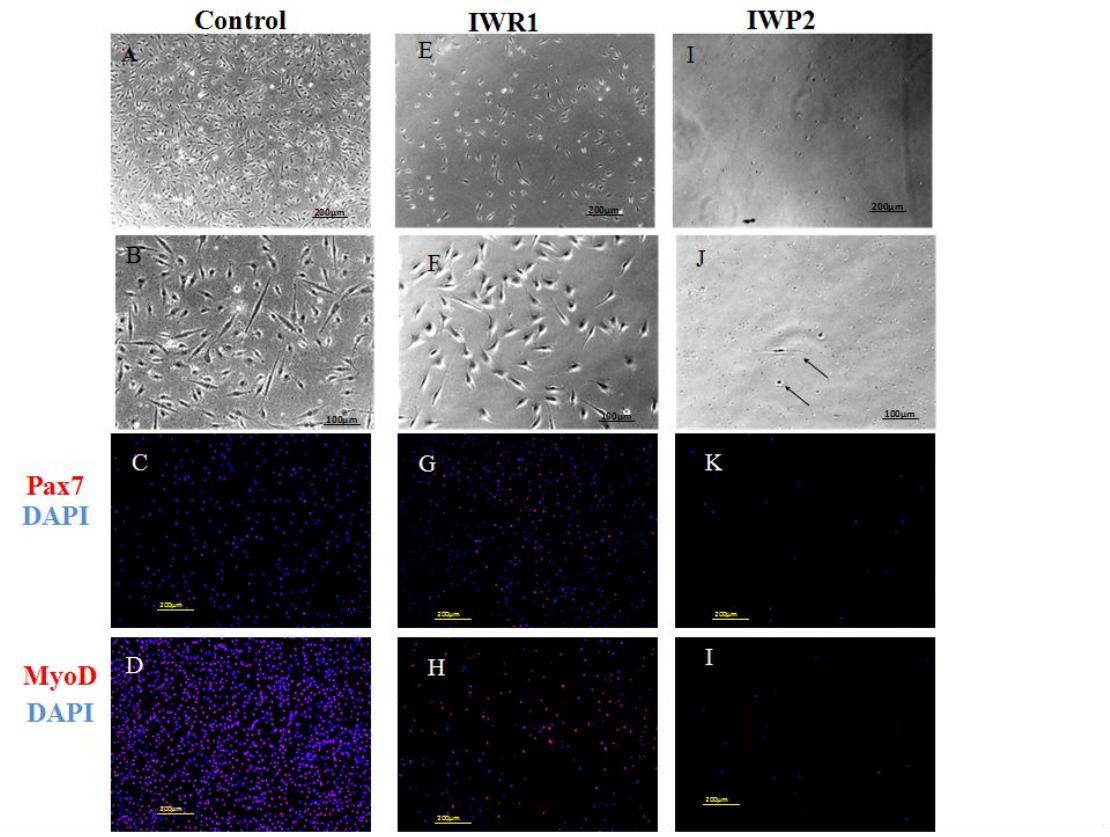


Figure (29). Effect of IWR1, IWP2 on cultured SCs. The upper 2 panels are showing cell morphology through phase contrast images. The lower 2 panels are showing Pax7 and MyoD immunostaining (red) with DAPI counter staining of the nuclei. (A,B,C&D) control, (E,F,G&H) IWR1, (I,J,K&L) IWP2. Scale bar is 200µM for immunofluorescent images and 200,100 µM for 10x phase contrast images. (n=1).

Effect of SR1 on cultured satellite cells and established myoblasts

SR1 inhibited differentiation of satellite cells on matrigel (figure 26D). It also inhibited the expression of MyoD gene in cultured satellite cells (figure 26F).

SR1 has been previously described to function as a blocker of **aryl hydrocarbon receptor**. So, SR1 has been assessed through:

a) **Testing the effect of SR1 on cultured satellite cells and cultured myoblasts** i.e. effect on proliferation, effect on differentiation, expression of Pax7, MyoD, Myogenin by immunofluorescence and by QRT-PCR for RNA analysis. In addition these effects have been linked to an action on AHR.

SR1 inhibited differentiation of myoblasts into myotubes in 2% horse medium. (figure 30&31).

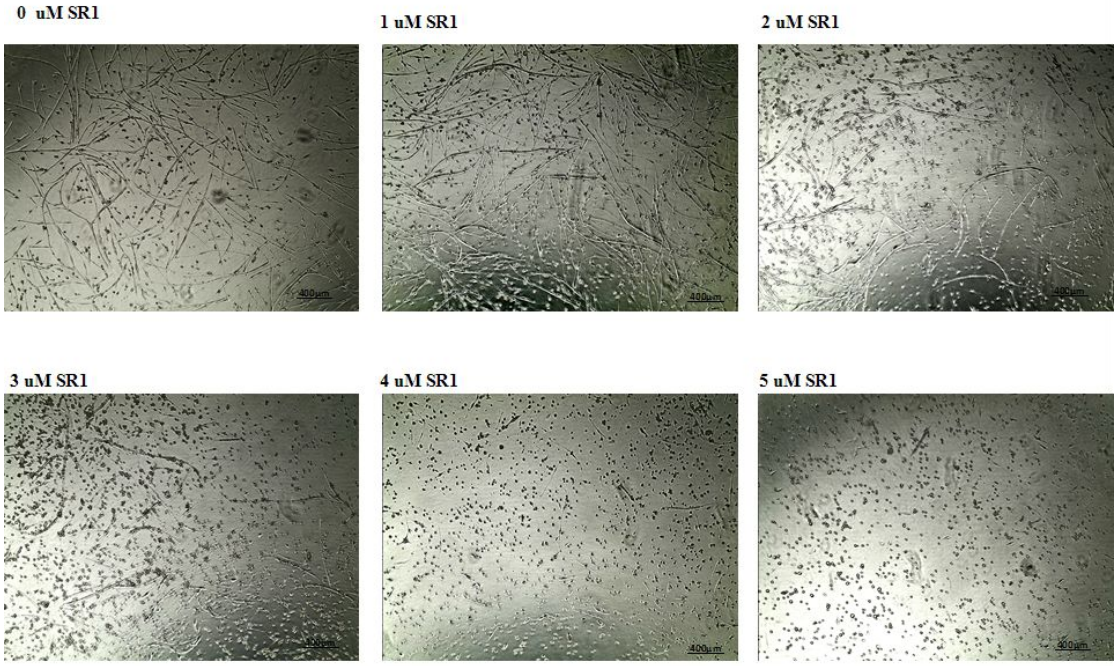


Figure (30) Phase contrast images showing dose response effects of SR1 on the differentiation of myoblasts in 2% Horse serum medium. (A) 0 µM sr1, 2% HS, (B) 1µM SR1, 2% HS medium (C) 2µM SR1, 2% HS medium (D) 3µM SR1, 2% HS medium (E) 4µM SR1, 2% HS medium (F) 5µM SR1, 2% HS medium. Scale bar 400 µM. (n=3).

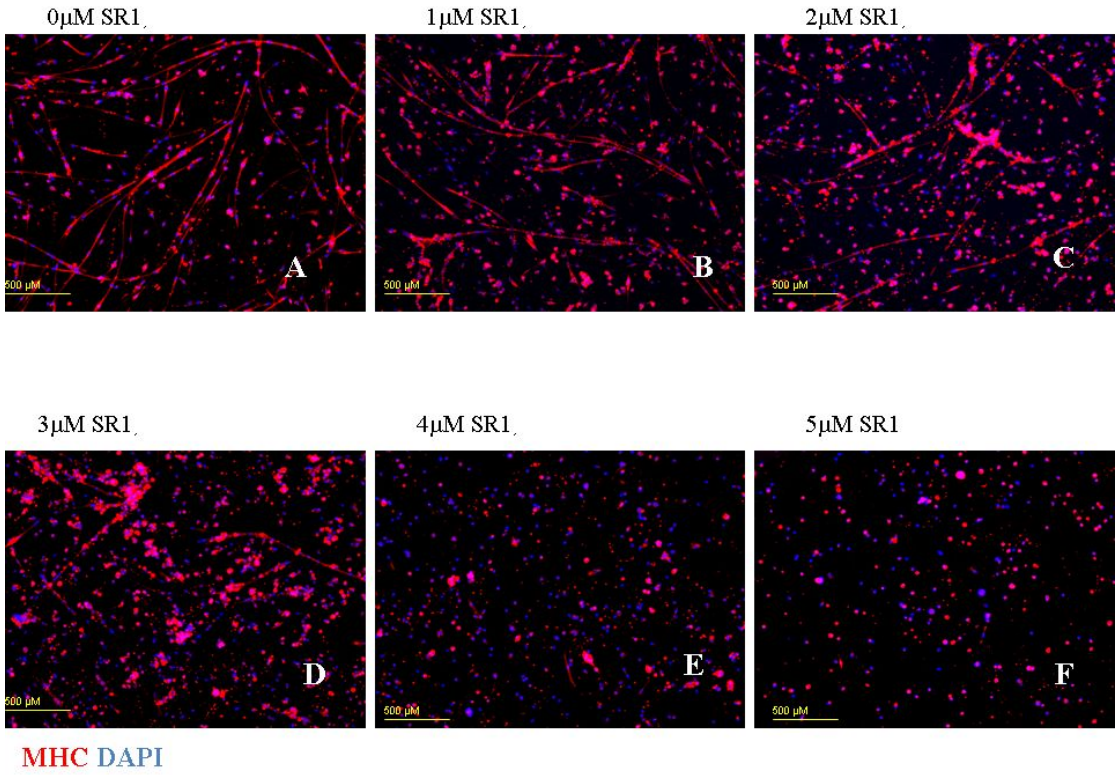


Figure (31). Immunofluorescence for MHC (red) and DAPI (blue) showing dose response effects of SR1 on the differentiation of myoblasts in 2% Horse serum medium. (A) 0 μM sr1, 2% HS, (B) 1μM SR1, 2% HS medium (C) 2μM SR1, 2% HS medium (D) 3μM SR1, 2% HS medium (E) 4μM SR1, 2% HS medium (F) 5μM SR1, 2% HS medium. Scale bar 500 μM.

Though SR1 proved to have a strong negative effect on satellite cell differentiation, yet its marked inhibitory effect on cell proliferation was an obstacle toward its practical use. So, I used cytokines to stimulate satellite cell proliferation. 1 μM SR1 has been combined with the following cytokines (IL6, LIF, EGF, IGF1) (figure.32). The numbers of the cells increased slightly, but they are still below the numbers that can allow practical use of SR1. There is no advantage in keeping the cells undifferentiated in culture if they are not proliferating.

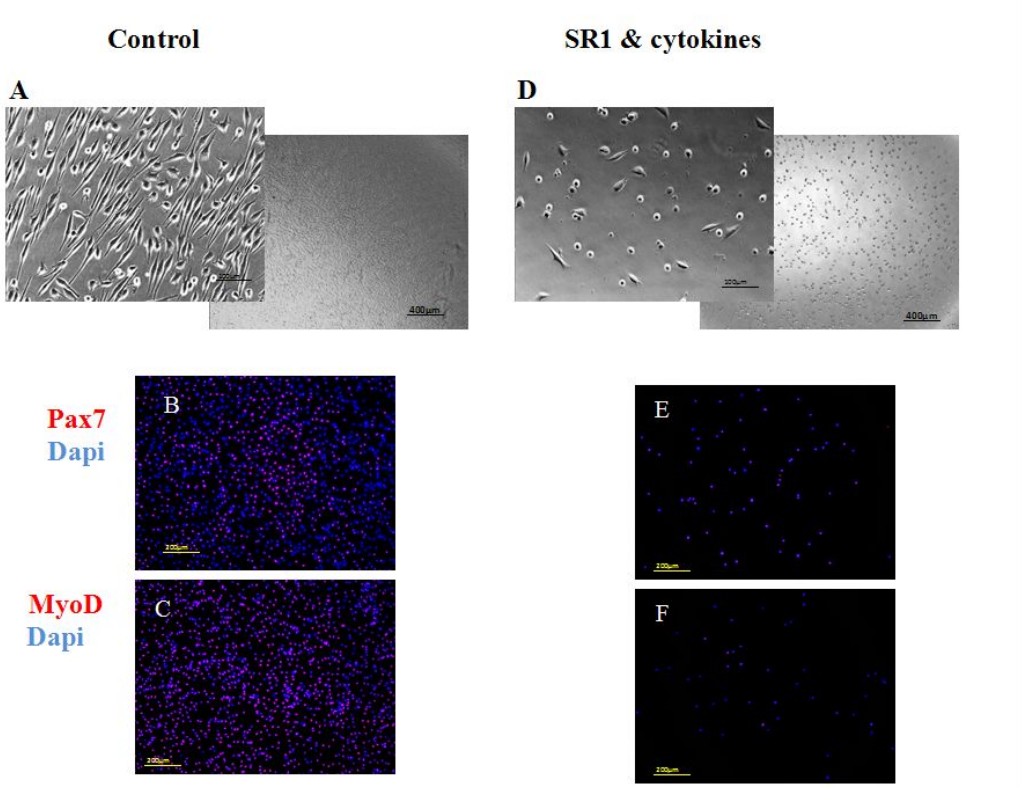


Figure (32). Effect of SR1 combined with cytokines on cultured SCs. The upper panels are showing cell morphology through phase contrast images. The middle and lower panels are showing Pax7 and MyoD immunostaining (red) with DAPI counter staining of the nuclei. (A,B&C) control, (D,E&F) SR1 + cytokines. Scale bar 200µM for immunofluorescent images and 100,400 µM for phase contrast images. (n=1).

Next, I tested SR1 on proliferating myoblasts with already activated myogenic system in the form of MyoD protein expression. Incubation of myoblasts with 1µM concentration of SR1 in 0.5% FBS medium succeeded to convert the exposed myoblasts into a MyoD negative Myogenin negative phenotype (Figure 33). The RNA levels of MyoD, Myogenin, Myf5 and Pax7 were assessed in SR1 treated cells (Figure 34). SR1 treated cells have significantly lower levels of Myogenin expression denoting a less differentiated state. In addition these effects have been linked to an action on AHR through the ability of SR1 to down regulate Cyp1B1 gene (A down stream reporter gene to AHR) (Figure 34). FACS analysis of SR1 treated established myoblast cells showed that there is no significant difference in ZsGreen in SR1 treated versus untreated cells

(Figure.35). Being active on myoblasts allows us to use SR1. Huge numbers of myoblasts can be obtained from proliferation of cultured satellite cells within few days. If these big numbers of myoblasts can be turned to a MyoD negative phenotype by the help of SR1, then we can say that the net effect would be having a huge number of transplantable cells from a small starting number of cultured satellite cells. This later conclusion is actually based on the assumption that MyoD negativity yields the cells more stem cell like, more quiescent phenotype like and transplantable.

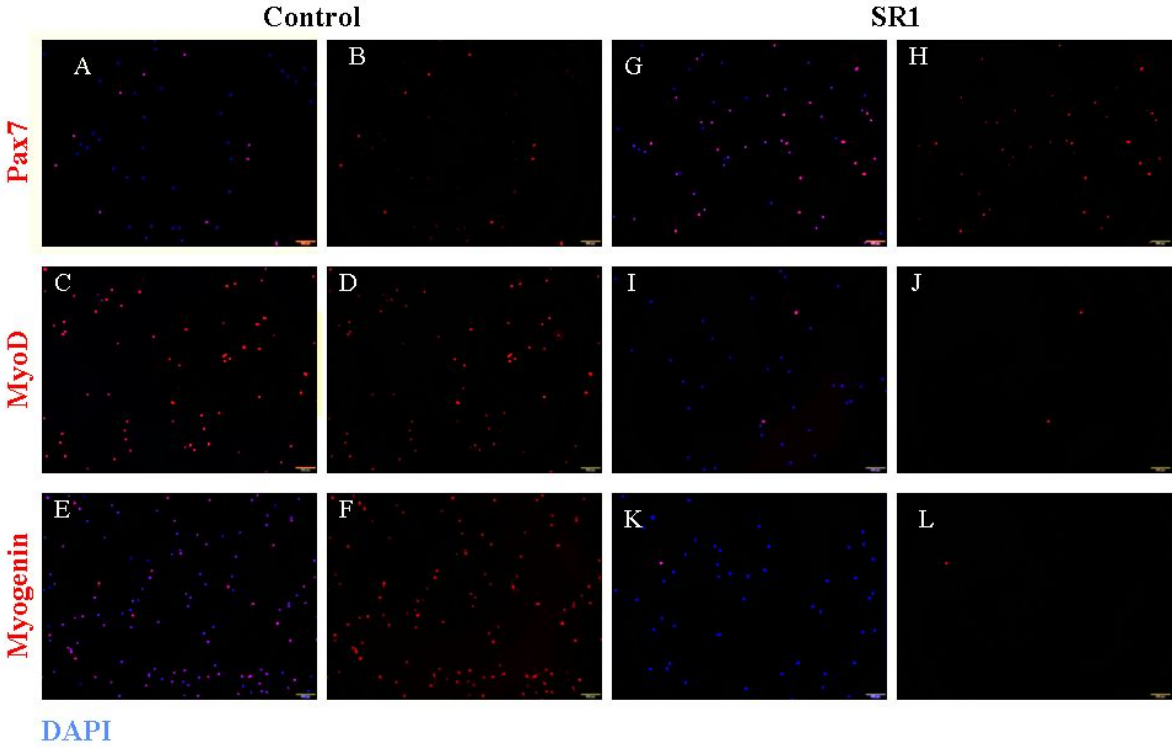


Figure (33). Effect of SR1 on expression of MyoD and Myogenin of cultured myoblasts. Immunofluorescence for Pax7 (A,B,G&F), MyoD (C,D,I&J), Myogenin(E,F,K&L) in red. DAPI (blue) was used for counterstaining the nuclei. 10X, Scale bar = 100 µm. (n>3). (A,C,E,G,I and K) are DAPI merged while the remaining figures show only the targeted protein staining.

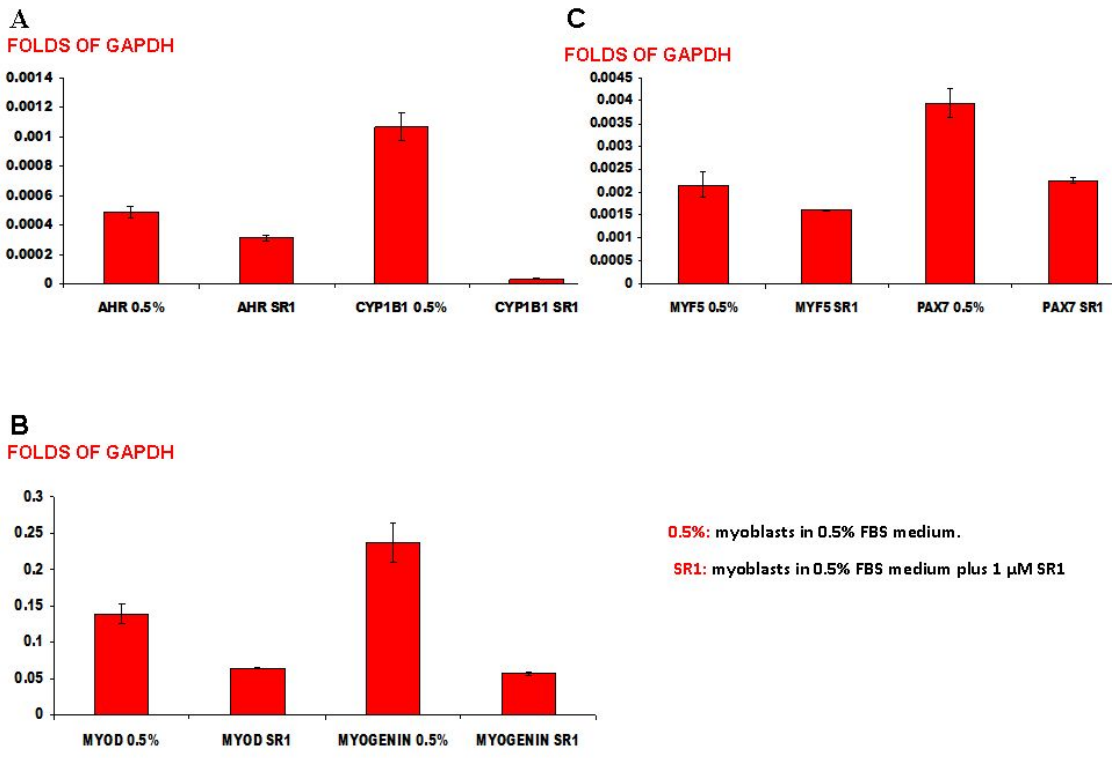
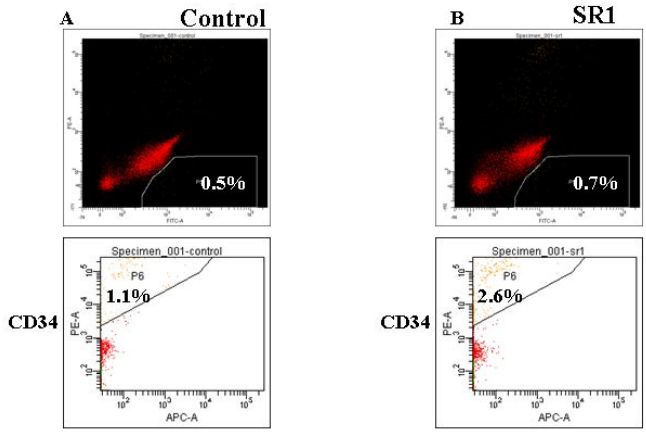


Figure (34). QRT-PCR of RNA levels of different myogenic factors in SR1 treated myoblasts. The values are normalized against GAPDH enzyme RNA levels. SR1 denote established myoblast cells treated with 1 µM SR1 for 4 days. 0.5% denotes 0.5% FBS control cells with no SR1 treatment. (n=3)



Figure(35). FACS analysis of SR1 treated cells. Upper panels for ZsGreen level of the cells. Lower panels for CD34 positive cells. The cells are established myoblast cells treated with 1 µM SR1 for 4 days in 0.5% FBS medium.

Myoblasts failed to proliferate in the presence of SR1. But, this effect is partly due to the type of medium in which the cells are incubated (0.5% FBS medium) which does not allow the cells to proliferate much.

b) Needed duration for SR1 to exert its full action on cultured SC MyoD expression. (Figure 36)

Myoblast cells have been incubated with SR1 for 24, 48, 72 and 96 hours. After each of the mentioned durations the cells were fixed and tested for MyoD. After 2 days the effect of the molecule started to appear with inhibition of MyoD expression. On day 3, more the percentage of MyoD negative cells increased. On day 4, the majority of cells were MyoD negative with very few positive cells. But, on day 4 an observed reduction in cell density happened most probably due to cell death as a result of arrest of cell proliferation over a long period of time. It will be discussed later that when SR1 treated myoblasts were transplanted; we incubated them with SR1 for 2 days only for fear of the possible toxic effect of the compound in case of longer durations of exposure.

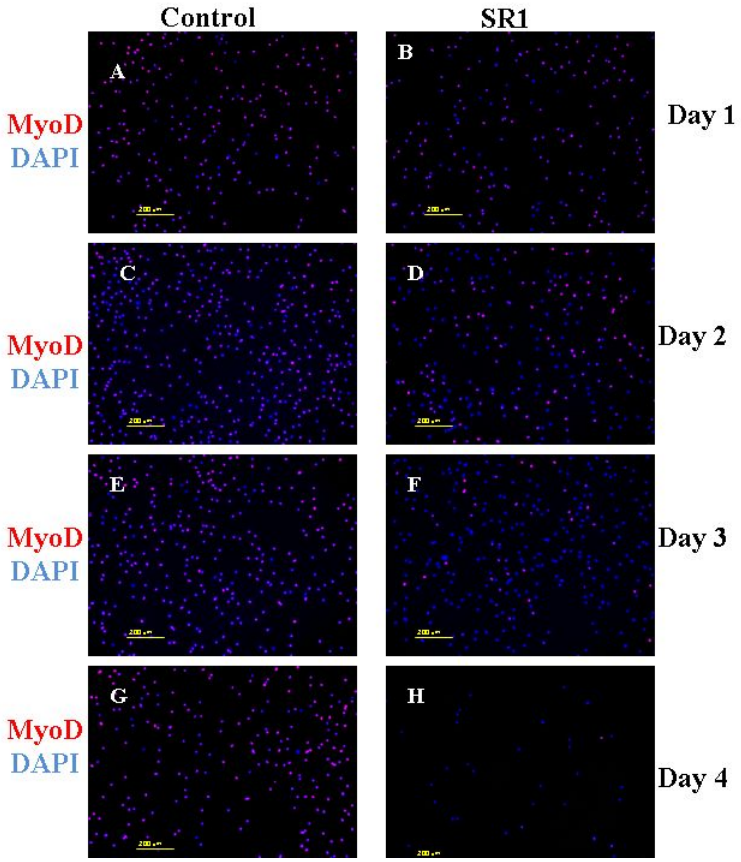


Figure (36). Verifying the required duration needed by SR1 to produce its full effect in MyoD inhibition. Cells are stained by immunofluorescence for MyoD (red), DAPI (blue). Scale bar 200 µm.

c) Searching the lowest effective dose of SR1. (Figure 37)

On testing 0.8 µM of SR1 MyoD positive cells started to appear in culture. The effect of 0.9 µM concentration was not different from that of the first adopted concentration of 1 µM. So, I continued to use the concentration of 1 µM.

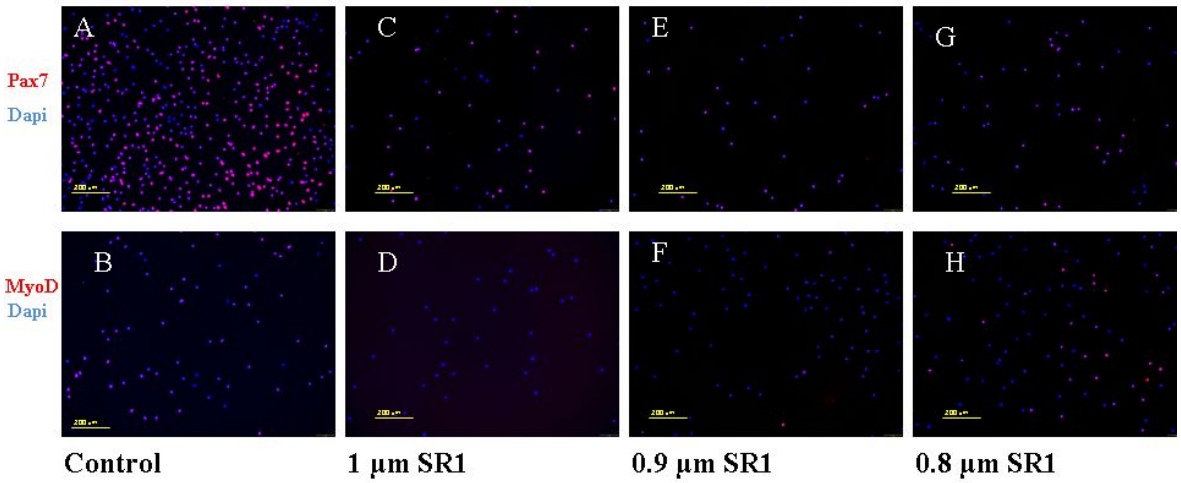


Figure (37). Searching the lowest effective dose of SR1. Cells are stained for MyoD and Pax7 (red) and DAPI was used fro counterstaining the nuclei. Scale bar 200 µm. (n=2).

d) Testing the effect of serum on its activity.

When SR1 was tested in 2.5% FBS medium, it was totally ineffective (Figure 38&39). No effect on cell proliferation or MyoD expression was observed with 2.5% FBS medium (Figure 38). So, I assessed the effect of serum on the activity of SR1. The activity of SR1 and its ability to inhibit matrigel induced differentiation decreased parallel to increasing the level of serum in the medium (Figure 39).

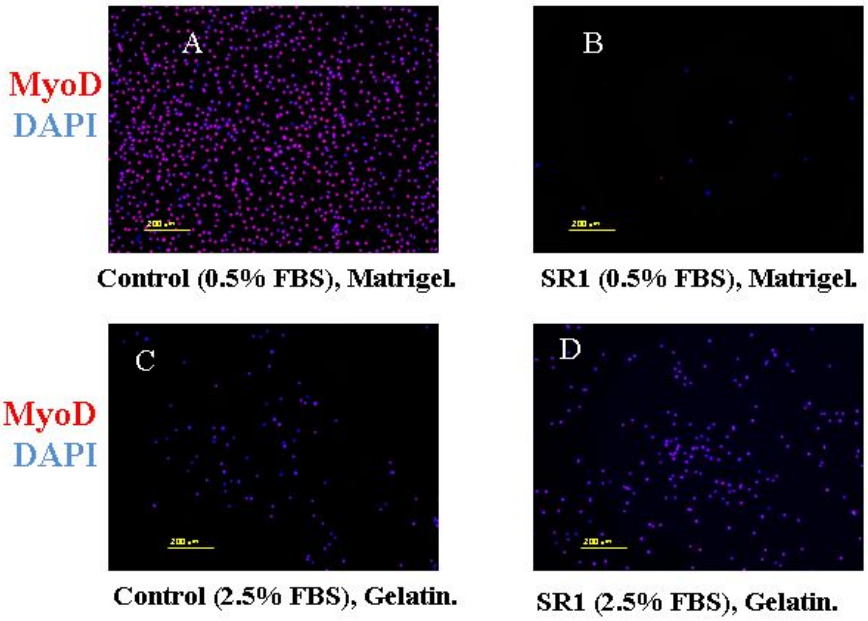


Figure (38). Lack of SR1 effect on myoblasts in the presence of 2.5% serum concentration. Cells are stained for MyoD (red) and DAPI (blue) was used for counterstaining of the nuclei. Scale bar200 µm.

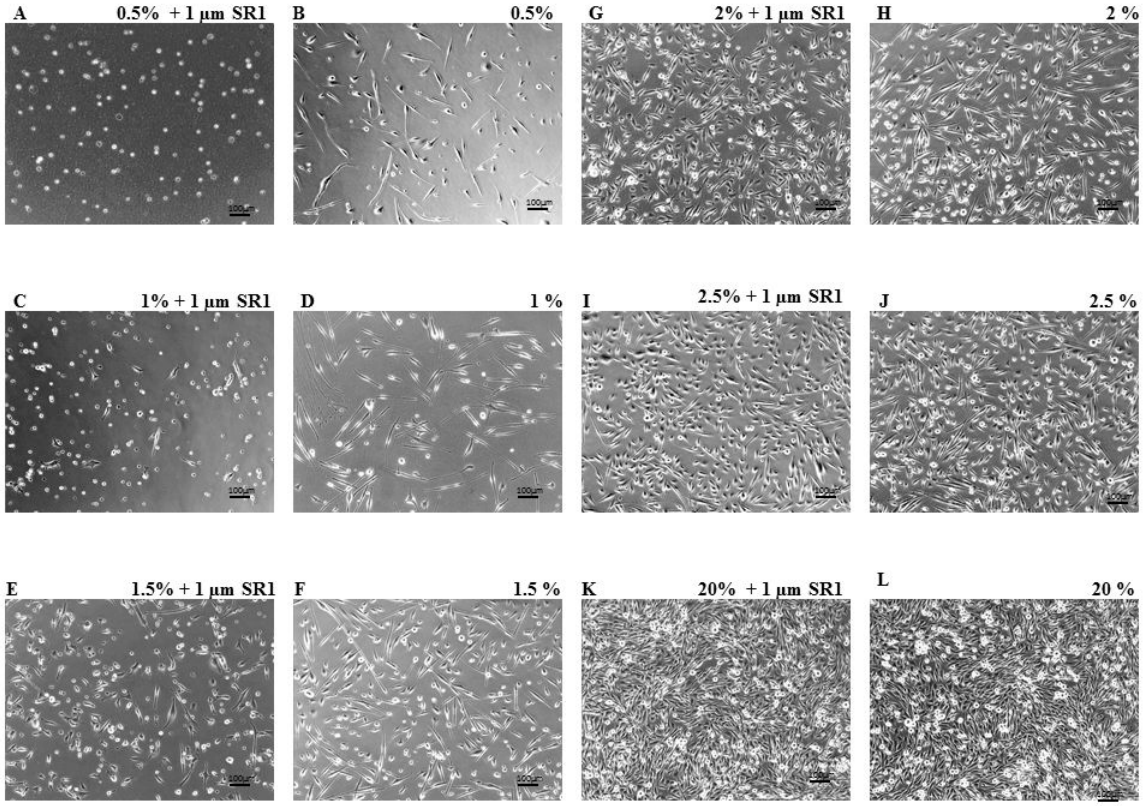


Figure (39) Effect of serum on the activity of SR1. Cultured myoblasts incubated with different concentration of FBS with and without 1 µm of SR1. (A&B) 0.5% FBS, (C&D) 1% FBS, (E&F) 1.5% FBS, (G&H) 2% FBS, (I&J) 2.5% FBS (K&L). Phase contrast images, Scale bar 100 µM. (n=1).

Since fetal bovine serum had a negative effect on the activity of SR1, I searched other methods to find a way where we can use higher serum concentration while preserving the activity of the compound. Charcoal stripped serum is a type of modified serum that has been absorbed with activated carbon that removes non-polar material such as lipophilic materials. This results in a more defined serum product with less variability in research results. So, I tried to use charcoal stripped serum to help grow myoblasts in a higher serum concentration while keeping SR1 active. But, slightly higher concentration of charcoal stripped serum was inhibitory to the effect of SR1. SR1 was not active except with a serum concentration of 0.5% even with charcoal stripped serum (Figure.40).

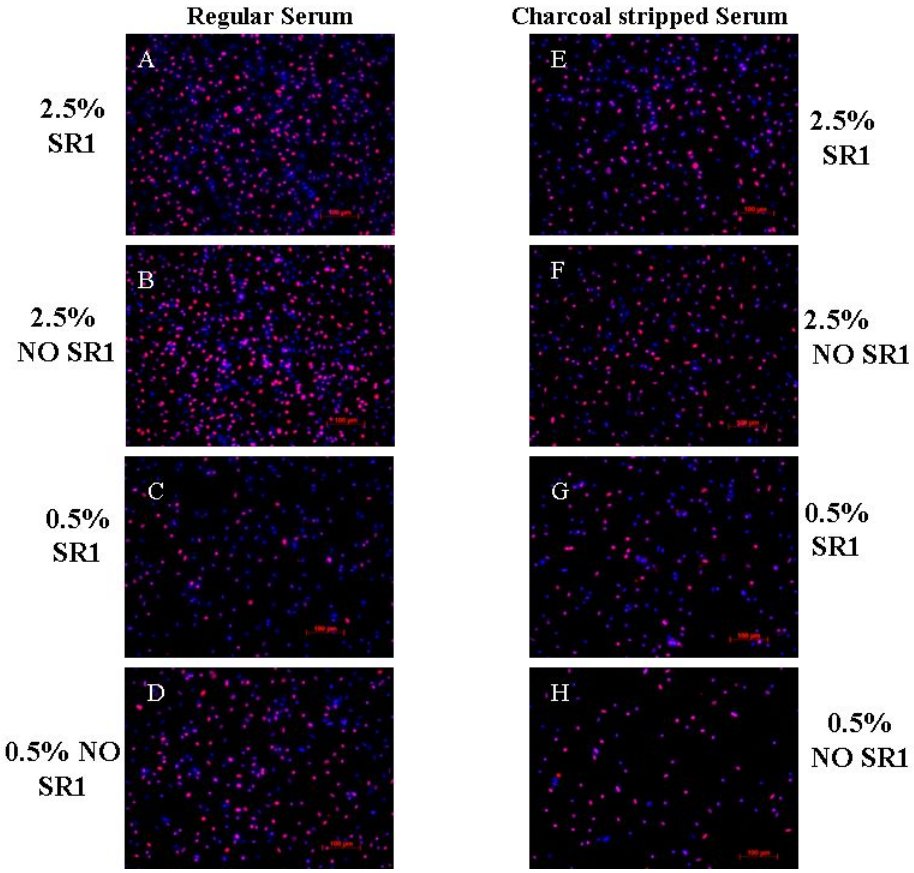


Figure (40). Charcoal stripped serum assessment.

MyoD (red), DAPI (blue) staining of different concentrations of regular serum and charcoal stripped serum in the presence and absence of SR1.

1- SR1 is not active in the presence of 2.5% concentration of charcoal stripped serum (E).No difference was observed either in cell growth rate or MyoD expression in comparison with the sample that was exposed to 1 µM SR1 in a serum concentration of 2.5% of charcoal stripped serum (F).

2- Rate of cell growth is lower with charcoal stripped serum than with regular serum. (E,F,G,H) in comparison with equal concentrations of regular serum (A,B,C,D).

e) Testing the ability of SR1 treated *in vitro* cultured satellite cells to successfully engraft after transplantation.

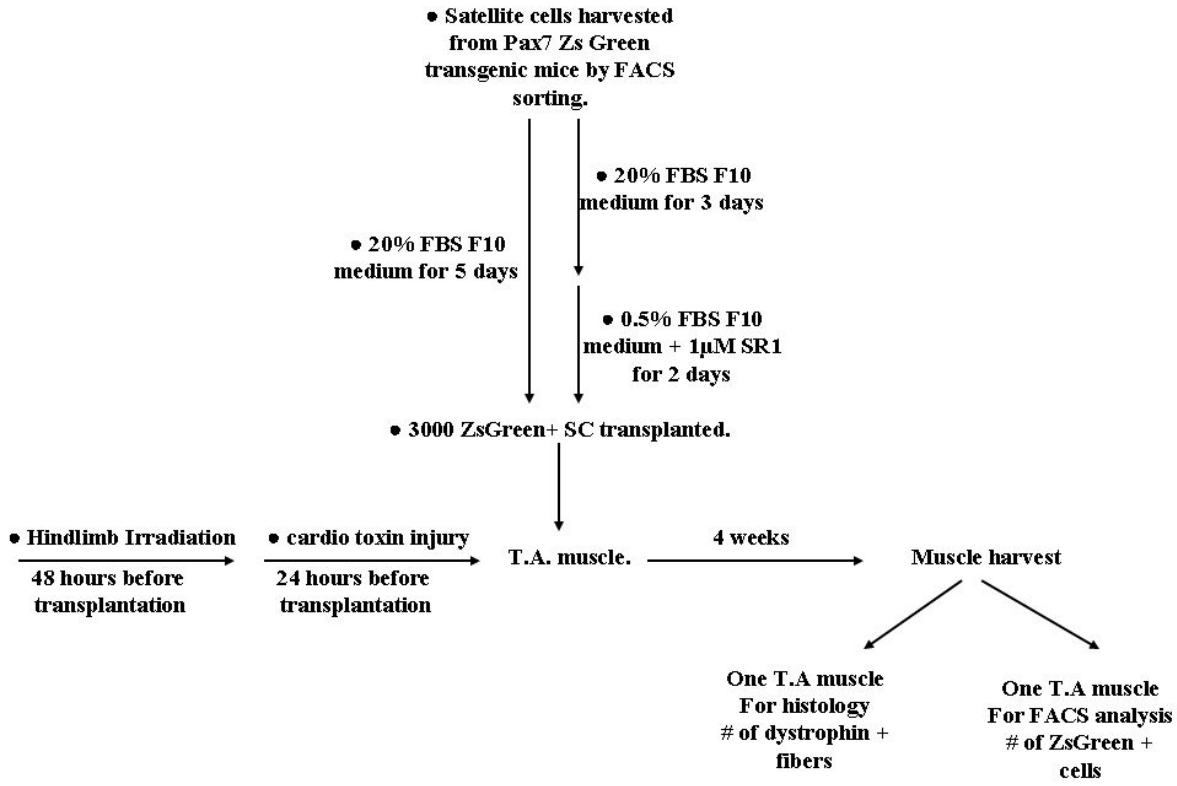


Figure (41). Design of SR1 treated myoblast transplant experiment.

The ability of SR1 treatment to improve transplantation efficiency was assessed by comparing 2 cell populations (Figure.41). Both are freshly isolated satellite cells that have been either.(a) incubated 3 days in 20% FBS F10 medium plus hbFGF followed by staying 2 days in 0.5% FBS F10 medium plus FGF + 1 μM SR1. (b) 5 days in 20% FBS F10 medium plus FGF. Number of transplanted cells: 3000 per tibialis anterior (TA) muscle. Duration before harvest, freezing and analysis: 4 weeks. After the 4 weeks one TA muscle was assessed by FACS analysis for the number of ZsGreen cells. The other TA was stained for the number of the number of donor derived dystrophin positive fibers. No significant difference in the number of the obtained donor fibers in the cells that have been treated with SR1 versus untreated cells (Figure 42). But, interestingly the number of ZsGreen positive cells was higher in the samples that were treated with SR1 (Figure 43). But, the number of ZsGreen positive cells was not uniform in the six samples that were SR1 treated. Broad range of ZsGreen positive cells was observed (Figure 43 A). This

may be denoting variability of the action of SR1 over different cells. I argued that the 48-hour exposure period was not sufficient to produce uniform effect on the cells (Figure 36). It has been proved that single satellite cells are engraftable (11) but, not every single satellite cell is engraftable.

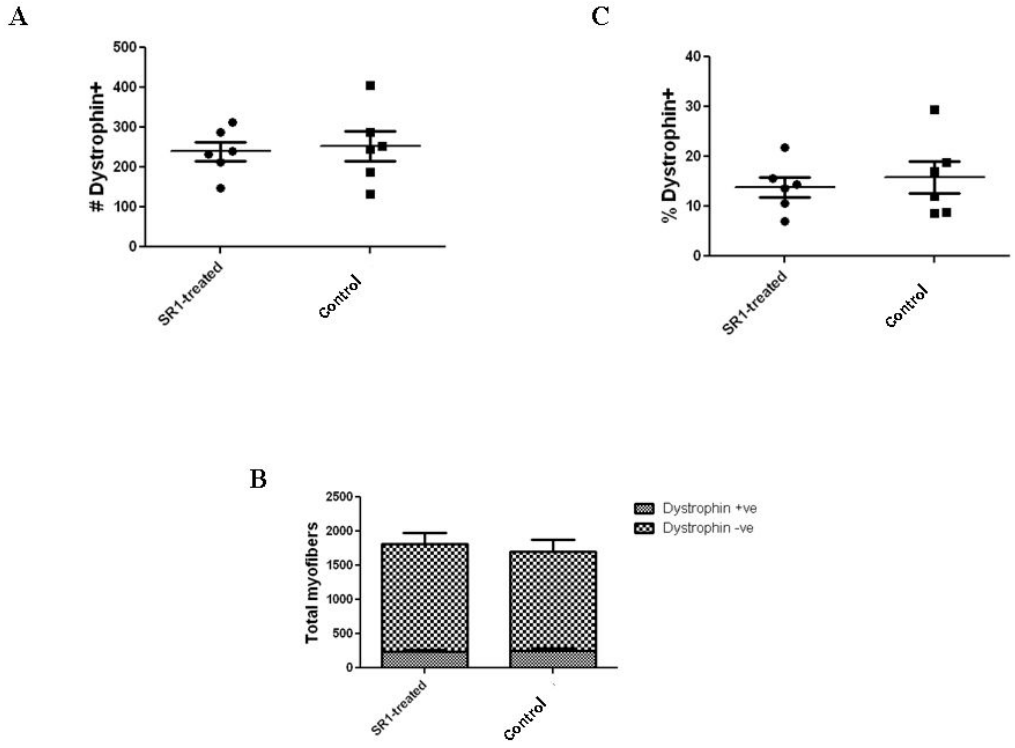


Figure (42) Fiber engraftment results of the SR1 treated versus untreated cells. (A) The absolute numbers of the Dystrophin positive fibers per TA of the transplanted mice. (B) Total numbers of dystrohin positive and dystrophin negative fibers (C) percentage of the dystrophin positive fibers per TA of the 12 mice that were transplanted. (n=6)

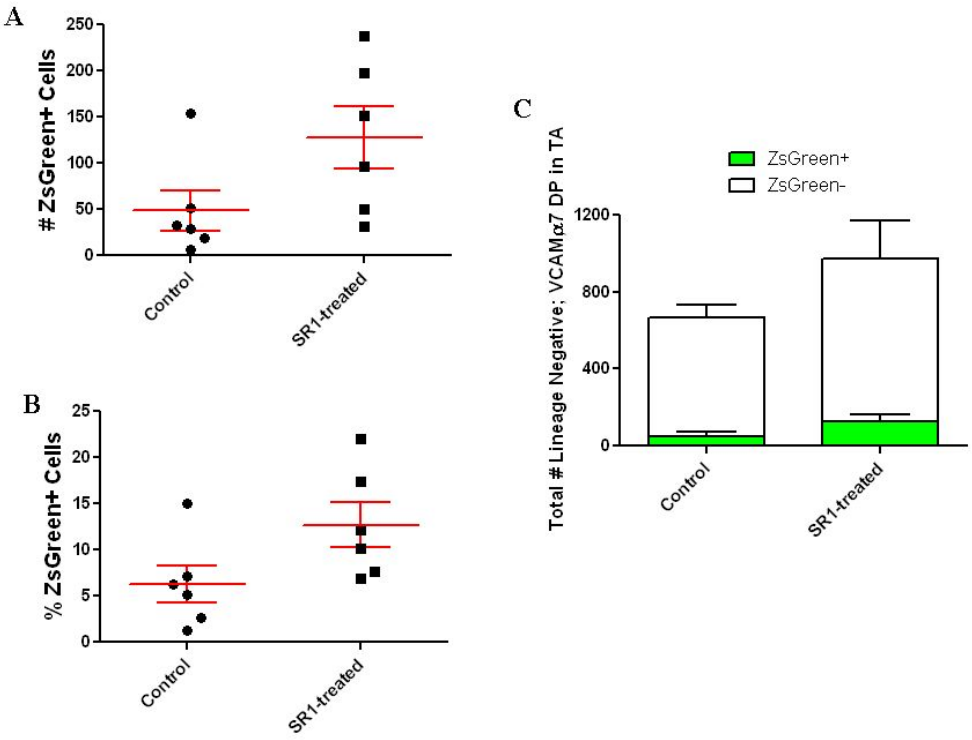


Figure (43). Satellite cell engraftment results of the SR1 treated versus untreated cells. (A) The absolute numbers of ZsGreen positive cells per TA of the transplanted mice. (B) Percentage of the ZsGreen cells of the total myogenic cell population of a TA of the 12 mice that were transplanted. (C) Total numbers of ZsGreen positive and ZsGreen negative fibers. (n=6)

f) Testing other compounds that have been described in literature to interact with AHR.

DIM (3,3′-diindolylmethane) has been described to be a ligand of Aryl Hydrocarbon Receptor (58). DIM inhibited matrigel induced differentiation (Figure 44). Using a concentration of DIM of 10 µM inhibited MyoD expression and Myogenin expression of the treated myoblast cells (Figure 45). The fore mentioned results indicate an inhibitory effect of DIM on myoblast differentiation and again refer to a possible role of AHR in satellite cell proliferation.

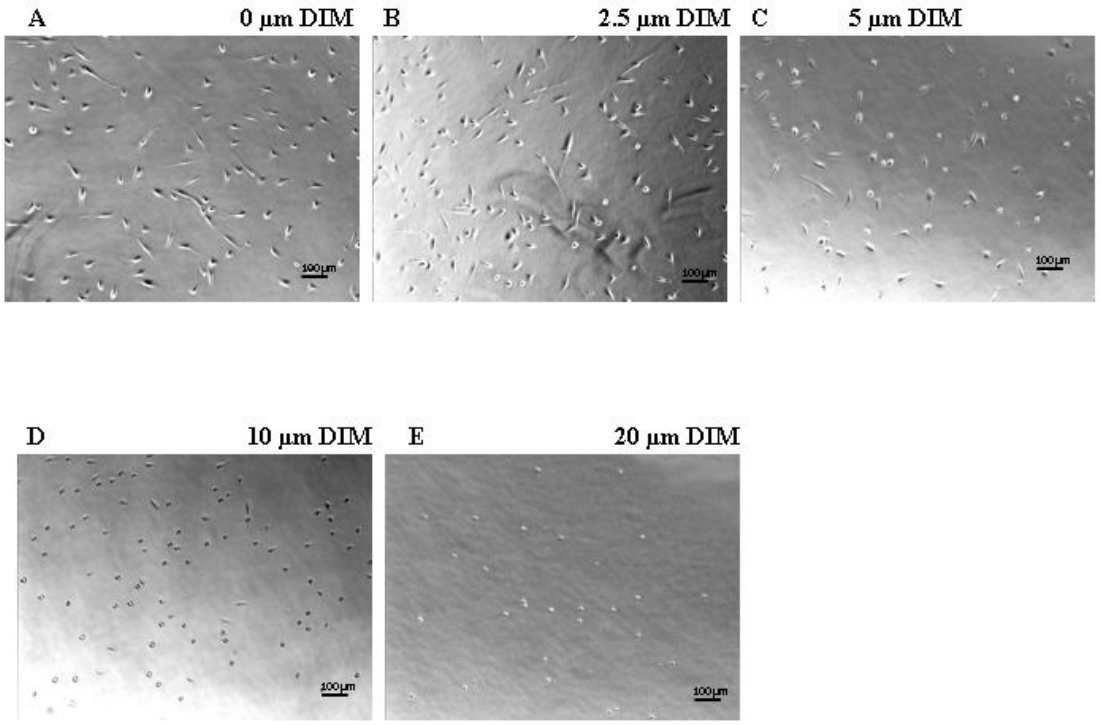


Figure (44) Effect of DIM on satellite cell differentiation on matrigel. (A) Control myoblasts incubated with 0.5% FBS medium alone for 2 days. (B) Testing 2.5 µM, concentration of DIM on cultured myoblasts. (C) Testing 5 µM, concentration of DIM on cultured myoblasts. (D) Testing 10 µM, concentration of DIM on cultured myoblasts. (E) Testing 20 µM, concentration of DIM on cultured myoblasts. Phase contrast images, Scale bar 100 µM.

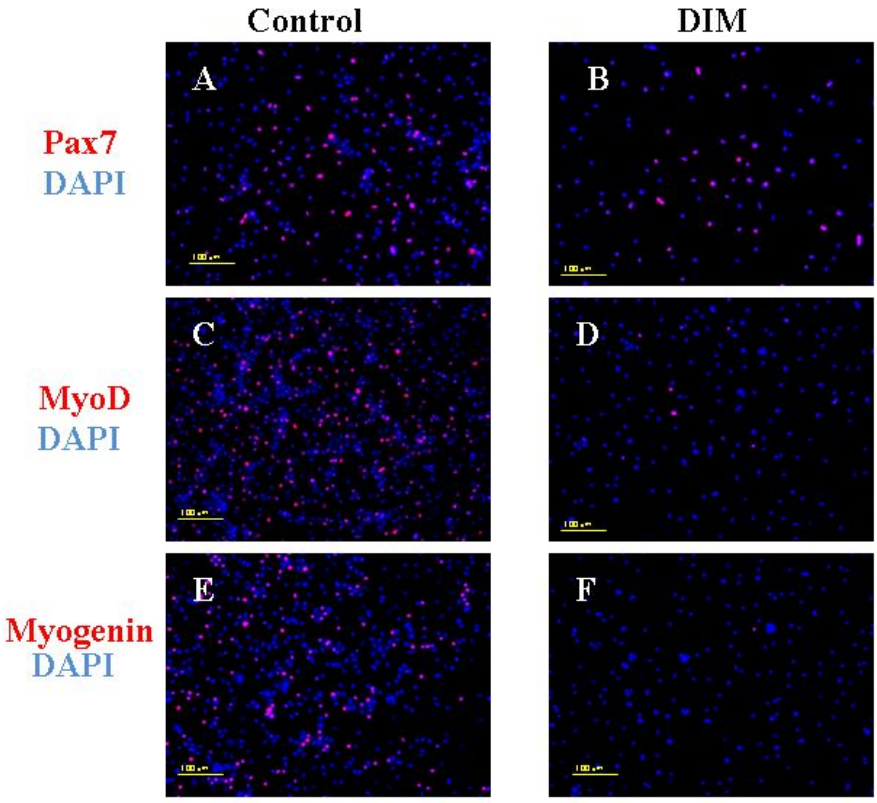


Figure (45). Effect of DIM on expression of MyoD and Myogenin of cultured myoblasts. Immunofluorescence for Pax7(A,B), MyoD (C,D), Myogenin(E,F) in red with DAPI (blue) used for counterstaining of the nuclei. Scale bar 100 µM.

g) Testing the effect of SR1 on other cell lines.

AHR has been described to be a ubiquitously expressed receptor. Fibroblast cells were stained for AHR and they were found to be positive, having cytoplasmic AHR staining pattern as evidenced by immunofluorescence (Figure 35). Following incubation of fibroblast cells with 1 µM concentration of SR1 in 0.5% FBS concentration was associated with an observed decline in fibroblast growth rate (Figure 46). The obtained result denotes a similar effect of SR1 on fibroblast cell proliferation to that on myoblasts and satellite cells.

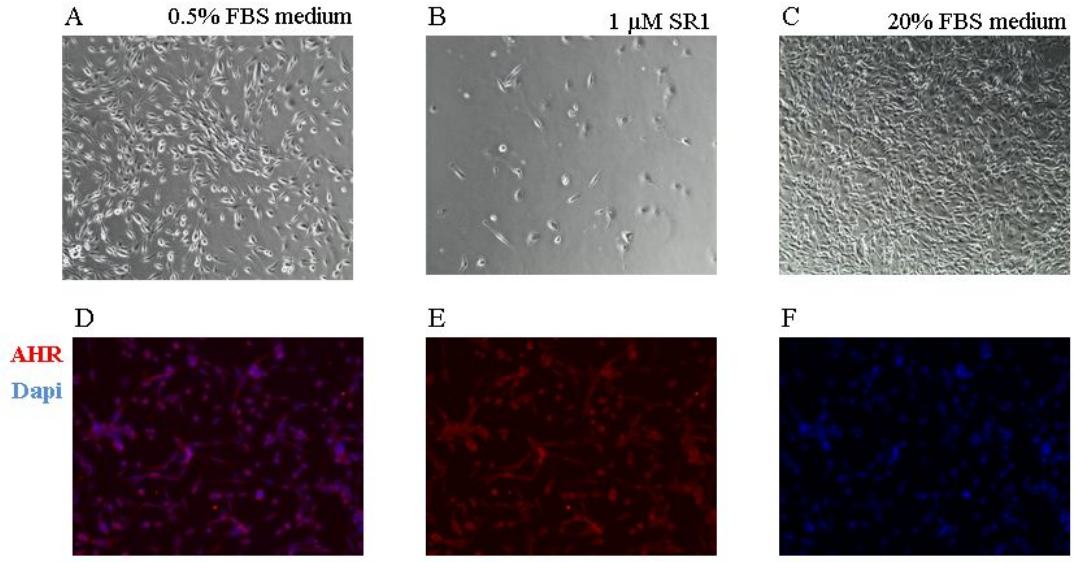


Figure (46). (A,B&C) Phase contrast images for testing the effect of SR1 on fibroblasts. (A) 0.5% FBS F10 plus FGF. (B) 0.5% FBS F10 plus FGF Plus 1 µM SR1 (C) Control of 20% FBS with no SR1. (D,E&F) Fibroblast cells stained by immunofluorescence for AHR (red) and DAPI (blue) indicating mostly cytoplasmic staining for Aryl Hydrocarbon receptor. Scale bar 100 µM.

Assessment of the expression of AHR in satellite cells

By immunofluorescence and RNA analysis, the expression of aryl hydrocarbon receptor has been tested in (cytospin preparation of freshly harvested satellite cells, early proliferating myoblasts, myoblasts that have been passaged several passages, early differentiating myoblasts and fully differentiated myotubes).

In quiescent satellite cells, AHR expression shows a cytoplasmic pattern of staining (figure 47). Staining of cross sections of wild type mouse T.A. muscle shows that AHR nuclear staining is missing in Pax7 positive satellite cells.

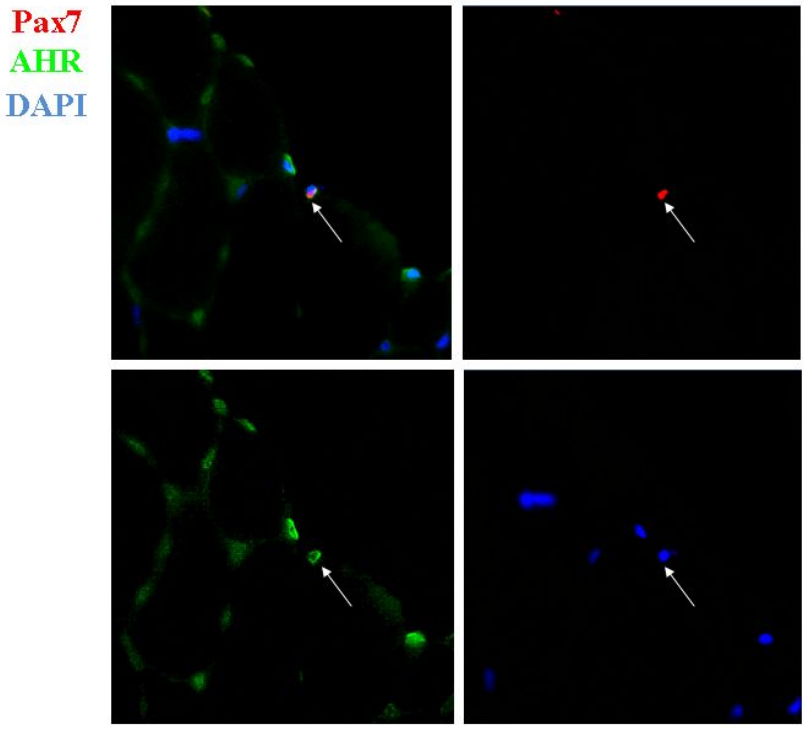


Figure (47). AHR expression in quiescent satellite cells. Immunostaining for AHR and Pax7 in cross sections of wild type TA muscle.

Cytospin preparation of **freshly isolated satellite cells** were stained for aryl hydrocarbon receptor. The cells were fixed around 6 hours following their activation as denoted by starting manipulation of the muscles. AHR staining of the cytospin preparation of SCs showed a cytoplasmic pattern of AHR staining indicating that AHR was not activated yet at this stage. (Figure 48)

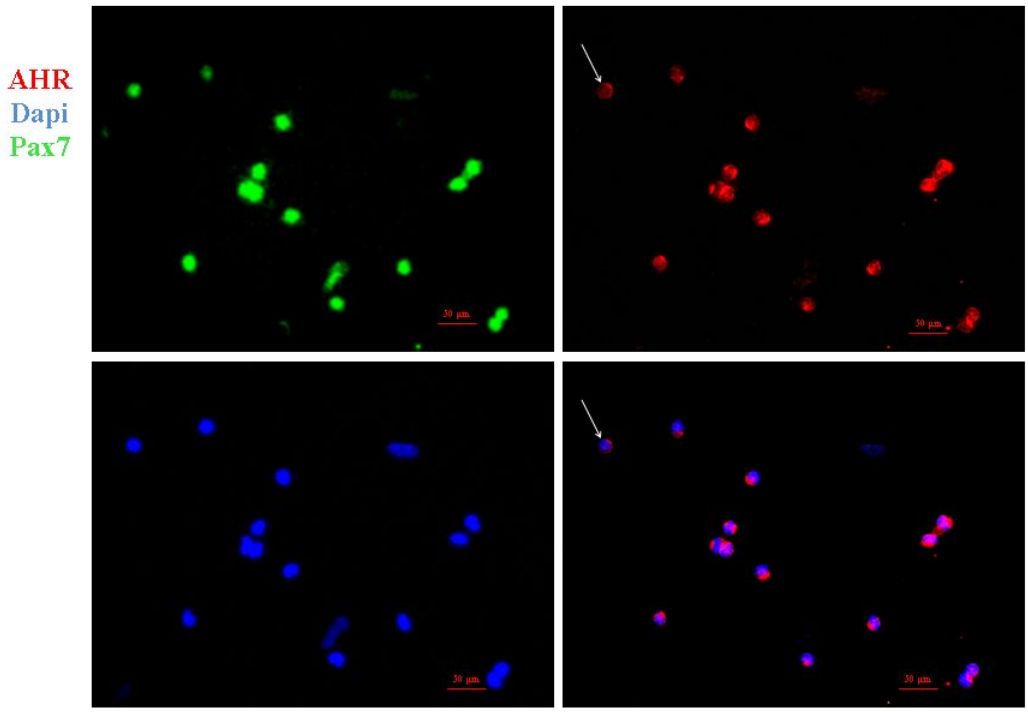


Figure (48). AHR expression in freshly isolated satellite cells. The cells are prepared by cytospin and stained for Pax7 (green), AHR (red) and DAPI (Blue). The arrow s pointing at a cell with clearly dominant cytoplasmic staining pattern of the receptor. Scale bar 50 µm.

In proliferating satellite cells, day-2 cultured satellite cells stained for AHR (Figure 49). AHR Showed, a) prominent cytoplasmic pattern of staining in the majority of the cells b) Decline in the level of AHR receptor expression in the differentiated cells (elongated myocytes) c) very few cells are showing positive nuclear and cytoplasmic staining of the receptor. The same pattern of AHR expression is maintained in day-4 cultured satellite cells (date not shown).

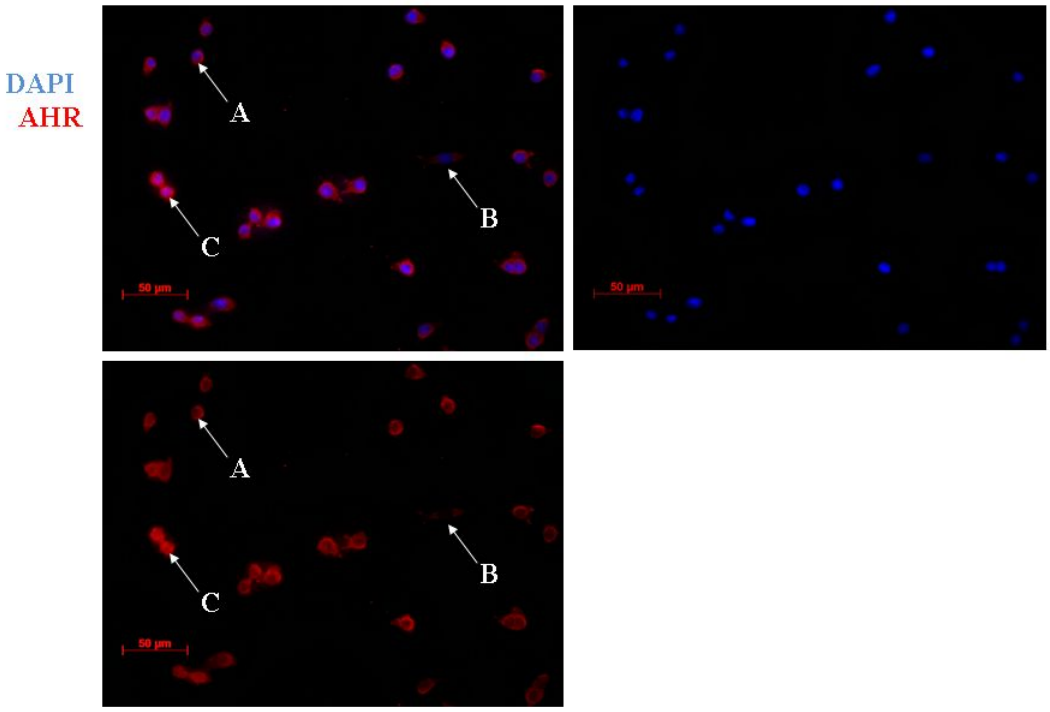
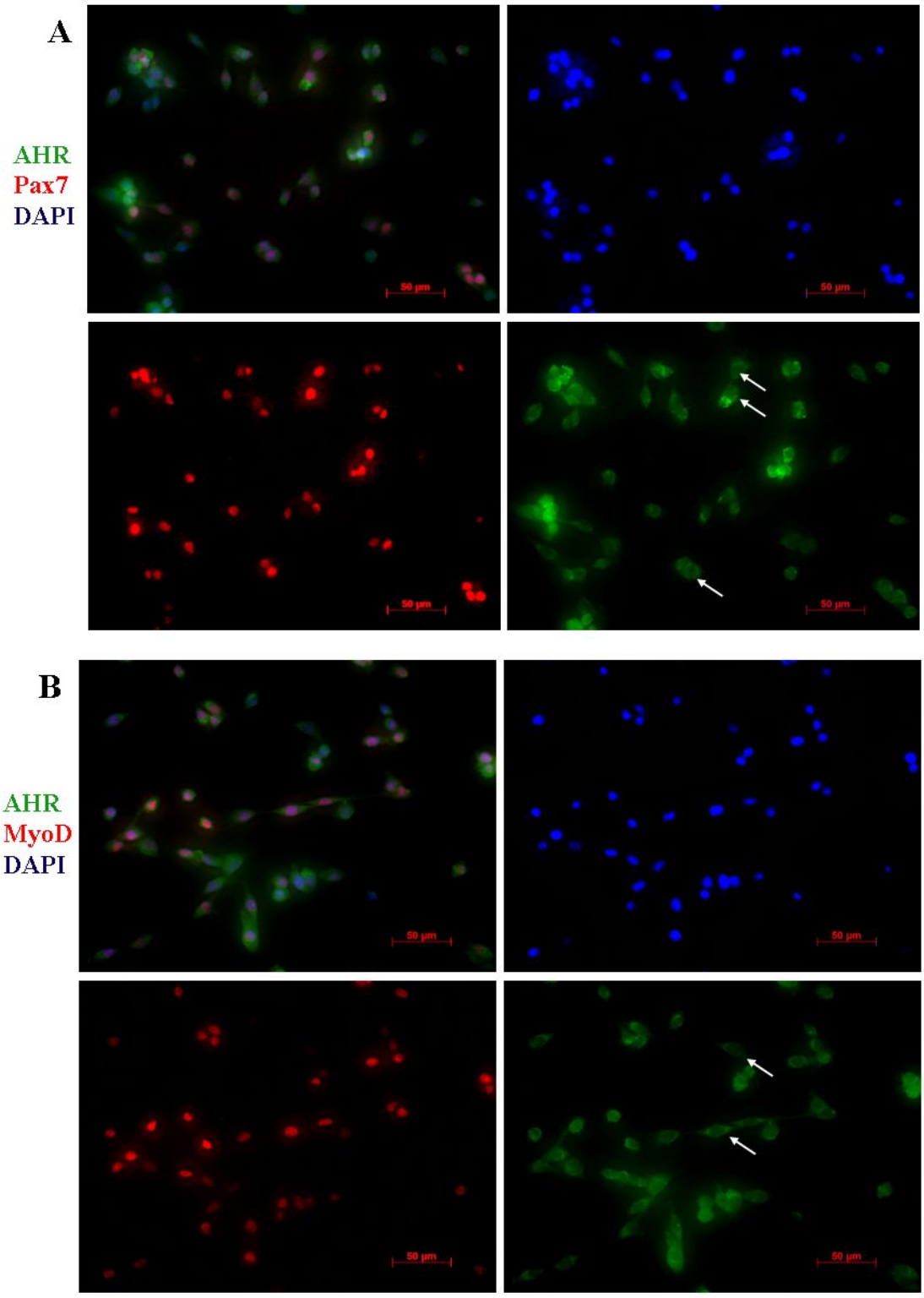


Figure (49).AHR expression in day 2 cultured satellite cells.

A) Cytoplasmic pattern of staining in some cells B) Down regulation of the AHR upon differentiation C) Some cells are showing Nuclear staining denoting activation. The cells were in proliferating medium (20% FBS medium).

In established myoblasts (cells that have been in culture for several weeks), the majority of the cells showed cytoplasmic pattern of AHR staining. Only few scattered cells were found positive for nuclear AHR (Figures 50).



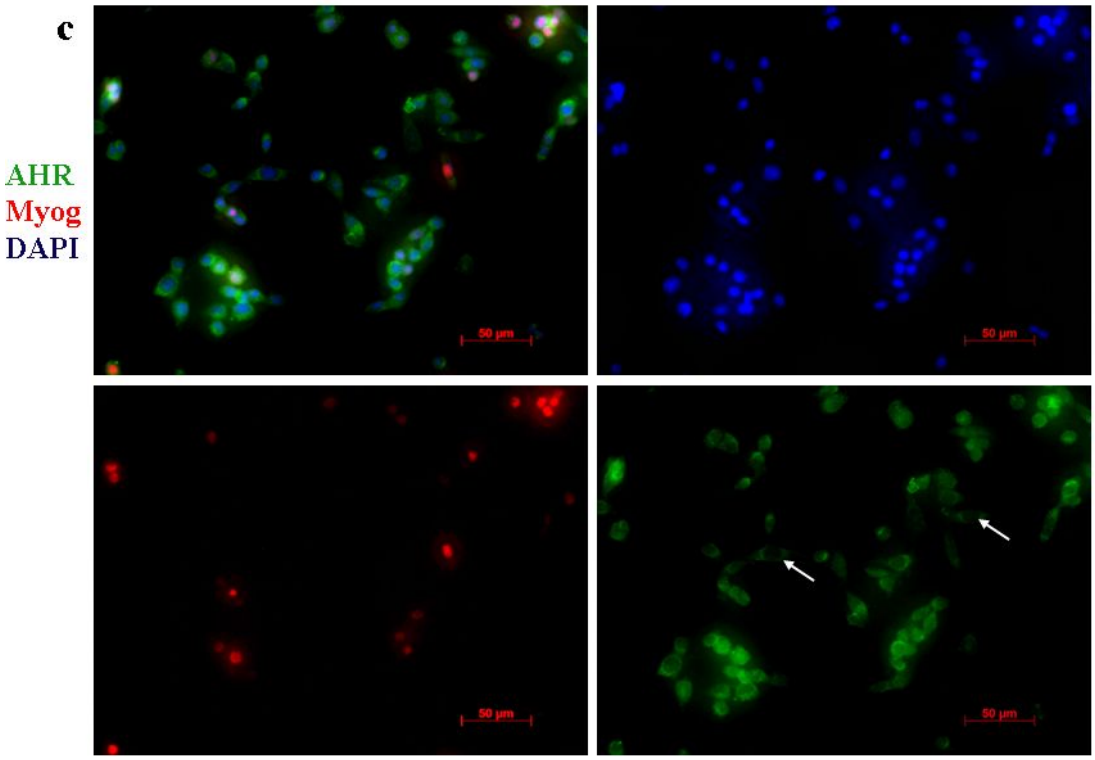
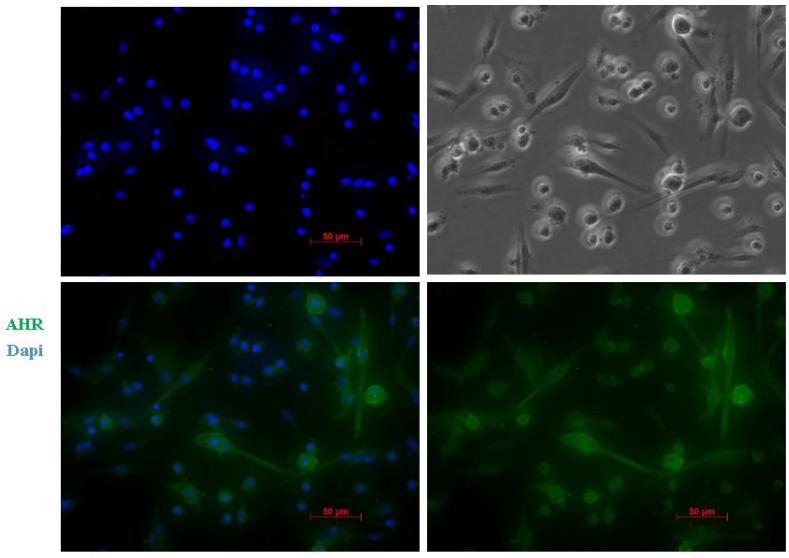


Figure (50). AHR expression in established myoblasts. The cells are proliferating myoblasts stained for pax7, MyoD, Myogenin (red), AHR (green) and DAPI (blue). The arrows refer to the predominant pattern of AHR expression in established myoblasts with a cytoplasmic staining pattern only. Scale bar 50 µm. The cells were in proliferating medium (20% FBS medium).

In differentiating Myoblasts, Myoblast cells were incubated in a differentiation medium for a period of 24/48 hours to stimulate the differentiation cascade. Following start of differentiation, the cells started to stimulate AHR as evidenced by acquisition of nuclear staining of the receptor that denotes nuclear translocation. When the cells were stained for AHR two points were observed (a) Increase in the percentage of cells that show nuclear AHR staining (b) the positive cells tend to be cells attain a large size (Figure 52). One hypothesis about these observations is that AHR activation as evidenced by nuclear translocation of the receptor may play a role in the process of myoblast differentiation. In addition, when I assessed the expression pattern of Cyp1B1 gene (a reporter gene which is known to be downstream of AHR (70)) in proliferating and differentiating myoblasts, I found a significant up-regulation (Figure 56). This is also indicative of the activity of the receptor which is suggestive of an implicated role in differentiation of cultured myoblasts. Stimulation of AHR was associated with loss of Pax7 staining and acquisition of Myogenin staining (Figure 52, 53). These two are amongst the major transcriptional changes that happen to myoblasts upon differentiation. When the cells were allowed to continue differentiation further increase in cell size was observed. In addition, the expression of AHR increased. This time AHR up-regulation was accompanied with loss of Myogenin (Figure 55). This later observation suggests a role of AHR in differentiation of myoblasts beyond that played by Myogenin.



- AHR staining of myoblsts that have been for 20 hours in 2% horse serum medium.

Figure (51). AHR expression in differentiating myoblasts. Myoblast cells that have been incubated in 2% HS differentiation medium for 20 hours. The cells are stained for AHR (green), DAPI (blue) and a phase contrast image was taken to show cell morphology. Scale bar 50 µm.

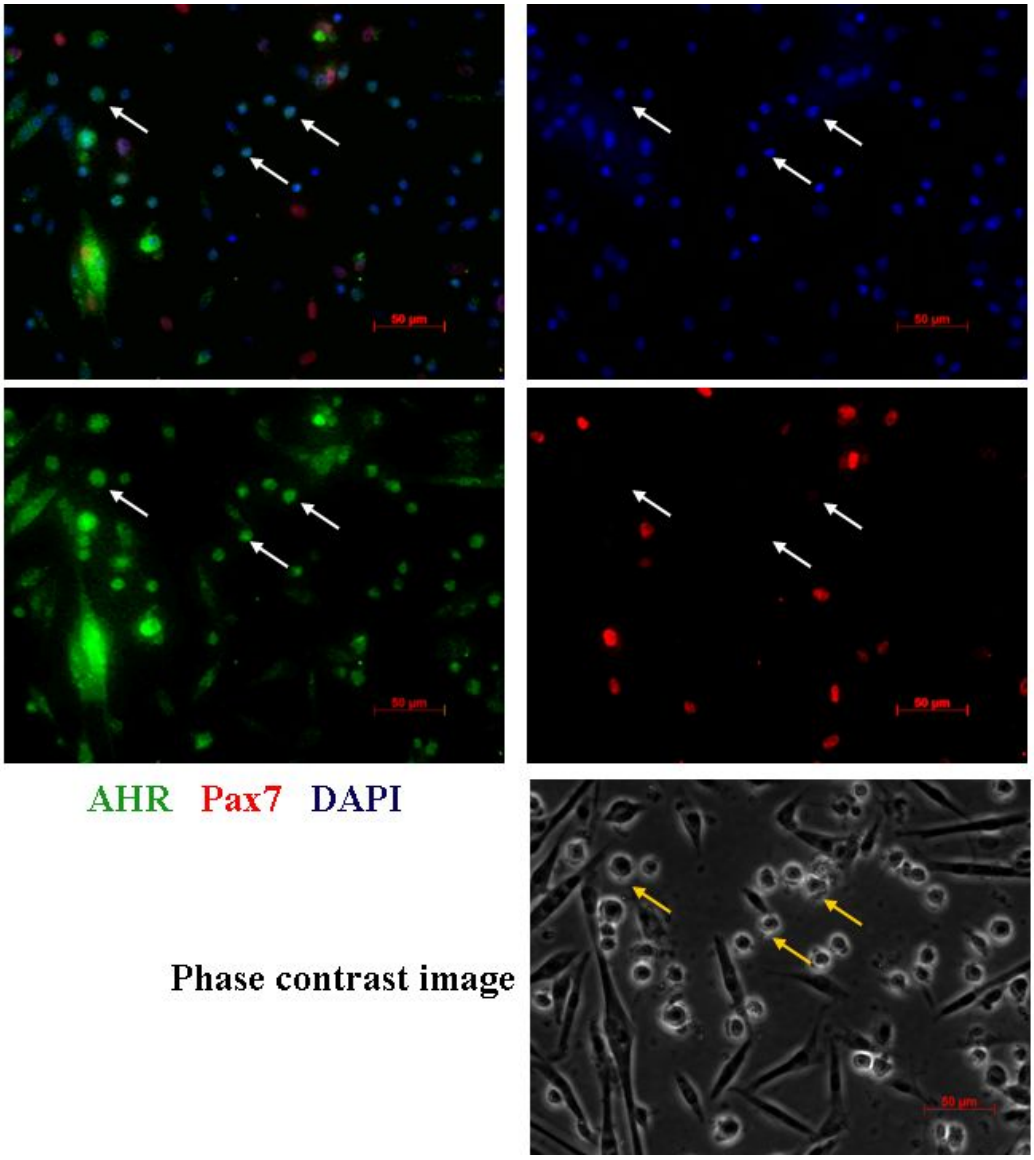


Figure (52). AHR expression in differentiating myoblasts (relationship with PAX7). Myoblast cells that have been incubated in 2% HS differentiation medium for 24 hours. The cells are stained for AHR (green), Pax7 (red), DAPI (blue) and a phase contrast image was taken to show cell morphology. Arrows refers to cells that have acquired nuclear AHR staining and losing Pax7.Scale bar 50 µm.

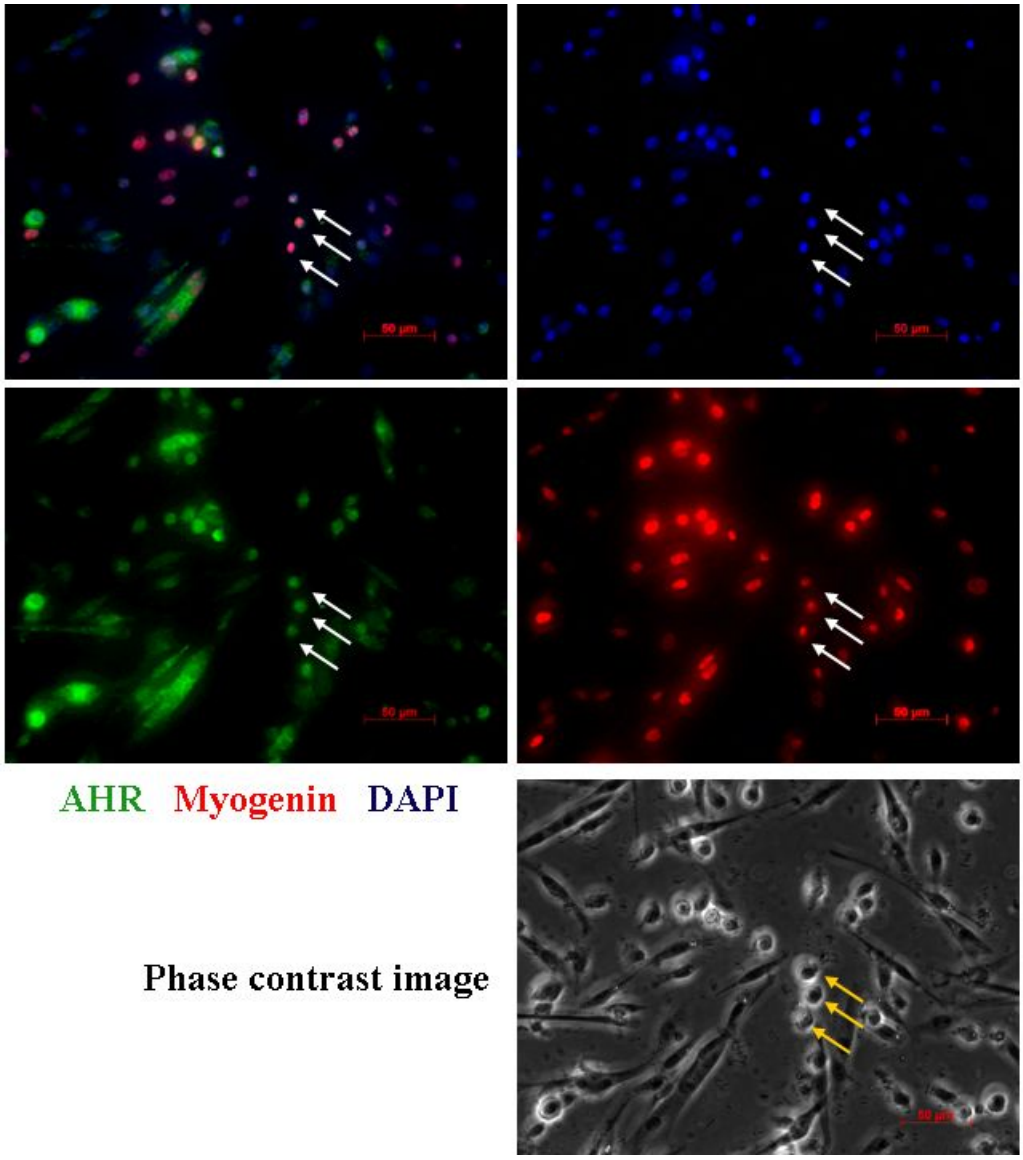


Figure (53). AHR expression in differentiating myoblasts (relationship with Myogenin). Myoblast cells that have been incubated in 2% HS differentiation medium for 24 hours. The cells are stained for AHR (green), Myogenin (red), DAPI (blue) and a phase contrast image was taken to show cell morphology. Arrows refers to cells that have acquired nuclear AHR staining and are expressing Myogenin. Scale bar 50 µm.

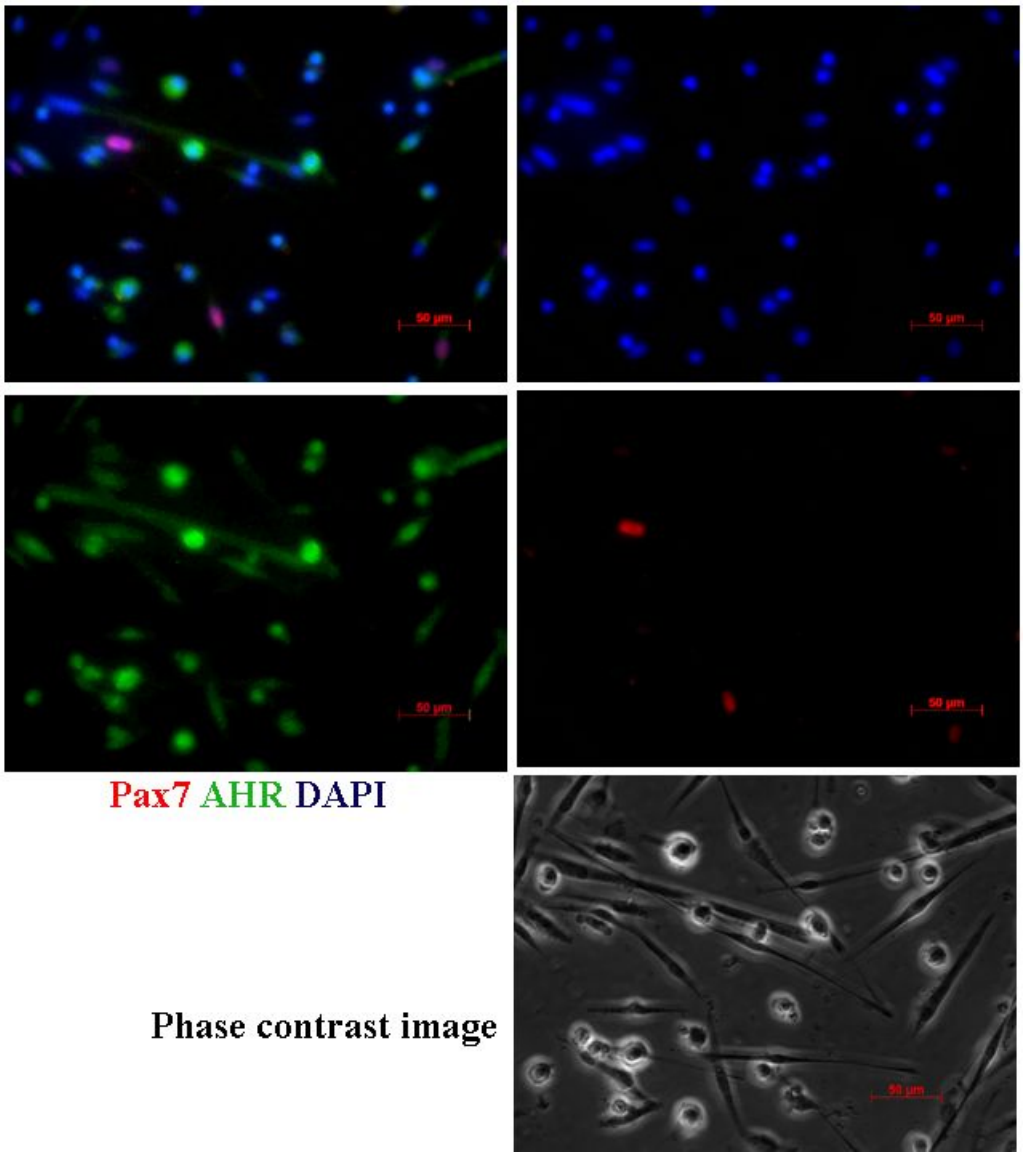


Figure (54). AHR expression in differentiating myoblasts (relationship with PAX7). Myoblast cells that have been incubated in 2% HS differentiation medium for 48 hours. The cells are stained for AHR (green), Pax7 (red), DAPI (blue) and a phase contrast image was taken to show cell morphology. Scale bar 50 µm.

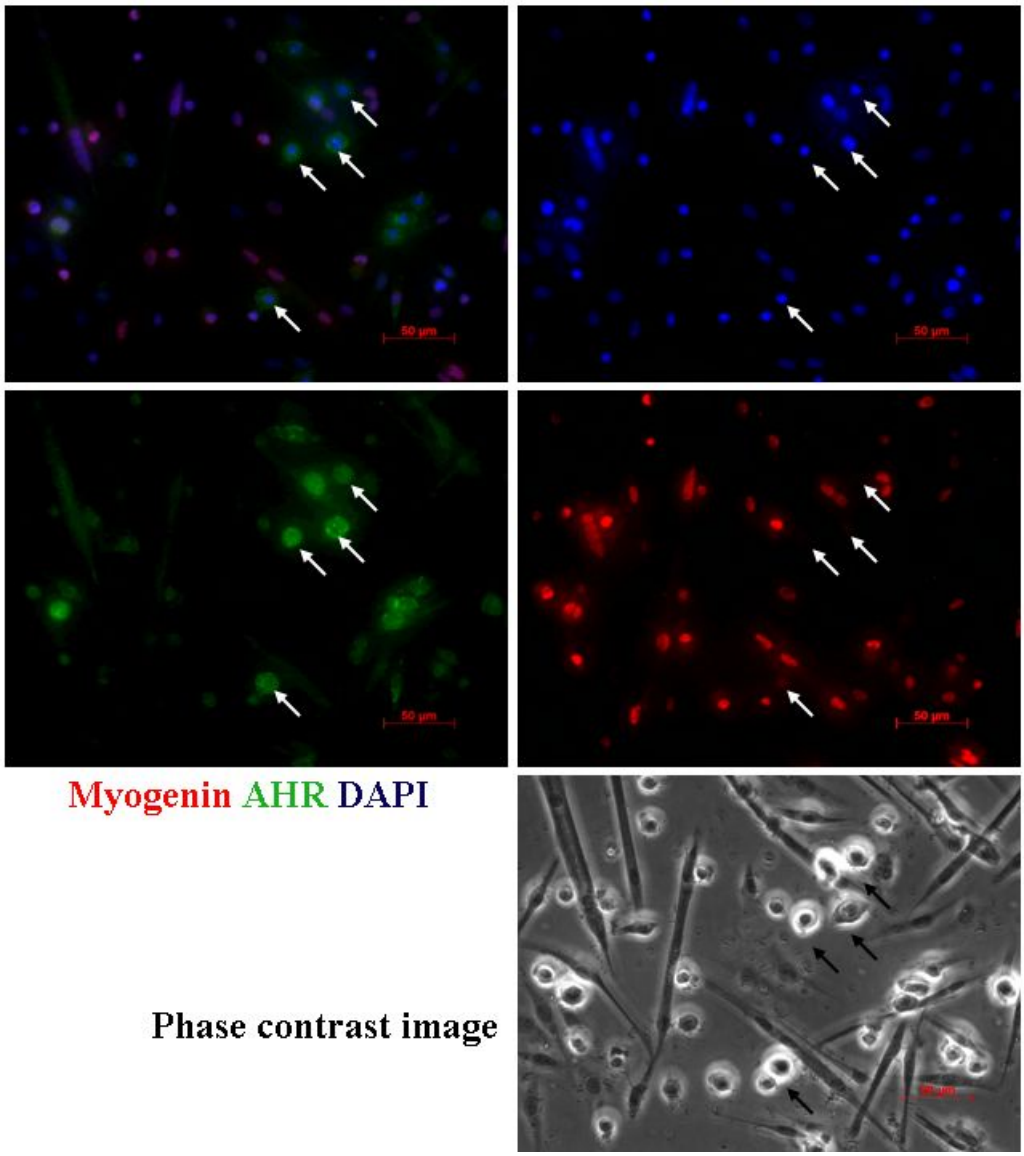


Figure (55). AHR expression in differentiating myoblasts (relationship with Myogenin). Myoblast cells that have been incubated in 2% HS differentiation medium for 48 hours. The cells are stained for AHR (green), Myogenin (red), DAPI (blue) and a phase contrast image was taken to show cell morphology. Arrows refers to cells that have acquired nuclear AHR staining and are lost Myogenin after a longer differentiation period was allowed. Scale bar 50 µm.

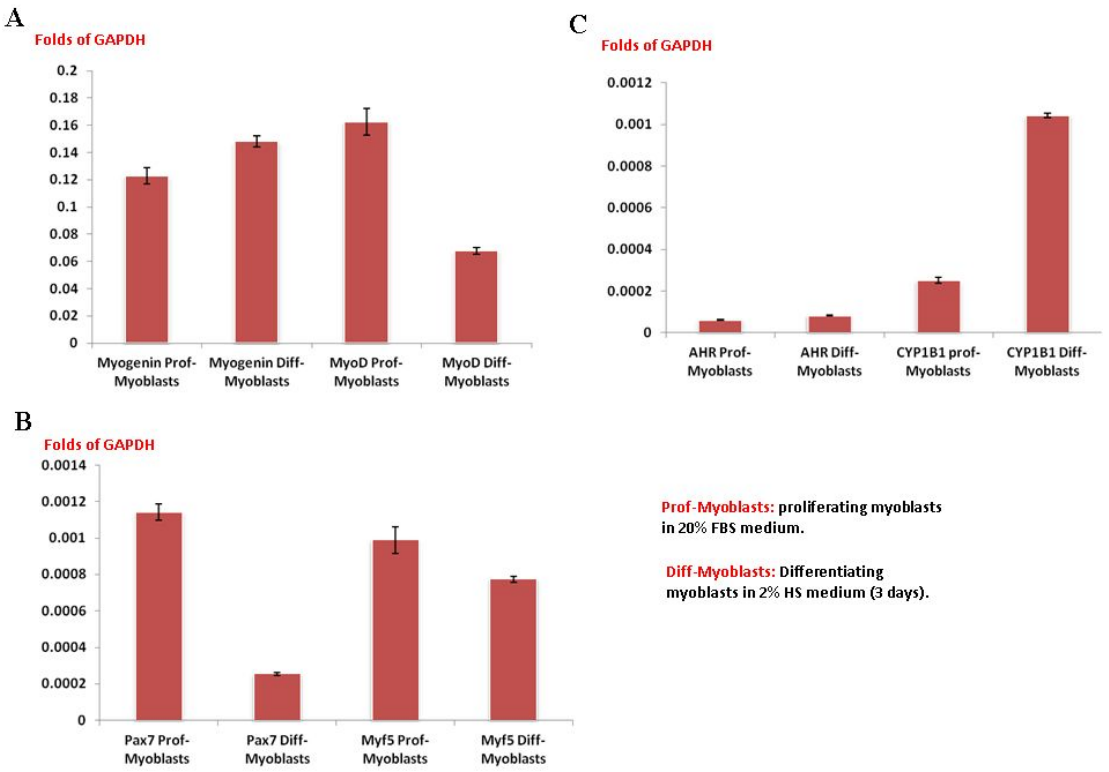


Figure (56) Activation of AHR upon differentiation of myoblasts. (n=3).

The cells were either in proliferating medium (20% FBS medium) or in differentiating medium (2% horse serum) for 3 days.

A) Up regulation of Myogenin and down regulation of MyoD following differentiation.

B) Down regulation of Pax7 and Myf5 following differentiation.

C) Up regulation of cyp1b1 indicative of AHR activation in response to differentiation.

In fully differentiated myotubes, the pattern of staining was totally a cytoplasmic pattern of staining (Figure 57). The expression of the receptor itself is down regulated as shown by Immunostaining for the receptor (Figure 57) and by RNA analysis of myoblasts following differentiation and complete differentiation. This down regulation of the receptor occurs simultaneously with its activation as evidenced by up regulation of cyp1b1 (figure 58). More over, rounded cells are still observed in differentiating cultures and are having markedly higher level of AHR expression (figure 57). These cells are hypothesized to be starting their differentiation cascade with increased propensity to form myotubes that would by followed with down regulation of the receptor.

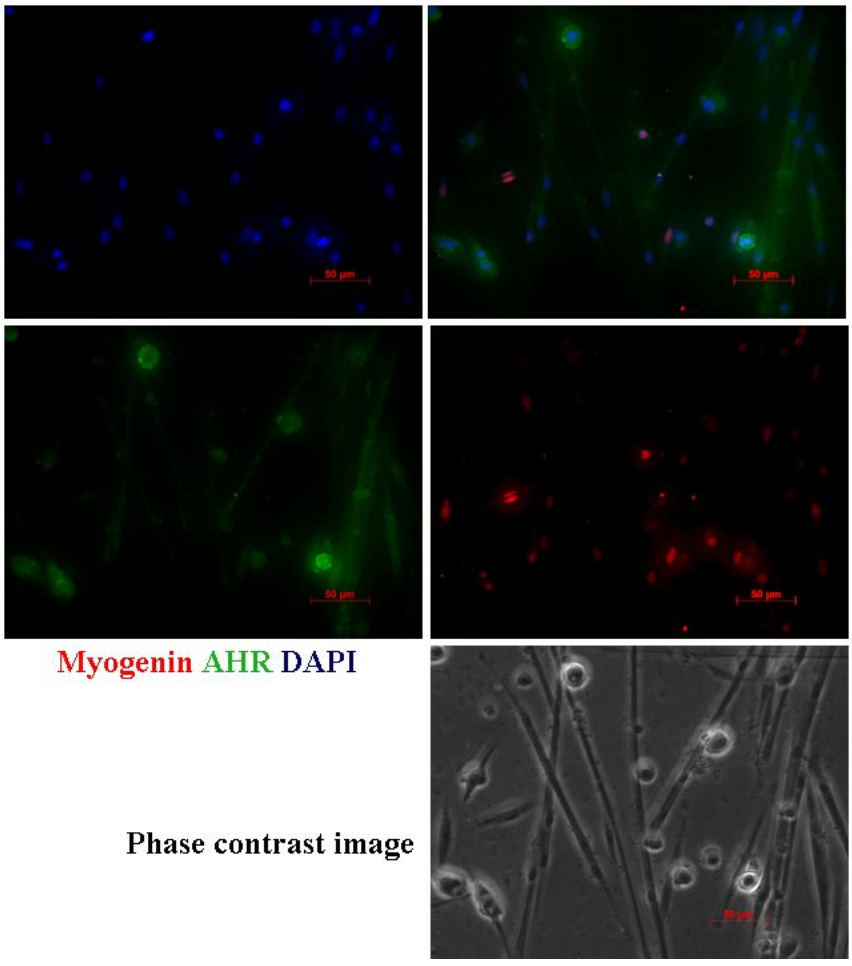


Figure (57). AHR expression in differentiating myoblasts (relationship with Myogenin). The cells are stained for AHR (green), Myogenin (red), DAPI (blue) and a phase contrast image was taken to show cell morphology. Scale bar 50 µm.

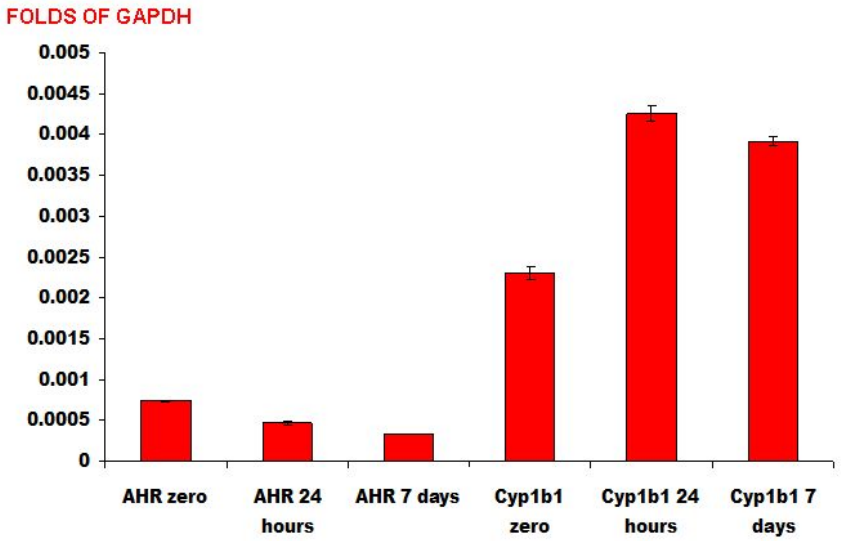


Figure (58). Down regulation of AHR expression after start of differentiation and activation of the receptor (evidenced by up regulation of cyp1b1). The figure shows RNA expression in established proliferating myoblasts (zero differentiation), cells that have been in 2% HS medium(differentiating medium) for 24 hours and for 7 days. (n=3).

So, the expression of AHR can be described to have a cytoplasmic pattern of staining in quiescent and early activated satellite cells. Nuclear translocation of the receptor occurs with start of differentiation. Nuclear translocation is associated with loss of Pax7 and acquisition of Myogenin expression. Upon full differentiation of the cells down-regulation of the receptor happens taking a cytoplasmic pattern of staining. To be noted that AHR activation has been previously described to be transient (69) and the receptor undergoes rapid translocation to the cytoplasm where it is degraded by cytoplasmic proteasome system (68). This is true about what I observed in AHR activation of being associated with active differentiation followed by down-regulation after differentiation is complete. In addition, AHR may be also activated during early satellite cell activation, but I did not follow the receptor close enough at this stage.

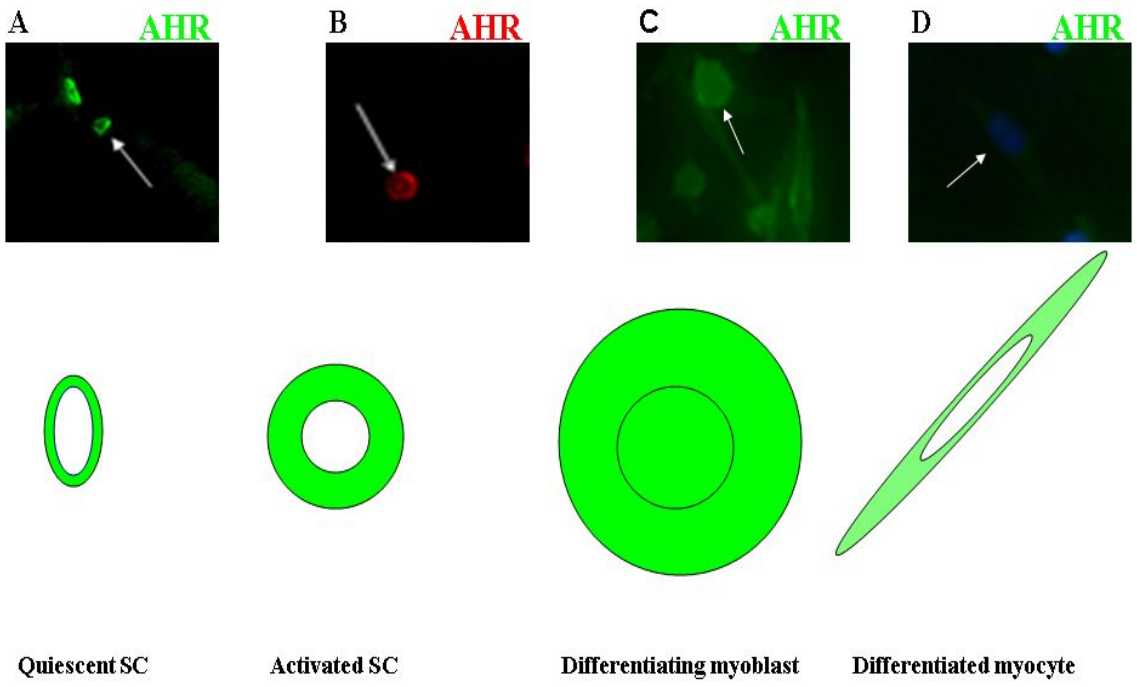


Figure (59). Expression of AHR in different stages of satellite cells.

A) cytoplasmic pattern of staining in quiescent satellite cells.

B) A predominant cytoplasmic pattern of staining in activated satellite cells (after 2 days in culture).

C) Nuclear translocation of AHR in differentiating myoblasts (20 hours in 2% horse serum).

D) A predominant cytoplasmic pattern of staining in differentiated elongated myocyte cells with decrease in staining intensity correlating with reduced expression of the receptor with differentaition.

Discussion

Potential applications of stem cells range from their use in drug discovery, disease modeling and cell transplantation therapy. Several obstacles must be overcome at first before any of these goals can be realized. In addition to the moral and ethical issues of using embryos, human embryonic stem cells have many other problems like genetic instability and cancer risk. Induced pluripotent stem cells developed by Yamanaka in 2006 (65), helped to solve the ethical problems of h-ESCs. Yet, concerns still exist for IPSCs like viral contamination, genetic instability and cancer development. Adult stem cells are different. Adult stem cells are highly committed cells (66). This makes adult stem cells more genetically stable; the liability for genetic deviation is much less, with resultant lower probability of cancer development. In addition, adult stem cells carry inherent ability to differentiate to their target cell line with no need for genetic manipulation or even cytokine stimulation. However, being highly committed cells makes adult stem cells a rare cell population. Proliferation of adult stem cells has always been at the core of researchers' thinking. Adult stem cells like Satellite cells (the adult stem cell which this thesis is investigating) are closer to their final fate. ESCs must first adopt mesodermal, ectodermal or endodermal fate before differentiating to their target cell line.

The aim of this project was to expand satellite cells for the aim of transplantation. I was trying to reach a way where I can proliferate satellite cells while conserving their transplantation efficiency. Trials of Myoblast transplantation have been carried out. These trials have succeeded to get the transplanted cells form new muscle fibers but failed to cause them repopulate satellite cell pool of recipient muscles. This is due to the difference in function between Myoblasts and satellite cells. Satellite cells proliferate in response to injury to achieve huge numbers of specialized myoblasts. In addition, they self renew to form new stem cells. On the other hand, Myoblasts fuse to form new muscle fibers. So, it is not the function of myoblasts to self-renew or at least late ones. Myoblasts' main function is to differentiate and fuse to form new muscle fibers. Myoblasts proliferate only *in vitro* when stimulated by growth factors. My results showed that in absence of h-FGF stimulation, Myoblast proliferation was markedly inhibited and

the cells started to differentiate. So, transplantation of Myoblasts may only be accompanied by new fiber formation that is still less efficient than uncultured satellite cells. Transplantation efficiency is reduced over several days of culture.

A strong relationship exists between satellite cell differentiation and proliferation. When the cells are proliferating they lose ZsGreen signal which means that they are going toward differentiation. This is depending on the fact that ZsGreen cells achieve efficient transplantation, although my data showed a disconnection between Pax7 and ZsGreen expression in cultured cells. It has been postulated that rapid proliferation of embryonic stem cells is associated with conservation of the undifferentiated state while ceasing of proliferation is associated with rapid differentiation. However, when satellite cells are not proliferating under certain conditions of the treated compounds, they remain undifferentiated (ZsGreen positive). Satellite cells have a number of states: quiescent state, activated state, proliferating state and differentiating state. In the quiescent state the cells are not dividing. They are residing beneath the basal lamina of skeletal muscle fibers waiting for an injury stimulus. So, when we say that satellite cells are not proliferating it may carry the possibility of being quiescent at least phenotypically, taking into consideration that there is an anatomical part in the definition of satellite cell quiescence. My analysis of this finding is that ZsGreen accumulates in the cells in vivo due to Pax7 expression of quiescent satellite cells. ZsGreen production stops in cultured satellite cells as evidenced by FACS analysis of cultured cells. In culture, the amount of ZsGreen protein in the cells is simply diluted by cell division which will not happen if the cells are not proliferating.

Epigenetics are thought to be responsible for control and regulation of the transcriptional state of many cell types. Epigenetic changes occur to satellite cells when they are activated into proliferating myoblasts. SAHA, a deacetylase inhibitor, was associated with a positive result in terms of the number of ZsGreen cells obtained after 4 days of stay in culture. Histone acetylation removes the positive charge from histones which is responsible for the interaction between the N termini of histones and the negatively charged phosphate of DNA leading to a more relaxed chromatin resulting in a greater level of transcription. This action is prevented by HDACs like SAHA. Exposing

satellite cells to SAHA should result in a more condensed chromatin with less active transcription resulting in a higher state of quiescence in the form of more ZsGreen cells.

The quiescence state of satellite cells has been shown to be an active process (54). Quiescent satellite cells are ready to respond to different stimuli by a multitude of actions (55). But, these cells are known to be not or rarely dividing during their quiescent state. So, we can collectively say that specialized myoblasts do not engraft efficiently and that quiescent satellite cells do not divide. So, manipulation of the cells with division capability (Myoblasts) to reach the phenotype of the cells of engraftment capability (quiescent satellite cells) is an effective approach to reach the aim of my project. One previous study (63) succeeded to prove that Myoblasts lacking Myod gene (MYOD -/- myoblasts) are conserving their stem cell properties and are having much higher engraftment properties. In addition, MyoD -/- myoblast derived satellite cells have been detected beneath the basal lamina of skeletal muscle fibers. This may refer the central commitment role that MyoD gene plays in satellite function. MyoD is a distinctive transcription factor that differentiate between satellite cells that are MyoD negative and Myoblasts that are MyoD positive. Demethylation of MyoD promoter is a required signal to initiate the activation of quiescent satellite cells (70).

Down regulation of MyoD in myoblasts may be the key to increase engraftment of myoblasts to a satellite stem cell like fashion. My design is not based on any genetic manipulation of the cells. I found that SR1 treatment of myoblasts converted them to a quiescent-like, MyoD negative phenotype. The treated cells have also been found to be Myogenin negative at a point where their untreated controls have a considerable proportion of Myogenin positivity. SR1 treatment also prevented formation of myotubes in low serum medium. This refers to a considerable inhibitory effect of SR1 on satellite cell and myoblast differentiation. So, SR1 treatment of myoblasts is expected to yield less differentiated cells that are hypothesized to achieve better engraftment.SR1 treated myoblasts (Day 5 cultured satellite cells) were transplanted to mouse models of muscle dystrophy. The engraftment results revealed that the number of dystrophin positive fibers (reflecting differentiation ability of the transplanted cells) were the same in the treated and the untreated myoblasts. However, satellite cell engraftment data was promising. The

total number of ZsGreen satellite cells of the SR1 treated samples was higher than the untreated cells. In addition, the mean of the number of ZsGreen positive cells was significantly higher in SR1 treated cells. This is an indicator of the better self renewable capacity under the effect of SR1 treated cells which is the main problem of cultured satellite cell transplantation.

Stemregenin 1 has been described to produce its action through AHR (aryl hydrocarbon receptor) antagonism (44). Studying the expression of AHR expression in different stages of satellite cells suggested a possible role for the receptor in the process of satellite cell differentiation. A nuclear translocation of the receptor in the cells occurs following differentiation. AHR nuclear translocation is accompanied with tendency to differentiate as evidenced by losing Pax7 expression and acquisition of myogenin. In addition, stimulation of myoblast differentiation by low serum medium is accompanied by up regulation of Cyp1b1 (a downstream gene of AHR that is used as a marker of its activity). Both the activation of AHR simultaneously with start of differentiation together with block of differentiation process by blocking the receptor suggests a role for AHR in the process satellite cell differentiation. Moreover, Myoblasts that activate AHR tend to increase in size and to have more than one nucleus at some times. Both conditions indicate that cells are differentiating, being ready to attain the elongated shape of a myocyte and to fuse with one another to form myotubes. The effect of AHR block on down regulating MyoD tends to be an indirect effect produced through overall block of one of the most important functions of a myoblast which is differentiation. Yet, being an indirect effect may add an advantage to the whole process because it leads to a change in the whole epigenetic and transcriptional state of the cell without affecting its vital functions needed to maintain it viable.

So, AHR has a strongly suggested role in satellite cell differentiation. But, The exact function of it in the differentiation pathways of satellite cells or the expression of different myogenic regulatory factors is in need to be further elucidated. Researchers dealing with satellite cells have been involved for along time in a continuous search for factors governing satellite cell differentiation. But, most of the previous trials as those involved with MyoD family or Pax7 family were actually dealing with transcription

factors. The idea about AHR is that we are dealing with a receptor with a known wide variety of ligands whether agonists or antagonists. This would definitely allow a much easier manipulation of the receptor which is a major advantage. In addition the transient character of AHR expression and activation is very much in harmony with changes occurring to satellite cells as they are activated, being time dependent and are also transient.

References

(1) The muscular dystrophies, Prof Alan EH Emery, FRCP , The lancet, Volume 359, Issue 9307, 23 February 2002, Pages 687–695

(2) Nelson Textbook of Pediatrics. 18th ed. Philadelphia, Pa:Saunders Elsevier; 2007:chap 608.Kliegman RM, Behrman RE, Jenson HB, Stanton BF. Muscular dystrophies. In: Kliegman RM, Behrman RE, Jenson HB, Stanton BF.

(3) Satellite cell of skeletal muscle fibers, Alexander Mauro. From The Rockefeller Institute. J Biophys Biochem Cytol. Feb 1, 1961; 9(2): 493–495.

(4) Satellite Cells as the Source of Nuclei in Muscles of Growing Rats , F. P. Moss, C. P. Leblond, Article first published online: 27 JAN 2005, DOI: 10.1002/ar.1091700405.

(5) Highly efficient, functional engraftment of skeletal muscle stem cells in dystrophic muscles. Cerletti M, Jurga S, Witczak CA, Hirshman MF, Shadrach JL, Goodyear LJ, Wagers AJ. Source Section on Developmental and Stem Cell Biology, Joslin Diabetes Center, One Joslin Place, Boston, MA 02115, USA. Cell. 2008 Jul 11;134(1):37-47. doi: 10.1016/j.cell.2008.05.049.

(6) Isolation of adult mouse myogenic progenitors: functional heterogeneity of cells within and engrafting skeletal muscle. Cell 119:543–554 Sherwood RI, Christensen JL, Conboy IM, Conboy MJ, Rando TA, Weissman IL, Wagers AJ (2004).

(7) Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J Cell Biol 151:1221–1234 Beauchamp JR, Heslop L, Yu DS, Tajbakhsh S, Kelly RG, Wernig A, Buckingham ME, et al. (2000).

(8) Cell heterogeneity in the myogenic lineage. Curr Top Dev Biol 23:185–208Cossu G, Molinaro M (1987).

(9) Dynamics of myoblast transplantation reveal a discrete minority of precursors with stem cell-like properties as the myogenic source. Beauchamp, J.R., Morgan, J.E., Pagel, C.N., and Partridge, T.A. J. Cell Biol. 144, 1113, 1999.

(10) Cell therapy for muscle regeneration and repair. Cao, B., Deasy, B.M., Pollett, J., and Huard, J. Phys. Med. Rehabil. Clin. N. Am. 16, 889, 2005.

(11) Self-renewal and expansion of single transplanted muscle stem cells. Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM Nature. 2008 Nov 27; 456(7221):502-6.

(12) Cell therapies for muscular dystrophy. Blau HM. N Engl J Med. 2008;359:1403–1405.

(13) Dynamics of myogenesis *in vitro*. COOPER WG, KONIGSBERG IR. Anat Rec. 1961 Jul;140:195-205.

(14) McLoon LK, Rowe J, Wirtschafter J, McCormick KM (2004) Continuous myofiber remodeling in uninjured extraocular myofibers: myonuclear turnover and evidence for apoptosis. Muscle Nerve 29:707–715.

(15) Porter JD, Merriam AP, Khanna S, Andrade FH, Richmonds CR, Leahy P, Cheng G, et al. (2003) Constitutive properties, not molecular adaptations, mediate extraocular muscle sparing in dystrophic mdx mice. FASEB J 17:893–895.

(16) Pavlath GK, Thaloor D, Rando TA, Cheong M, English AW, Zheng B(1998) Heterogeneity among muscle precursor cells in adult skeletal muscles with differing regenerative capacities. Dev Dyn 212: 495–508.

(17) Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells Relaix F, Montarras D, Zaffran S, Gayraud-Morel B, Rocancourt D, Tajbakhsh S, Mansouri A, et al. (2006). J Cell Biol 172:91–102.

(18) HGF/SF Is Present in Normal Adult Skeletal Muscle and Is Capable of Activating Satellite Cells Ryuichi Tatsumi,* Judy E. Anderson,† Cedrine J. Nevoret,‡ Orna Halevy,§ and Ronald E. Allen,1 DEVELOPMENTAL BIOLOGY 194, 114–128 (1998) ARTICLE NO. DB978803.

(19) Effects of transforming growth factor-beta (TGF-β1) on satellite cell activation and survival during oxidative stress. Rathbone CR, Yamanouchi K, Chen XK, Nevoret-Bell CJ, Rhoads RP, Allen RE. J Muscle Res Cell Motil. 2011 Sep;32(2):99-109. doi: 10.1007/s10974-011-9255-8. Epub 2011 Aug 8.

(20) Pleiotropic effects of sonic hedgehog on muscle satellite cells. Koleva M, Kappler R, Vogler M, Herwig A, Fulda S, Hahn H. Source Institute of Human Genetics, Heinrich-Dueker-Weg 12, Goettingen, Germany.

(21) β-catenin promotes self-renewal of skeletal-muscle satellite cells Ana Perez-Ruiz1, Yusuke Ono1, Viola F. Gnocchi1,2 and Peter S. Zammit1.

(22) Myogenic satellite cells: physiology to molecular biology Thomas J. Hawke1 and Daniel J. Garry1,2.

(23) The FGFR1 inhibitor PD 173074 selectively and potently antagonizes FGF-2 neurotrophic and neurotropic effects. Skaper SD, Kee WJ, Facci L, Macdonald G, Doherty P, Walsh FS. Source Department of Neuroscience Research, SmithKline Beecham Pharmaceuticals, Essex, England. Stephen_Skaper-1@sbphrd.com.

(24) Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer Baozhi Chen,^,1 Michael E. Dodge,^,1 Wei Tang,1

Jianming Lu,2 Zhiqiang Ma,2 Chih-Wei Fan,1 Shuguang Wei,2 Wayne Hao,2 Jessica Kilgore,2 Noelle S. Williams,2 Michael G. Roth,2 James F. Amatruda,3 Chuo Chen,2 and Lawrence Lum1.

(25) Notch-Mediated Restoration of Regenerative Potential to Aged Muscle. Irina M. Conboy1, Michael J. Conboy1, Gayle M. Smythe1, Thomas A. Rando1,2,*

(26) Kinetics of Myoblast Proliferation Show That Resident Satellite Cells Are competent to fully regenerate Skeletal Muscle Fibers Peter S. Zammit,1 Louise Heslop, Vale´rie Hudon, J. David Rosenblatt, Shahragim Tajbakhsh,† Margaret E. Buckingham,* Jonathan R. Beauchamp, and Terence A. Partridge.

(27) Asymmetric Self-Renewal and Commitment of Satellite Stem Cells in Muscle Shihuan Kuang,1 Kazuki Kuroda,1 Fabien Le Grand,1 and Michael A. Rudnicki1,2.

(28) Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells' Naoko Yoshida1,2,*, Shosei Yoshida1,*,†, Kyoko Koishi3, Kanjiro Masuda2 and Yo-ichi Nabeshima1,4

(29) Pax7 is required for the specification of myogenic satellite cells. Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. Source Department of Biology, McMaster University, Hamilton, Ontario, Canada.

(30) Myf5-Positive Satellite Cells Contribute to *Pax7*-Dependent Long-Term Maintenance of Adult Muscle Stem Cells Stefan Günther1, Johnny Kim1, Sawa Kostin1,
Christoph Lepper2, Chen-Ming Fan2, Thomas Braun1.

(31) Prospective isolation of skeletal muscle stem cells with a Pax7 reporter. Bosnakovski D, Xu Z, Li W, Thet S, Cleaver O, Perlingeiro RC, Kyba M. Source

Lillehei Heart Institute and Department of Pediatrics, University of Minnesota, Minneapolis, Minnesota, USA.

(32) Duchenne muscular dystrophy: pathogenetic aspects and genetic prevention. Moser H. Hum Genet. 1984;66(1):17-40.

(33) Evidence-based path to newborn screening for Duchenne muscular dystrophy. Mendell JR1, Shilling C, Leslie ND, Flanigan KM, al-Dahhak R, Gastier-Foster J, Kneile K, Dunn DM, Duval B, Aoyagi A, Hamil C, Mahmoud M, Roush K, Bird L,Rankin C, Lilly H, Street N, Chandrasekar R, Weiss RB. Ann Neurol. 2012 Mar;71(3):304-13. doi: 10.1002/ana.23528.

(34) J. D. Gumerson and D. E. Michele, "The dystrophin-glycoprotein complex in the prevention of muscle damage," Journal of Biomedicine and Biotechnology, vol. 2011, Article ID 210797, 13 pages, 2011.

(35) In Memoriam: Robert A. Good May 21, 1922–June 13, 2003, by Max D. Cooper, The Journal of Immunology, 2003, 171: 6318-6319.

(36) D. N. Landon, "Skeletal muscle: normal morphology, development and innervation," in Skeletal Muscle Pathology, pp. 1–87, Churchil Livingstone, Edinburgh, UK, 1982.

(37) A. M. Neumeyer, D. M. DiGregorio, and R. H. Brown Jr., "Arterial delivery of myoblasts to skeletal muscle," Neurology, vol. 42, no. 12, pp. 2258–2262, 1992.

(38) T. A. Partridge, "Invited review: myoblast transfer: a possible therapy for inherited myopathies?"Muscle & Nerve, vol. 14, no. 3, pp. 197–212, 1991.

(39) Lindstrom M, Thornell LE: New multiple labelling method for improved satellite cell identification in human muscle: application to a cohort of power-lifters and sedentary men. Histochem Cell Biol 2009, 132:141–157.

(40) Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal Hugo C. Olguin, Bradley B. Olwin http://dx.doi.org/10.1016/j.ydbio.2004.08.015

(41) Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'.Yoshida N1, Yoshida S, Koishi K, Masuda K, Nabeshima Y.
J Cell Sci. 1998 Mar;111 (Pt 6):769-79.

(42) A new immuno-, dystrophin-deficient model, the NSG-mdx(4Cv) mouse, provides evidence for functional improvement following allogeneic satellite cell transplantation. Arpke RW, Darabi R, Mader TL, Zhang Y, Toyama A, Lonetree CL, Nash N, Lowe DA, Perlingeiro RC, Kyba M. Stem Cells. 2013 Aug;31(8):1611-20. doi: 10.1002/stem.1402.

(43) Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? Zammit PS1, Golding JP, Nagata Y, Hudon V, Partridge TA, Beauchamp JR. J Cell Biol. 2004 Aug 2;166(3):347-57. Epub 2004 Jul 26.

(44) Aryl Hydrocarbon Receptor Antagonists Promote the Expansion of Human Hematopoietic Stem Cells. Anthony E. Boitano, Jian Wang, Russell Romeo, Laure C. Bouchez, Albert E. Parker, Sue E. Sutton, John R. Walker, Colin A. Flaveny, Gary H. Perdew, Michael S. Denison, Peter G. Schultz,1, and Michael P. Cooke. Science. Sep 10, 2010; 329(5997): 1345–1348.

(45) Serum alters the uptake and relative potencies of halogenated aromatic hydrocarbons in cell culture bioassays. Hestermann EV1, Stegeman JJ, Hahn ME.Toxicol Sci. 2000 Feb;53(2):316-25.

(46) Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. Bushby K1, Finkel R, Birnkrant DJ, Case LE, Clemens PR, Cripe L, Kaul A, Kinnett K, McDonald C, Pandya S, Poysky J, Shapiro F, Tomezsko J, Constantin C;DMD Care Considerations Working Group. Lancet Neurol. 2010 Jan;9(1):77-93. doi: 10.1016/S1474-4422(09)70271-6. Epub 2009 Nov 27.

(47) Myoblast transfer in the treatment of Duchenne's muscular dystrophy.Mendell JR, Kissel JT, Amato AA, King W, Signore L, Prior TW, Sahenk Z, Benson S, McAndrew PE, Rice R. N Engl J Med. 1995 Sep 28; 333(13):832-8.

(48) In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. Cooper RN1, Tajbakhsh S, Mouly V, Cossu G, Buckingham M, Butler-Browne GS. J Cell Sci. 1999 Sep;112 (Pt 17):2895-901.

(49) Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. Burbach KM1, Poland A, Bradfield CA. Proc Natl Acad Sci U S A. 1992 Sep 1;89(17):8185-9.

(50) Identification of functional domains of the aryl hydrocarbon receptor. Fukunaga BN1, Probst MR, Reisz-Porszasz S, Hankinson O. J Biol Chem. 1995 Dec 8;270(49):29270-8.

(51) Involvement of the xenobiotic response element (XRE) in Ah receptor-mediated induction of human UDP-glucuronosyltransferase 1A1. Yueh MF1, Huang YH, Hiller A, Chen S, Nguyen N, Tukey RH. J Biol Chem. 2003 Apr 25;278(17):15001-6. Epub 2003 Feb 3.

(52) Evidence for an aryl hydrocarbon receptor-mediated cytochrome p450 autoregulatory pathway. Chiaro CR1, Patel RD, Marcus CB, Perdew GH. Mol Pharmacol. 2007 Nov;72(5):1369-79. Epub 2007 Aug 24.

(53) The aryl hydrocarbon receptor: studies using the AHR-null mice. Gonzalez FJ1, Fernandez-Salguero P. Drug Metab Dispos. 1998 Dec;26(12):1194-8.

(54) Chromatin modifications as determinants of muscle stem cell quiescence and chronological aging. Liu L1, Cheung TH, Charville GW, Hurgo BM, Leavitt T, Shih J, Brunet A, Rando TA. Cell Rep. 2013 Jul 11;4(1):189-204. doi: 10.1016/j.celrep.2013.05.043. Epub 2013 Jun 27.

(55) Lying low but ready for action: the quiescent muscle satellite cell. Montarras D1, L'honoré A, Buckingham M. FEBS J. 2013 Sep;280(17):4036-50. doi: 10.1111/febs.12372. Epub 2013 Jul 12.

(56) Selective glycogen synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilization *in vitro* and in vivo. Ring DB, Johnson KW, Henriksen EJ, Nuss JM, Goff D, Kinnick TR, Ma ST, Reeder JW, Samuels I, Slabiak T, Wagman AS, Hammond ME, Harrison SD. Diabetes. 2003 Mar;52(3):588-95.

(57) The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin. Butler LM, Zhou X, Xu WS, Scher HI, Rifkind RA, Marks PA, Richon VM. Proc Natl Acad Sci U S A. 2002 Sep 3;99(18):11700-5. Epub 2002 Aug 20.

(58) Toxic and chemopreventive ligands preferentially activate distinct aryl hydrocarbon receptor pathways: implications for cancer prevention. Okino ST1, Pookot D, Basak S, Dahiya R. Cancer Prev Res (Phila). 2009 Mar;2(3):251-6. doi: 10.1158/1940-6207.CAPR-08-0146. Epub 2009 Feb 17.

(59) Skeletal muscle satellite cell proliferation in response to members of the fibroblast growth factor family and hepatocyte growth factor. Sheehan SM, Allen RE. J Cell Physiol. 1999 Dec;181(3):499-506.

(60) Matrigel, but not collagen I, maintains the differentiation capacity of muscle derived cells *in vitro*. Grefte S1, Vullinghs S, Kuijpers-Jagtman AM, Torensma R, Von den Hoff JW. Biomed Mater. 2012 Oct;7(5):055004. doi: 10.1088/1748-6041/7/5/055004. Epub 2012 Jul 13.

(61) Muscle satellite cells are primed for myogenesis but maintain quiescence with sequestration of Myf5 mRNA targeted by microRNA-31 in mRNP granules. Crist CG1, Montarras D, Buckingham M. Cell Stem Cell. 2012 Jul 6;11(1):118-26. doi: 10.1016/j.stem.2012.03.011

(62) Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? Zammit PS1, Golding JP, Nagata Y, Hudon V, Partridge TA, Beauchamp JR. J Cell Biol. 2004 Aug 2;166(3):347-57. Epub 2004 Jul 26.

(63) Increased survival of muscle stem cells lacking the MyoD gene after transplantation into regenerating skeletal muscle. Atsushi Asakura, Hiroyuki Hirai, Boris Kablar, Shigeru Morita, Jeff Ishibashi, Bryan A. Piras, Amanda J. Christ, Mayank Verma, Karin A. Vineretsky, and Michael A. Rudnicki. Published online Oct 10, 2007. doi: 10.1073/pnas.0708145104.

(64) Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Collins CA1, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, Morgan JE. Cell. 2005 Jul 29;122(2):289-301.

(65) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Takahashi K1, Yamanaka S. Cell. 2006 Aug 25;126(4):663-76. Epub 2006 Aug 10.

(66) Plasticity of adult stem cells. Wagers AJ1, Weissman IL. Cell. 2004 Mar 5;116(5):639-48.

(67) Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. Kristen J. Nowak and Kay E. Davies. EMBO Rep. Sep 2004; 5(9): 872–876. doi: 10.1038/sj.embor.7400221

(68) Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytosplasmic proteasome following nuclear export. Davarinos NA1, Pollenz RS. J Biol Chem. 1999 Oct 1;274(40):28708-15.

(69) Timing is everything: consequences of transient and sustained AhR activity. Mitchell KA1, Elferink CJ. Biochem Pharmacol. 2009 Mar 15;77(6):947-56. doi: 10.1016/j.bcp.2008.10.028. Epub 2008 Nov 6.

(70) The epigenetic network regulating muscle development and regeneration Daniela Palacios and Pier Lorenzo Puri. Article first published online: 9 SEP 2005 DOI: 10.1002/jcp.20489