# Derivation and Characterization of Pax7 Positive Skeletal Muscle Precursor Cells from Control and HGPS-derived induced Pluripotent Stem Cells

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## ABSTRACT

Hutchinson-Gilford Progeria Syndrome (HGPS) is a rare genetic disorder associated with premature aging in various tissues and organs of the afflicted individuals, including accelerated skeletal muscle atrophy. Classical HGPS manifests due to single-base substitution in the LAMNA gene which encodes Lamin A/C proteins. As a result of the mutation, a truncated form of Lamin (known as Progerin) is produced which undergoes persistent farnesylation during post-translational modification. Accumulation of Progerin in the nucleus has been linked to various cellular abnormalities including abnormal nuclear morphologies and altered chromatin organization, among others. However, the exact molecular mechanisms leading to skeletal muscle atrophy have not yet been elucidated. In this study, the iPSC approach was implemented in order to study the skeletal muscle phenotype of HGPS by generating and characterizing a population of Pax7 positive skeletal muscle precursor cells (SMPs).

During the course of this project, we have demonstrated the need for excessive optimization of the previously developed directed differentiation protocol for successful application on induced Pluripotent Stem Cells. Furthermore, we have successfully modified the protocol to allow for a more rapid expansion of the SMPs through regular passaging of the myogenic cells starting on day 20 of differentiation. Additionally, this new method produced more uniform distribution of the myogenic cells and allowed for successful freezing/thawing of the myogenic cells.

When compared to the controls, the HGPS-derived SMPs did not appear to be defective in formation, proliferation or differentiation. Abnormal nuclear morphology and DNA damage, documented in HGPS fibroblasts and vascular smooth muscle cells, were not detected the in myogenic cells. Furthermore, we were not able to detect Progerin protein accumulation in the

generated myogenic cultures, offering an explanation for the absence of these phenotypes in the skeletal muscle system.

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# COLLABORATOR CONTRIBUTIONS

***Sections 3.1.1***

- All experiments were performed by myself

***Sections 3.1.2***

- I performed the differentiations for panel A and took images for day 0, 1 and 2 cells. For Panels B and C, I performed the IF staining, image capture and qPCR analysis for the 167-1J iPSCs, while Michael Shelton did the same for the H9 ESCs.

***Sections 3.1.3-3.1.7***

- All experiments were performed by myself.

***Sections 3.2.1-3.2.7***

- All differentiations and analysis were performed by myself.

***Sections 3.2.8-3.2.9***

- Differentiations and immunofluorescence staining were performed by myself. Zhaoyi Chen assisted me with confocal image capture. Julien Yockell-Lelievre designed the ImageJ MACRO used to analyse/quantify DNA damage and nuclear morphology defects.

This thesis was written by myself and edited by Dr. William Stanford.

# CHAPTER 1 – INTRODUCTION

## *1.1. Pluripotent Systems.*

### *1.1.1. Pluripotency in vivo.*

Pluripotency refers to the transient, yet unrestricted ability of a cell to give rise to all three germ layer-derived tissues (ectoderm, mesoderm and endoderm), thus having the potential to produce virtually every cell found in the adult organism [1]. *In vivo,* pluripotent cells can be found in the in the inner cell mass of an early embryonic structure, known as the blastocyst [2]. Following the developmental program, these cells undergo successive rounds of proliferation and differentiation, progressively losing their pluripotent state, as they form the cells of the adult organism.

### *1.1.2. Embryonic Stem Cells.*

Transition of pluripotent cells into an *in vitro* environment was a key milestone in developmental biology research. This was first achieved through the harvest of pre-implanted blastocysts, followed by dissection and plating of inner cell mass cells onto gelatin-pretreated Petri dishes containing mitomycin C inactivated STO fibroblasts [3]. Through subsequent expansion of these cells, embryonic stem cell (ESC) lines were established. ESC lines from mouse (mESCs) were the first ones to be characterized in the early 1980s [3][4]. Successful human ESC (hESC) lines were established approximately 20 years later [5]. Since then, a number of hESC lines have been created and characterized [6][7]. These cells, unlike their *in vivo* counterparts, can be maintained pluripotent indefinitely, as well as expanded through the implementation of proper tissue culture and passaging techniques. Thus, ESCs possess limitless self-renewing ability while at the same time maintaining their pluripotency. Due to these properties, the immense therapeutic potential of these cells was immediately recognised in the

scientific community. Further development in stem cell technologies also saw the emergence of another method for creation of ESC line which allows to preserve the embryos. In this method, known as single blastomere isolation, an ESC line is established through a single cell isolation from the morula stage embryo (8 cells), followed by culture and expansion [8][9]. The embryo is left essentially intact, with no effect on its developmental potential.

### ***1.1.3. Generation of Pluripotent Cells through Somatic Cell Reprograming.***

More recently, pluripotency has been achieved and maintained *in vitro* through alternative methods, avoiding the use of embryos altogether [reviewed in [10]]. These include: somatic cell nuclear transfer (SCNT) and transcription factor-mediated epigenetic reprogramming of somatic cells. In the first method, applied to multiple species over the past 20 years, the nucleus of a somatic cell is extracted and inserted into a de-nucleated unfertilized egg cell [11][12][13][14]. The factors stored in the cytoplasm of the egg cell, in concert with specific *in vitro* culture conditions, facilitate the age-independent reprogramming of the adult somatic cell into a pluripotent cell [15]. Though implementation of SCNT has resulted in the creation of several hESC lines, the requirement for quality ova donations and precise microsurgical techniques has prevented this method from being widely adopted in the field [10]. Perhaps the most revolutionary technique developed in the stem cell field involved the discovery and subsequent use of the so called pluripotency transcription factors on somatic cells. It was demonstrated in 2006 that retroviral transduction of four transcription factors Oct3/4, Sox2, Klf4 and c-Myc into mouse fibroblasts converts them into stem cell-like cells, referred to as induces pluripotent stem cells (iPSCs) [16]. One year later, human iPSCs were generated for the first time by the same group [17]. Subsequently, a different group demonstrated that iPSCs can be generated without the use of c-Myc [18][19], a proto-oncogene, which has been shown to cause

apoptosis and differentiation in ESCs [20]. More studies have since followed to demonstrate the feasibility of using different combination of transcription factors for reprogramming purposes [21][22][23].

### ***1.1.4. Pluripotent Cells in Therapeutics.***

The potential for the use of PSCs in regenerative medicine was recognised immediately after creation of the first ESC lines. The subsequent development and improvement of the various differentiation protocols, allowing for efficient generation of specialised cells *in vitro,* opened the possibility of performing transplantations as a replacement for damaged or diseased tissues. In fact, in 2010, the first phase one clinical trial was initiated to test the feasibility of using hESC-derived oligodendrocyte precursors for spinal cord injury [10]. In the same period, trials were conducted testing hESC-derived retinal pigment epithelium cells (hESC-RPEs) as a treatment for age-related muscular degeneration in the eye and Stargardt disease. Results showed that hESC-RPEs were well tolerated, with no indication of hyperproliferation, tumor formation or rejection, 4 to 22 months after injection, and indicated signs of engraftment even after removal of perioperative immunosuppressants [24][25]. Signs of improvement in vision/visual acuity was also reported for some of the patients. These promising findings have opened the avenue to other groups, who since have received permission to conduct clinical trials with the aim of replenishing tissues affected by other conditions, such as diabetes and ischemic heart disease [10]. These studies are currently in progress.

The development of iPSC technologies has overcome some of the limitations of using ESCs, as well as opened new exciting avenues for development of treatment strategies. Through the iPSC approach, for example, patient cells can be reprogrammed, corrected for the disease and differentiated for eventual transplantation, thereby overcoming complications such as immune

rejections [26]. In addition, the iPSC approach allows for the generation of an appropriate model systems for essentially any genetic disorder with the ultimate goals of studying these diseases on a cellular basis and developing novel therapeutics for these conditions through *in vitro* drug screens or gene correction techniques [27][28].

## *1.2. Vertebrate Embryonic Skeletal Myogenesis.*

### *1.2.1. Early Embryonic Development: From Conception to Gastrulation.*

In order to understand the origin and mechanisms of skeletal muscle development, comprehensive knowledge of embryology is required. Mammalian embryonic development involves many key events, the proper unfolding and timing of which is vital for the production of a viable fetus [reviewed in [29]]. Embryogenesis starts immediately after conception, which denotes the fusion of the sperm cell with the ovum to produce the zygote. After a few rounds of division, the zygote, by now referred to as the morula, comprises eight cells in a tightly packed, spherically shaped structure [30]. Further proliferation of the cells produces the blastocyst, which contains a fluid filled cavity inside the structure, as well as, three distinct cell populations: the inner (ICM), outer (OCM) cell mass cells and extraembryonic endoderm (EE) cells [31]. The ICM cells found inside one side of the blastocysts form the embryo, whereas the OCM cells (a.k.a. trophoblast), positioned in the periphery, contribute to the formation of the placenta and other related structures [32]. The EE cells form the yolk sack of the embryo. Cells from ICM subdivide to form the hypoblast cells which are in contact with the blastocyst cavity and the epiblast cells, which can be found sequestered away from it [33][34]. The next important stage in the developmental program is gastrulation, which sees the transition of the embryo from a bilaminar to a trilaminar state and involves the migration of epiblast cells [reviewed in [35]]. This highly coordinated movement in the tail region of the embryo, known as the primitive

streak, involves the invasion of epiblast cells into the region between the epiblast and hypoblast, in the cranio-lateral direction [36][37][38]. In the process, the three germ layers are generated. As a result, early mesoderm, one of the germ layers, is located sandwiched between the ectoderm and the newly formed endoderm layer. Later, in a process called neurulation, the top midline ectoderm layer differentiates to produce the neural plate which subsequently folds and fuses to give rise to the neural tube [39]. The subsequent development of the neural tube occurs along the anteroposterior axis of the embryo and forms the brain and the spinal cord [39][40]. The top ectoderm, now detached from the neural tube, gives rise to the epidermis [40]. The endoderm cells will form the lining of the gastrointestinal, respiratory and urogenital tracts [41]. The early mesoderm further differentiates to specialise into paraxial, intermediate and lateral plate mesoderm. Intermediate mesoderm gives rise to the gonads and the kidneys whereas lateral plate mesoderm further subdivides to generate blood cells, smooth muscle, and adipose tissue amongst others [42]. Mesoderm patterning is highly coordinated and controlled by a variety of signalling pathways originating in different parts of the embryo, including the notochord and the neural tube [43][44][45][46].

### ***1.2.2. Embryonic Origin of Skeletal Muscle.***

The entire skeletal musculature, with the exception of craniofacial, originates from a region in the developing embryo known as the paraxial mesoderm which can be found on both sides, immediately adjacent to the forming neural tube [47][48]. Along the course of the developmental program, this region undergoes epithelization and condensation to produce repetitive ball-like structures called somites, which form along the anteroposterior axis of the embryo [49][50]. As the embryo matures, the dorsal part of the somites develops into the dermomyotome [51], whereas the ventral side undergoes epithelio-mesenchymal transition to

generate the sclerotome, which gives rise to the skeletal system and the axial cartilage [51][52][53][54]. The dermomyotome compartmentalizes further into the ventrolateral lip (VLL) and the dorsomedial lip (DML) sections [55] (**Figure 1.1.**). The cells of the VLL dermomyotome delaminate to gives rise to the hypaxial myotome which subsequently generates the musculature of the ventrolateral body wall including the limbs, whereas the DML section of the dermomyotome generates the epaxial myotome which is the source of back musculature [55][56]. The hypaxial muscle, possesses two distinct myogenic precursor cell populations, depending on the somite's position along the anteroposterior axis of the embryo. Hypaxial myotomes and dermomyotomes, present at the interlimb, cervical and cranial levels, have a population of myogenic cells which differentiate to produce the ventral, cervical and abdominal muscles that make up the body wall musculature [reviewed in [55]][57][58]. The other population of cells, present at the occipital, fore- and hind limb somite levels, comprises the premyogenic progenitors capable of migration. Along the course of development, these cells detach from the VL dermomyotome and travel towards their target locations in the hindlimbs, forelimbs or the tongue, depending on the somite location along the developmental axis [reviewed in [55]][59][60][61]. Interestingly, these migratory cells also give rise to, what is called the secondary trunk musculature, through retrograde migration from the limb buds towards the thorax and, subsequently, generate the perineal and pectoral gridle muscles of the adult body [62][63][64][65][66].

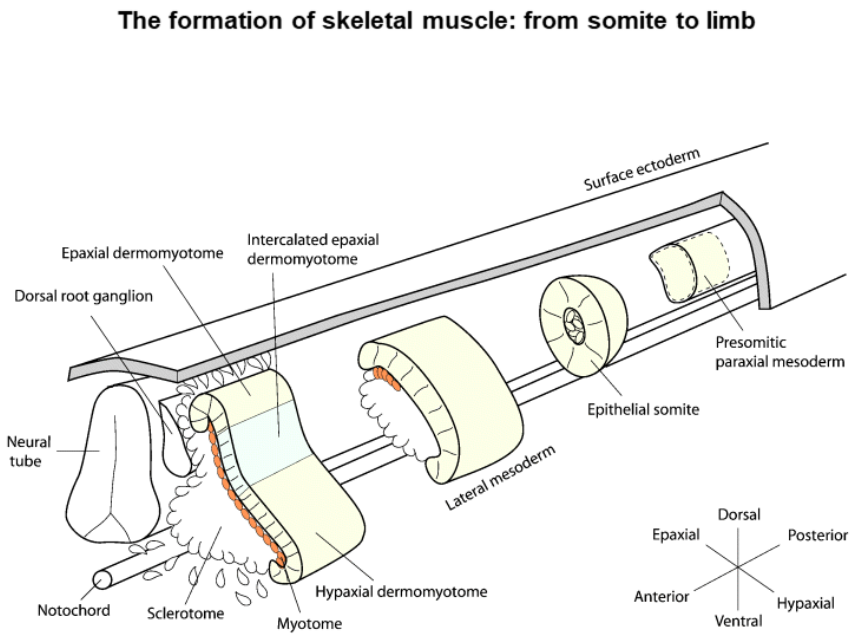


**Figure 1.1. Embryonic origin of skeletal muscle.** All muscle with the exception of craniofacial is derived from paraxial mesoderm, which, during development, condenses to form ball-like structures on either side of the neural tube and notochord. Subsequently, somites give rise to the dorsal dermomyotome and the ventral sclerotome. The dermomyotome differentiates to give rise to the ventolateral (VLL) and the dorsomedial (DML) lips which produce the hypaxial and the epaxial myotomes, respectively. The myotome is the site where the first differentiated myofibers can be found. Figure adapted and reproduced from *Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., Montarras, D., Rocancourt, D., and Relaix, F. (2003). The formation of skeletal muscle: from somites to limb. J Anat 202:59-68* with permission from John Wiley and Son's ©.

### *1.3. Molecular Control of Skeletal Myogenesis.*

Embryonic myogenesis describes the generation of all skeletal muscle during development. This immensely complex process is governed by a multitude of genetic pathways involving many transcription factors and signalling molecules [67]. The correct spatial as well as temporal regulation of the members of these pathways is crucial for the proper development and function of muscle. In this section, the interplay as well as the hierarchy of these factors will be discussed.

It is important to keep in mind that regulation of skeletal muscle generation can be dependent on its anatomical origin, where, for example, molecular factors controlling the aspects of limb muscle formation may not necessarily play the same role in craniofacial or trunk musculature development [67][68]. An example of this can be observed in mice harbouring the Pax3 splotch allele, which introduces a mutation in this transcription factor, resulting in the ablation of the hypaxial-derived muscles of the limb buds but having a less drastic effect on the epaxial-derived back musculature [67][69]. A general conservation of the key molecular players of myogenesis does, however, exist for all muscle development processes.

On a cellular level, embryonic myogenesis can be divided into a series of events which can be thought of as checkpoints. In order to become a functional myocyte, the pluripotent cell must successfully pass through each of these checkpoints, progressively narrowing its developmental pathway choices. As the pluripotent cells progress through the developmental program of myogenesis, they undergo cell identity changes which drive the morphological changes in the embryo. The myogenic checkpoints can be monitored through the expression of a specific set of molecular factors which essentially define the myogenic event and hence the new cell identity.

### *1.3.1. Mesodermal Markers of Myogenesis.*

In the embryonic developmental program, the first set of factors that indicate a path choice towards the muscle lineage are those that mark the mesodermal germ layer. Indeed, as all muscle is mesoderm-derived, every muscle cell can be assumed to have expressed mesodermal markers early during development. One such marker is T (Brachyury T), a member of T-box family of transcription factor which is expressed in early, uncommitted mesoderm [70][71]. The importance of T in mesoderm was demonstrated in mice, where homozygous T mutant animals showed a number of mesodermal abnormalities and died post gastrulation [72]. Markers of a more specified mesoderm include Mesogenin and Tbx6, which denote the presence of paraxial (myogenic) mesoderm [73][74][75]. Formation, maintenance and pattering of embryonic mesoderm has been shown to be controlled by multiple signalling pathways which include Wnt, BMP, FGF and Nodal acting in a somewhat overlapping manner [reviewed in [76]][77]. Signals from these pathways, which originate externally, bind to membrane surface receptors of their target cells and through various mechanisms influence expression of gene(s) involved in cell fate decisions [76]. One such mechanisms involves the canonical Wnt pathway, where the Wnt signalling molecule binds to a cell surface receptor called Frizzled leading to the inhibition of a destruction complex that otherwise degrades beta-catenin. Thus, when Wnt is bound to the receptor, beta-catenin, a transcriptional coactivator, accumulates in the cytoplasm and translocates to the nucleus where, in concert with TCF/LEF transcription factor family, it is able to influence gene expression [78]. One gene that is activated in this manner is T, which denotes mesodermal identity in the cells [79][80].

### *1.3.2. Premyogenic Mesoderm: Pax3 and Pax7.*

Another crucial set of factors, that orchestrate early as well as post-natal myogenesis are the Paired Homeobox (Pax) family of transcription factors, namely Pax3/7 [reviewed in [81]]. Initially, Pax3 is detected in the mesoderm prior to somite formation [82] and subsequently, after somitogenesis, its expression is maintained in the entire somite. As embryonic development proceeds, somite compartmentalization sees the localization of Pax3 expression to the dermomyotome [83], where it is particularly high in the ventrolateral (hypaxial) domain [84][85]. These regions, as reviewed earlier, constitute a rich source of muscle in the body and thus, Pax3/7 appears to mark a population of myogenic progenitor cells (MPCs) which subsequently generate most of the organism's musculature [86][87]. Pax3 expression is also maintained in the migratory MPCs which delaminate from the ventrolateral dermomyotome and migrate to various sites, such as the limbs, where they proliferate and differentiate as needed to form muscle [88][89]. It is noteworthy, however, to mention that Pax3 expression is not exclusive to the muscle lineage and has been shown to be present during organogenesis in other tissues, including the dorsal neural tube [90][91]. Functionally, Pax3 has been shown to play a role in the migration as well as survival of MPCs, though the mechanism of the latter is not entirely clear [81][92][93][94]. Pax7 is also expressed early during the development in the central dermomyotome [81][95]. Evidence suggests some degree of overlap (though not complete) between the functions of Pax3 and Pax7 as, Pax3 mutants do not display central dermomyotomal defects, whereas Pax7/Pax3 double mutants have a severe somite phenotype and muscle deficiency [96]. Absence of Pax7 in mice does not appear to initially affect embryonic muscle [97]. However, the significance of Pax7 in muscle is revealed after birth, where it plays an important role in the maintenance of adult muscle stem cells, otherwise known

as the satellite cells (discussed in section 1.4) [98]. Pax3 has been shown to be regulated by a number of upstream molecular factors, which include Dach2, Six1, Six4 and Eya1/2 [99][100][101]. Successively, Pax3 has been shown to have a role in the direct and indirect control of downstream targets of myogenesis [67][81].

### ***1.3.3. Master Regulators of Myogenesis: The MRFs.***

The next key factors in myogenic genetic hierarchy are a set of four basic helix-loop-helix (bHLH) transcription factors collectively referred to as the myogenic regulatory factors (MRFs). To exert their function, these factors heterodimerize with ubiquitously expressed E proteins and bind the E box sequence (CANNTG) on DNA [reviewed in [102]][103][104][105]. MRFs include Myf5, MyoD, MRF4 and Myogenin which drive myogenesis in the myotome [67]. Having a role in the activation of muscle specific genes [106][107][108][109][110], the MRFs act as myogenic determination and differentiation factors, expression of which first and foremost denotes commitment of the MPCs to the skeletal muscle lineage and thus, confers skeletal muscle identity [111]. Committed skeletal muscle precursor cells are referred to as myoblasts, which subsequently terminally differentiate to form myocytes. Moreover, the ability of MRFs to transform various other cell types into muscle, through transfection experiments, confirms the notion that these factors are key drivers of myogenesis [112][113][114][115][116]. The role of MRFs has been studied through gene targeting experiments in order to gain insight into the function as well as priority of each member. In these studies, MRF members were knocked out, individually or in pair, in order to observe the effect on muscle formation processes [117]. Generally, inactivation of a single MRF member does not appear to have a drastic impact on myogenesis. For example, inactivation of either MyoD or Myf5 alone does not interfere with muscle formation [118][119]. Moreover, the absence of MRF4 does not appear to produce a

significant muscle defect due to a possible compensatory role of Myogenin [120], however animals lacking functional MyoD and Myf5 need MRF4 as a compensatory determination factor [121]. An exception to this appears to be Myogenin, since knockout mice for this MRF alone show severe reduction in muscle and die at birth due to respiratory problems [122][123]. Further study of these samples revealed affected differentiation and fusion of mononucleated cells to form muscle fibers. Triple knockout mice for Myogenin, MRF4 and MyoD appear to have myoblast but fail to produce differentiated muscle fibers, indicating that Myf5 alone is not able to promote differentiation [124]. On the other hand, mice with dysfunctional MyoD Myf5 and MRF4 [121][125] die shortly after birth and possess no skeletal muscle or myoblasts. Thus, these experiments collectively demonstrate that the MRFs can be viewed as having a somewhat redundant roles in orchestrating the myogenic differentiation program. In terms of hierarchy, MyoD and Myf5 appear to be more upstream of Myogenin and regulate skeletal muscle lineage determination and commitment, whereas Myogenin is responsible for terminal differentiation. MRF4 appears to play a role in both of these events. Variety of factors act upstream of the MRFs with the capability to either directly or indirectly activate the members of its family. The identity of these factors, however, is also dependent on the location of myogenesis. To mention a few, Wnt signalling, the Six proteins, Pax3/7, Sonic Hedgehog signalling (through Gli1), among others have all been shown to be upstream and exert control over MRF expression [67][126][127][128][129].

## ***1.4. Adult Muscle Stem Cells.***

Adult (or somatic) stem cells are multipotent cells that can be found in many tissues of an adult organism [130]. An important aspect of myogenesis that was mentioned earlier but not expanded upon is the generation of the adult muscle stem cells, or as they are also known

satellite cells. These cells serve a crucial role in postnatal muscle repair and thus, their proper formation is vital for normal muscle maintenance and function after birth [131]. Satellite cells were first discovered by Alexander Mauro in 1961 by means of electron microscopy [132]. These cells were found to be situated between the basement membrane and sarcolemma of muscle fibers in varying numbers, largely dependent on the anatomical location and type of the muscle [133][134][135]. In addition to serving as the primary repair mechanism in the adult skeletal muscle, satellite cells also account for postnatal muscle growth which explains the discrepancy in numbers of satellite cells present in the aged versus young organisms [136][137][138][139][140].

### ***1.4.1. Markers of Satellite Cells.***

Cell morphology of non-activated satellite cells in an uninjured muscle fiber conveys quiescence, as these cells possess few organelles, a high nuclear-to-cytoplasmic volume ratio, small nuclear size and a large quantity of heterochromatic DNA compared to the neighbouring myocytes [141]. The most established biomarker of the satellite cells, conserved amongst multiple species, is Pax7, which is expressed in all or most activated as well as dormant muscle stem cells [98]. Other biomarkers include Pax3, Myf5, cluster of differentiation CD34 and tyrosine kinase c-met, amongst a number of others [reviewed in [140]][142][143][144][145]. It is important to note, however, that many of these markers are not exclusive to satellite cells and can be found in the cells of surrounding tissues. Moreover, the heterogeneity that exists among the satellite cell population, which will be discussed next, makes it unlikely that all of these biomarkers can be found in one cell [140].

### *1.4.2. Satellite Cell Heterogeneity.*

Originally thought to comprise a homogenous population of cells [146], satellite cell populations were subsequently discovered to possess variability in myogenic differentiation capacity, stemness, gene/marker expression, and lineage potential to assume nonmyogenic fates (multipotency) [extensively reviewed in [140]]. Differentiation potential discrepancy was first demonstrated through transplantation experiments, where satellite cells from tibialis anterior (TA) were shown to contribute less to the repair of irradiated muscles of mdx/nude mice, when transplanted, than those from extensor digitorium longus (EDL) or soleus muscles [140][147]. Furthermore, variance in the satellite cell population was found between the expression of some surface markers, such as CD34 and M-cadherin [148] along with other non-surface markers, such as Pax3 and Myf5 [140][148][149][150], further dividing the satellite cell population. Propensity for multipotency also appears to create a division in the satellite cells. Although the majority of satellite cells have a myogenic fate, some minor side populations were found to be more prone to enter a non-myogenic lineage and differentiate into osteocytes, fibroblasts and adipocytes in an *in vitro* environment [140][151][152]. Thus, taking all this evidence of heterogeneity into account, adult muscle stem cells can be categorised and divided into "sub populations" which, currently, are still poorly defined and understood, due to the lack of specific markers [140].

### *1.4.3. Satellite cell origin.*

Question about the developmental origin(s) of satellite cells has been a particularly intriguing one to the skeletal muscle research community. Evidence from immunofluorescence labelling and lineage tracing experiments [reviewed in [140]] suggest that satellite cells of the trunk and limb muscles arise from a population of Pax3/7 positive muscle precursors that can be

traced back to the dermomyotome [96][153][154][155]. Along embryonic development, proportion of these cells, through migration, established their niche under the basal lamina of embryonic myofibers and post-natally became the adult muscle stem cells [96][153]. On the other hand, craniofacial satellite cells have been shown to have cranial mesoderm origin [156]. Thus, it appear, satellite cells and the muscle fiber in which they reside have a common embryonic origin, whereby a subset of proliferating muscle progenitors that contributes to the growing muscle, along the course of development, finds an appropriate niche and becomes the adult stem cell population [157].

### ***1.4.4. The Role of Satellite Cells.***

Satellite cells function as the primary repair mechanism of the post-natal muscle injury, being it either physiological, like extensive muscle exercise [158] or pathological involving, for example, an injury due to a toxin [140][159]. Repair is initiated when injury to the muscle fiber triggers activation of the quiescent cells, which is followed by their subsequent proliferation and differentiation [160][161]. Each of these events are crucial for proper muscle regeneration (**Figure 1.2**). Differentiated myocytes then fuse into the muscle fiber to replace the damaged tissue [160][161]. Complex mechanisms govern the maintenance of quiescence as well as their activation. A key regulator of these events is thought to be the microenvironment (niche) of satellite cells, where many of the signals originate [162]. Activation has been shown to be triggered by a variety of growth factors and cytokines generated by macrophages, interstitial cells, fibroblasts and microvasulature-related cells, as well as necrotic cues and adhesion molecules released from the surrounding injured muscle tissue [140][162][163][164][165][166].

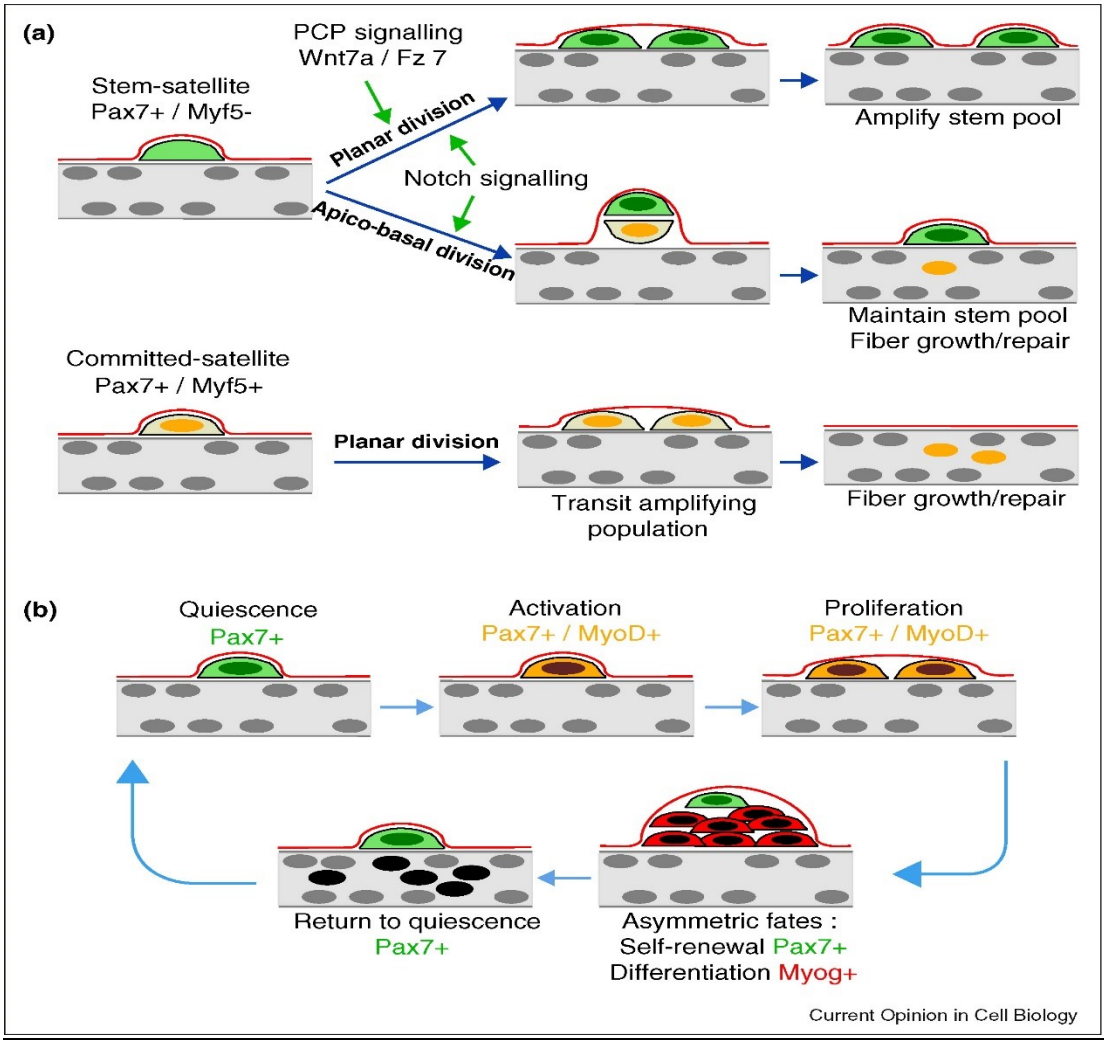


**Figure 1.2. Skeletal muscle stem cells (Satellite Cells) during muscle repair.** Initially quiescent, adult muscle stem cells are marked by the expression of Pax7. Upon injury to the muscle, satellite cells activate, undergo proliferation and differentiate to replenish damaged tissue. A subset of these cells reverts back to quiescence in order to maintain the satellite cell pool. Panels a and b illustrate the hypothesised mechanisms of satellite cell function further discussed in the proceeding sections. Figure adapted and reproduced from *Relaix, F., Marcelle, C. (2009). Muscle stem cells. Curr Opin Cell Biol 21(6):748-53* with permission from Elsevier ©.

### *1.4.5. Mechanisms of Satellite Cell Function.*

All quiescent satellite cells are Pax7 positive and lack the expression of two of the four MRFs which include MyoD and Myogenin [143]. Interestingly, however, a high percentage of these quiescent cells also express Myf5 indicating their pre-commitment to the muscle lineage [140][148]. Activation marks the entry of the satellite cell into the cell cycle which sees the upregulation of Myf5 and MyoD expressions [143][167][168][169]. Once these "early" MRFs are expressed, the satellite cells become referred to as myoblasts, as they become committed to the myogenic lineage. After activation, satellite cells have two choices: to differentiate or to proliferate. According to one hypothesis [reviewed in detail in [140]], the ratio of Myf5/MyoD prevalence in these cells controls the interplay between these two events, where Myf5 expression is associated with proliferation, whereas MyoD - with differentiation. Yet, it is important to note that the aforementioned model for the mechanisms of satellite cell activation are still fluid in nature, as some MyoD/Pax7 positive proliferating satellite cells have been shown to possess the ability to return to quiescence by becoming MyoD negative [140][170][171]. Moreover, MyoD has also been shown to play a role in myoblast proliferation [172]. Orienting on these findings, another mechanism of satellite cell fate control is thought be through the ratio of Pax7 to MyoD expression in the cells [140][173]. In this model, cells with high Pax7 levels and low MyoD expression maintain quiescence. Equal levels of Pax7 and MyoD expression are found in proliferating myoblasts, whereas high levels of MyoD and low levels of Pax7 expression trigger differentiation [140]. The expression of Myogenin, a "late" MRF, during differentiation, has a less fluid meaning and has been shown to be crucial for the terminal differentiation of satellite cells into myocytes [174].

### *1.4.6. Satellite Cell Self-Renewal.*

An important aspect of satellite cells is their ability to self-renew. Indeed, after activation and proliferation a subset of the cells is able to return to quiescence, maintaining the stem cell pool in the muscle [161]. This was demonstrated through transplantation experiments, where satellite cells transplanted into scid-mdx mice with irradiated muscles differentiated to contribute to muscle fibers as well as persisted undifferentiated in the satellite cell niche of the host [175]. Molecular mechanisms ensuring this process are complex and multi-step, as satellite cells have been shown to undergo both symmetric as well as asymmetric cell division [86]. In the case of the former, a cell divides to produce two identical daughter cells, whereas the later sees the production of two distinct daughter cells [176][177]. Symmetric division has been observed in two populations of satellite cells: the Pax7+ve/Myf5-ve and the Pax7+ve/Myf5+ve ones (**Figure 1.2**). In the case of asymmetric division of satellite cells, one daughter cell retains stemness, whereas the other one becomes poised to differentiate. It was demonstrated by Dr. Michael Rudnicki's group that the decision to undergo symmetric versus asymmetric division depends on the orientation of the mitotic spindle of the dividing cells relative to myofiber axis [86]. Furthermore, only the Pax7+ve/Myf5-ve cells have been shown to undergo symmetric as well as asymmetric divisions. Interestingly, satellite cells have also been shown to return to quiescence through lineage regression. This was demonstrated in *ex vivo* cultured myofibers, where a subset of myoblasts (Pax7+ve/MyoD+ve), was shown to revert back to quiescence through repression of MyoD and maintenance of Pax7 expression [178]. These cells are sometimes referred to as the reserve cells.

## *1.5. In Vitro Myogenesis Model.*

Theoretically, stem cell myogenesis can be thought of as a model system for the *in vivo* process, though perhaps with different kinetic rate and a more simplified cellular environment. Thus, in order to guide the pluripotent cell into the skeletal muscle lineage *in vitro,* a detailed understanding of *in vivo* myogenesis is required. This knowledge allows for the development of tissue culture conditions for the cells, similar to what they would encounter in an *in vivo* setting. The closer this environment is reconstituted *in vitro,* the more efficiently myogenic cultures can be generated in a tissue culture system.

In a tissue culture dish myogenesis can roughly be divided into four general stages, which include: mesoderm induction, premyogenic mesoderm specification, commitment to myoblasts and finally terminal differentiation into myocytes (**Figure 1.3**) [179]. Each of these stages can be monitored by the expression of specific sets of markers through q-RT-RCR or immunofluorescence staining techniques. The first stage, mesoderm induction, is monitored through the expression of Brachyury T, an early mesoderm marker. The presence of more specialised mesoderm can be tracked by the expression of Mesogenin and Tbx6, denoting the presence of paraxial mesoderm, which possesses the myogenic potential. Efficient induction of mesoderm early on during differentiation is crucial for the subsequent generation of pure, myogenic-rich cultures at the endpoint of differentiation. Pax3/7 expression marks the presence of premyogenic mesoderm as well as the existence of highly proliferative muscle precursor cell pool. Commitment of MPCs into myoblasts, sees the cells expressing a set of four genes collectively referred to as myogenic regulatory factors (MRFs), discussed earlier. Finally, the endpoint of differentiation is marked by the upregulation of muscle structural and functional genes such as the myosin heavy chain (MyHC) proteins and muscle creatine kinase enzyme.

Mature muscle cells can also be easily distinguished in the cultures due to their unique bipolar cell morphology.

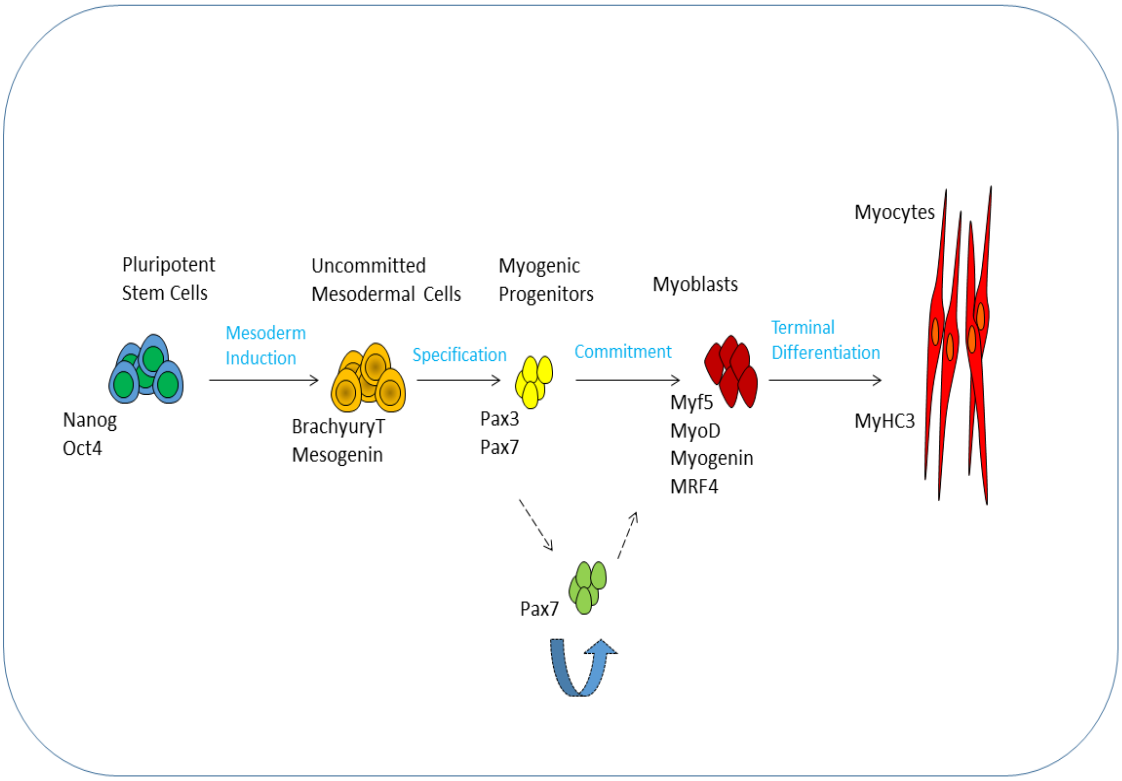


**Figure 1.3. Schematics of skeletal muscle generation *in vitro*.** The four check points along with their respective markers outlining the path of a pluripotent cell differentiating into a skeletal myocyte.

### *1.5.1. Evolution of Skeletal Muscle Differentiation Protocols.*

A number of protocols, detailing the efficient differentiation of pluripotent cells into various tissues, including heart muscle, neurons, smooth muscle and many others, have been developed and described ever since the introduction of ESC and iPSC lines [180][181][182][183]. Until recently, however, a particular challenge has been the differentiation of pluripotent cells into skeletal muscles, as until several manuscripts were published in the last 3 years, skeletal muscle differentiation protocols relied on cell aggregation into embryoid bodies which yielded low percentages (less than 10%) of myogenic cells [184]. Moreover, lot to lot variability of undefined serums used in the myogenic medium contributed to highly inconsistent differentiation. The past decade saw incredible advances in the understanding of embryonic myogenesis, which have brought about several new protocols capable of producing myogenic-rich cultures through a serum-free, directed differentiation approach.

One particular protocol developed and published by *Shelton et al.* has been shown to robustly and efficiently produce a highly myogenic-rich population in a course of 50 days [179]. In this method, pluripotent cultures are dissociated into single cells, plated in a monolayer at a particular density and treated with a potent Wnt pathway activator CHIR99021 (CHIR) for two days to induce mesoderm. The cells are subsequently cultured in serum-free, chemically defined E6 medium until day 12 to facilitate the survival, proliferation and differentiation of the cells. StemPro-34 medium supplemented with FGF2 is used from days 12 to 20 of differentiation to support the expansion of a satellite-cell-like population of Pax7 positive cells, termed, skeletal muscle precursor cells (SMPs) in the cultures. The media is switched back to E6 on day 20 and cultured in it until day 35 at which point the media is changed to N2-ITS. The cells are kept in this media until the day 50 (endpoint) to facilitate terminal differentiation.

Implementing this protocol, allows for the creation of a rich population of Pax7 positive SMPs by day 20 of differentiation. Moreover, this population is maintained throughout the differentiation, much like the population of muscle precursor cells (MPCs) in the embryonic myogenesis that eventually gives rise to the satellite cells. By day 50 of differentiation, SMPs comprise 40% and myocytes comprise 50% of the total cell population indicating a 90% myogenic rich culture at the endpoint of differentiation.

Subsequently, I have discovered that the population of SMPs on day 20 of differentiation is sufficiently dense to allow for passaging of the cultures and re-plating the cells at lower density. Culturing the cell in E6 supplemented with FGF2 for two week following the passage, produces SMPs and myocytes at levels comparable to the intact cultures of the original protocol [185]. Some of the advantages that this offers include allowing for a faster expansion of the SMPs and creation of a more uniform distribution of the myogenic cells. Moreover, we have discovered that the cultures can be maintained for up to 90 days through regular re-passaging, allowing us to essentially age the cells *in vitro.* The modified protocol also allows for the cells to be frozen. We have observed that the Pax7 positive SMPs can be successfully recovered and expanded following thawing. Schematic representation of the original and the modified protocols is illustrated in **Figure 1.4**.

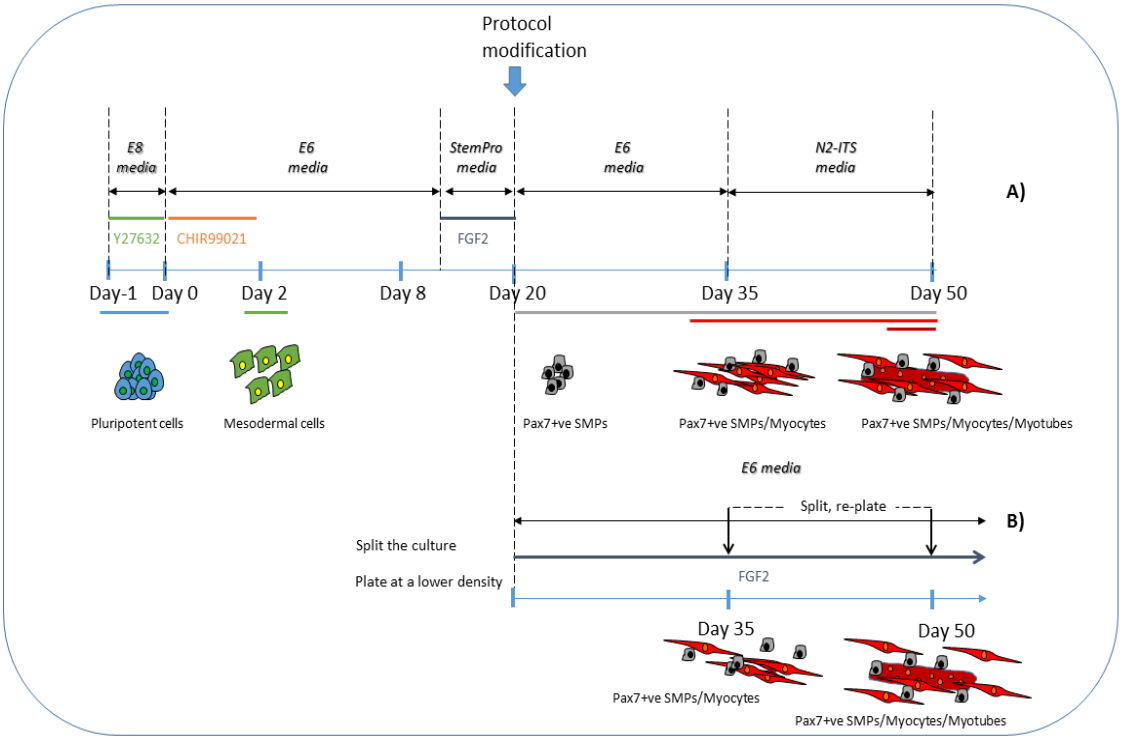


**Figure 1.4. Overview of the original and modified directed differentiation protocols.** A) One day prior to the start of differentiation, pluripotent colonies are enzymatically dissociated and plated in monolayer. Cells are then treated with CHIR for two days to induce mesoderm and subsequently cultured in E6, StemPro-34 and N2-ITS media until day 50. B) Alternatively, the cells can be split and plated at a lower density on day 20 of differentiation. Culture of these cells in E6 media supplemented with 5ng/ml FGF2 yields myogenic rich cultures every 15 days. This method allows for a more rapid amplification of Pax7 positive SMPs, removes 3D overgrowth areas in cultures allowing for better visualization/analysis of the cells and supports successful freezing/thawing of the myogenic cultures.

## *1.6. Hutchinson-Gilford Progeria Syndrome.*

### *1.6.1. The Nuclear Lamin Proteins.*

Lamins belong to a family of fibrous proteins found in the nucleus of most metazoan cells [186][187]. These class V intermediate filaments interact with each other and other protein members to make up a dense fibrillar network inside the cell nucleus, the nuclear lamina, whose function includes mechanical support, anchoring of the nuclear pore complexes, mediating chromatin modifications, among others [188][189][190][191]. Two types of Lamins exist in humans: A-type and B-type which are grouped in this manner according to their structural similarities and isoelectric points [192]. Major A-type Lamins include Lamin A and C, whereas the main B-type Lamins comprise Lamin B1 and Lamin B2, though other variations of both types exist. All A-type Lamins are encoded by the LMNA gene located on chromosome 1, while their B-type counterparts are the products of two genes LMNB1 and LMNB2, located on chromosomes 5 and 19, respectively [193][194][195][196][197]. While Lamin B1/B2 can be found in all cell types throughout development, including undifferentiated stem cells [198], Lamins A/C are expressed later, in differentiated cells [199][200]. In fact, the absence of A-type Lamins in pluripotent nuclei is hypothesised to be critical for their plasticity [201][202]. The two major forms of the A-type proteins, Lamins A and C, arise from alternative splicing of the messenger RNA transcribed from the LMNA locus [192]. As a result, an important distinction between these two proteins becomes the absence of a cysteine-aliphatic-aliphatic-any (CaaX) motif in the C isoform [203]. CaaX is, however, also present in the B-type Lamins [203]. This motif has been demonstrated to play an important role in the post translational modifications of the Lamin proteins [204]. Structurally, Lamins are made up of an N-terminal head, long central alpha-helical rod and C-terminal tail domains which are involved in the Lamins self-assembly

into higher-order structures [202]. Moreover, A-and B-type Lamins have also been demonstrated to interact with each other [205][206]. Yet, the polymeric structure and composition of Lamins inside the nuclear lamina still remains largely unknown [202]. Lamin protein function/role has also been studied using gene targeting.

Gene knockout studies conducted in mice, targeting LMNA and LMNB1 genes have revealed a great deal about the role of Lamins during embryonic development. Ablation of both Lamin A/C in the animals resulted in post-natal growth retardation and muscular dystrophy, though pre-natal development was not shown to be affected [207], consistent with the notion that A-type lamin expression is found in terminally differentiated cells. Interestingly, mice null for only Lamin A, but still capable of expressing Lamin C, were demonstrated to be healthy [208]. Animals lacking functional Lamin B1, contrastingly, display abnormal bone as well as lung tissue and generally expire shortly after birth [209], reinforcing the importance of B-type Lamins in pluripotent ESCs. Furthermore, investigation of cell morphology on mouse embryonic fibroblasts extracted from these animals revealed irregularly shaped nuclei.

### ***1.6.2. Laminopathies.***

Hutchinson-Gilford Progeria Syndrome (HGPS) is part of a wide range of disorders collectively known as laminopathies [210][192]. As the name suggests, these conditions are primarily associated with autosomal-dominant mutations in the lamin proteins and comprise such disorders as Emery-Dreifuss muscular dystrophy (EDMD), congenital muscular dystrophy, Atypical Werner syndrome, Dunningan-type familial partial lipodystrophy, among others [192]. Laminopathies can be further subcategorized based on the tissues and organs that they affect. These include disorders that affect adipose tissue (lipodystrophies), striated muscle (myopathies), peripheral nervous system (neuropathies) or multiple tissues with accelerated aging phenotype

[reviewed in [192]][211]. It is important to note, however, that these subdivisions, though considered fairly robust, do contain some degree of overlap [192]. Myopathies were the first to be discovered among these subgroups [212] and shown to be the result of mutations resulting in amino acid substitutions, small in-frame mutations or RNA splicing defects in the A-type Lamins [192]. One example, EDMD, reported in 1999 as a laminopathy, displays progressive skeletal muscle weakness and wasting in various anatomical locations of the body, including a heart muscle pathology in the form of dilated cardiomyopathy [212]. It is of importance to note, however, that EMDM can also develop due to a mutation in the *EMD* gene encoding Emerin, an interacting partner of Lamin A inside the inner nuclear membrane [213]. Some lipodystrophies were linked to laminopathies for the first time roughly 15 years ago, when a connection between LMNA mutations and adipose tissue disease was established [192][214][215][216]. One example of such disorder, Dunningan-type familial partial lipodystrophy, results in loss of adipose tissue from the extremities during puberty. As a result, these patients also develop metabolic pathologies such as insulin resistance and diabetes mellitus among others. It is interesting to note that most of these disorders are associated with A-type lamin mutations, while few conditions have been linked to LMNB1/2 mutations. This can be reconciled by animal knockout models, where mice lacking B-type Lamins generally die soon after birth.

### ***1.6.3. Features of Hutchinson-Gilford Progeria Syndrome.***

A special subgroup of laminopathies, to which HGPS belongs, affects multiple systems and organs and exhibits features of accelerated aging [210]. This disorder results from a sporadic, autosomal dominant point mutation in LMNA gene, which produces a truncated form of Lamin A, referred to as Progerin [217]. HGPS is a very rare genetic disorder, occurring on average 1 in 4 million births [218], which is associated with premature aging features of the

afflicted individuals [210]. This condition affects a wide variety of tissues, including skeletal muscle, which undergoes accelerated muscle atrophy [218][219]. Initially, individuals harbouring this condition are born with no detectable symptoms and appear symptom-free during the first year of life. Afterwards, however, the patients fail to thrive and begin to acquire many geriatric features such as sclerotic skin, alopecia, hearing loss, bone abnormalities and growth impairment [220]. Though many of the symptoms of the condition resemble natural aging features, HGPS cannot be thought of as completely mimicking natural aging, as patients do not seem to possess all the characteristics of the natural aging process such as senile cataracts or cognitive impairments [221]. In individuals afflicted with HGPS, death generally occurs during the second decade of life due to myocardial infarction or stroke [222].

### ***1.6.4. Genetic and Molecular causes of Hutchinson-Gilford Progeria Syndrome.***

To understand the disease mechanisms of HGPS, it is important to know how LMNA gene products are processed post translation (**Figure 1.5**). Initially, the LMNA transcript is translated into a precursor protein known as Prelamin A, which undergoes extensive post-translational modifications to become the mature, functional Lamin A protein. The CaaX motif that is located at the C-terminus of the protein was shown to be responsible for these modifications [203][204]. During the processing of Prelamin A, the CaaX motif is first farnesylated by a farnesyltransferase enzyme after which the three last amino acids (aaX) are cleaved (catalyzed by ZMPST24 or RCE1 enzymes). Subsequently, carboxymethylation of the carboxyl-terminal occurs by the action of Isoprenylcysteine carboxyl methyltransferase which is followed by proteolytic cleavage of the last 15 amino acids to produce the mature, farnesyl-free Lamin A protein [223]. This is dissimilar to the final product of B-type Lamins, where farnesylation is retained at the end of post translational processing [224][225][226].

The most frequent mutation responsible for HGPS takes place in exon 11 of the LMNA gene at position 1824. This single-base substitution (C > T) results in the deletion of an internal 50 amino acid region from Prelamin A that contains the cryptic splice site which is responsible for the removal of the farnesylated part of the protein during the final post translational modification step (**Figure 1.5**). As a result, the farnesylated end along with the Caax motif of Prelamin A are retained in the final protein product which is referred to as Progerin [227]. Accumulation of Progerin in various tissues of HGPS patients is thought to be responsible for the symptoms, though a mechanistic link has not yet been fully established. On a cellular level, Progerin accumulation has been linked to a variety of abnormalities, ranging from irregular nuclear morphology and altered chromatin organization to delayed mitosis and growth arrest [227][228][229].

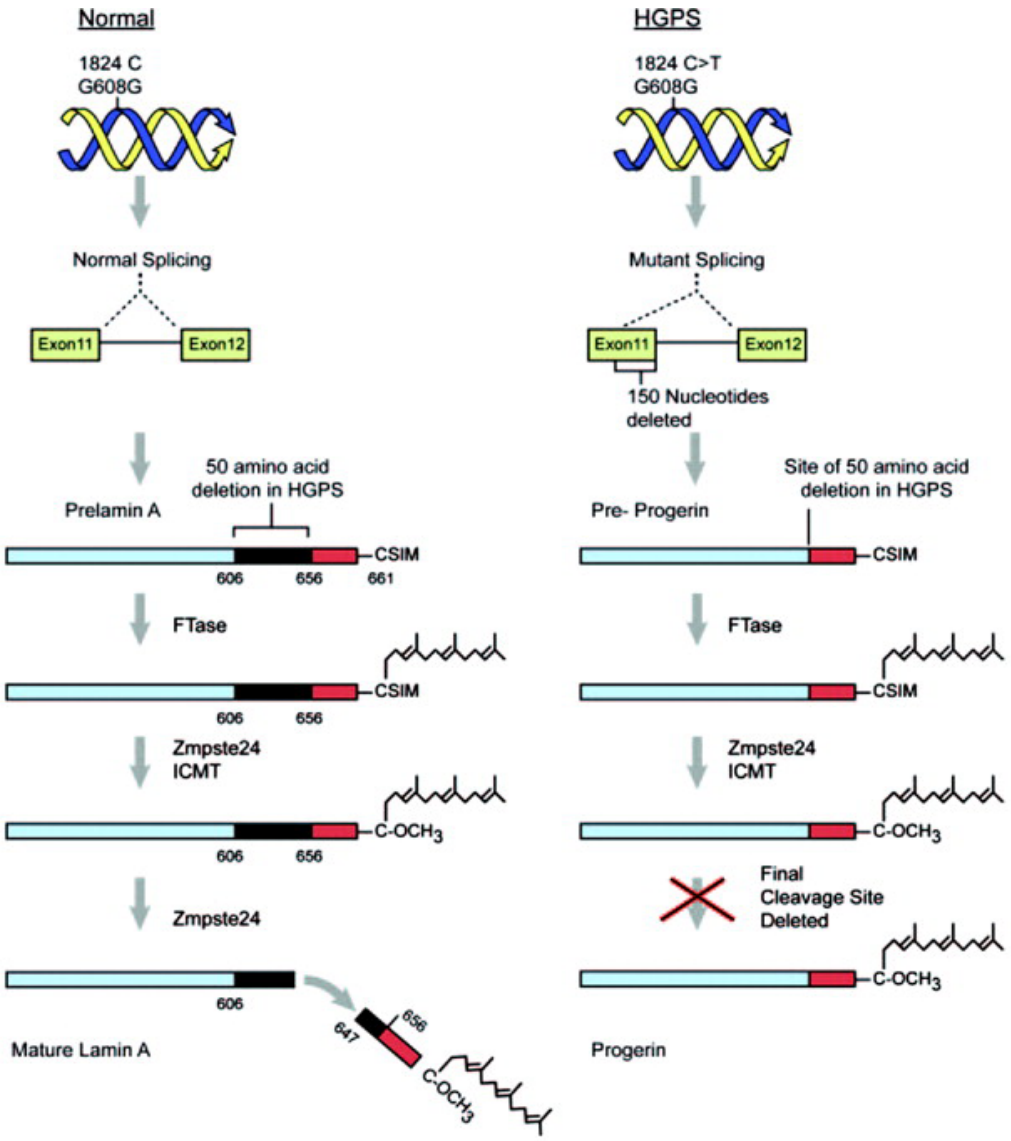


**Figure 1.5. Prelamin A post-translational processing in normal versus HGPS cells.** Normal post translational processig of Prelamin A involves farnesylation, decaboxymethylation and cleavage, which removes the farnesylated part of the protein from the final product (Mature Lamin A). Due to the putative HGPS mutation in the LMNA, the cleavege site of Zmpste24 is removed, and as a result, Prelamin A remains permanently farnesylated (reffered to as Progerin). Figure adapted and reproduced from *Capell, B.C., Collins, F.S., Nabel, E.G. (2007). Mechanisms of cardiovascular disease in accelerated aging syndromes. Circulation research 101(1):13-26* with permission from Wolters Kluwer Health, INC. ©.

## *1.7. Rationale.*

Gradual functional failures in an organism's tissues, followed by considerable increase in the likelihood of developing many chronic conditions are some of the consequences of biological aging in humans [230][231][232][233]. On a cellular level, oxidative damage [234], mitochondrial dysfunction [235], cellular senescence and elevated genome instability [236], amongst others have been shown to enhance the aging process. Thus, understanding the mechanisms of biological aging can help us develop strategies directed at combating these conditions with the ultimate aim of increasing the quality and length of life.

Sarcopenia is a condition that describes the loss of skeletal muscle mass, strength and tone due to the aging process [237]. One of the contributing factors to this condition has been long thought to be the inability of satellite cells to efficiently repair the accumulated damages in the muscle tissue occurring due to age [237]. In support of this viewpoint, multiple studies in mice have shown the decline of satellite cell number and function in aged animals [237][238][239][240]. Specifically, studies have shown aged satellite cells to be defective in activation, proliferation as well as differentiation [241][242][243][244][245].Yet other studies have demonstrated the failure of aged satellite cells to remain quiescence [246][247]. Some advances have also been made at dissecting the mechanisms involved in the dysregulation of aged satellite cells in mice, specifically, through the P16Ink4a axis [248]. In this study, satellite cells from geriatric and progeric mice have been shown to switch from a quiescent to a senescent state, resulting in their failure to activate and differentiate following injury to old muscle. However, the key question of whether the malfunction of aged satellite cells is caused trough cell-intrinsic or a cell-extrinsic mechanisms, or an interplay between the two, still remains open

for debate. More research needs to be done, preferentially using human cells, to answer this key question.

HGPS possesses many of the features of the natural aging process, and thus, has the potential to serve as a model for the study of human aging [249]. In addition, the development of iPSC technologies has theoretically opened the possibility of studying essentially any tissue affected by the disease. Indeed, somatic cells can be obtained from a patient and reprogrammed to pluripotency for differentiation into a tissue of interest in order to study the age-induced phenotypes. Following the iPSC approach also allows to specifically study human cells and thus, move away from the mouse models of HGPS which do not possess all of the disease phenotypes found in the humans [249].

The robust generation of Pax7 positive SMPs through the described protocols, allows for the development of a model to study the aging effect of HGPS in a satellite-cell-like system, opening avenues for the dissection of disease causing mechanisms, *in vitro* drug screens and ultimately, development of strategies to cure sarcopenia, muscle atrophies and HGPS.

## ***1.8. Hypothesis.***

Based on the rationale, we predicted HGPS-derived myogenic cells (in particular the SMPs) to exhibit defects in differentiation and proliferation characteristic of aged satellite cells. Further, consistent with phenotype of HGPS-derived fibroblasts, we hypothesised these cells to display DNA damage, abnormal nuclear morphology and senescence.

## ***1.9. Objectives.***

The first aim of this project was to generate control and HGPS patient-derived myogenic cells (including Pax7 positive SMPs, more committed Myogenin positive cells and terminally

differentiated Myosin Heavy Chain positive myocytes). To achieve this aim, the first part of the project encompassed the optimization of the *Shelton et al.* protocol for iPSCs. Furthermore, in this section, the protocol was modified to allow for regular passaging of the generated myogenic cells [185] to simulate *in vitro* aging and thus, facilitate the expression of HGPS phenotypes in the myogenic cells, as recently demonstrated in HGPS-derived vascular smooth muscle cells [250].

The second objective of the project was to characterize the HGPS-derived myogenic cells in terms of their ability to proliferate, and differentiate. Furthermore, these cells were to be assessed for DNA damage, senescence and nuclear morphology defects.

## ***1.10. Thesis Summary.***

Though some studies have looked at the progeroid skeletal muscle phenotype in mice [248], none have yet used human cells for this particular investigation. The general objective of this project was to explore the HGPS in the context of myogenesis using human iPSCs as a model system for the disease. A specific aim of the project was to investigate whether the aged satellite cell phenotypes (described in section 1.7) would be observed in the skeletal muscle precursor cells derived from HGPS iPSCs. As stated, in order to achieve this, the project was divided into two general parts.

The goal of the first part was to optimise the directed differentiation protocol published by *Shelton et al*. [179] in order to apply it to iPSC lines (as well as additional ESC lines). Furthermore, the protocol was to be modified for general, convenient use, to be applicable for large scale comparative studies between disease and control derived iPSCs. The successful completion of this goal would not only allow to investigate the skeletal muscle phenotype of

HGPS (second goal of the project), but also open avenues for investigations of other muscle disorders in the future, implementing the iPSC approach.

The second objective of the project was to implement the protocol in order to create Pax7 positive skeletal muscle precursor cells using control and HGPS-derived iPSC and subsequently characterize the HGPS-derived SMPs in terms of their ability to proliferate and differentiate as well as assess the prevalence of senescence and DNA damage in these cells. In case of an establishment of a myogenic defect in HGPS-derived myogenic cultures, the future objectives were to revolve around drug screening to alleviate the phenotype and the development of strategies aimed at dissecting the molecular mechanisms of the disease.

Though the first part of the project generated considerable difficulties, it was eventually achieved [185], as a new and improved protocol was published detailing several significant improvements over the old one (described in Chapter 3, [185] and section 1.5.1.).

The results from the second part of the project, demonstrated that the HGPS-derived myogenic cultures did not appear to be defective in formation, differentiation or proliferation. Also, no difference between HGPS and controls was detected in the expression of senescence, quiescence and DNA damage markers. These results leave open the possibility that the skeletal muscle wasting in HGPS patients is secondary to another pathology such as lipodystrophy.

# CHAPTER 2 – MATERIALS and METHODS

## *2.1. Induced Pluripotent Stem Cell Culture.*

Induced pluripotent stem cells (iPSC) were cultured in chemically defined, serum-free Essential 8 (E8) medium (DMEM/F12 medium [Gibco by Lifetech, Grand Island, NY, USA] supplemented with 64 µg/ml l-ascorbic acid [Sigma–Aldrich, St. Louis, MO, USA], 543 µg/ml $NaHCO_3$ [Sigma–Aldrich, St. Louis, MO, USA], 19.4 µg/ml insulin [Wisent, Saint-Jean-Baptiste, QC, Canada], 10.7 µg/ml transferrin [Sigma–Aldrich, St. Louis, MO, USA], 5 µg/ml gentamicin [Gibco by Lifetech, Grand Island, NY, USA], 14 ng/ml Sodium Selenite [Sigma–Aldrich, St. Louis, MO, USA], 100 ng/ml FGF2 [Gibco by Lifetech, Grand Island, NY, USA] and 2 ng/ml TGF-β [Gibco by Lifetech, Grand Island, NY, USA]) on 6 well Matrigel [Corning, New York, USA]-coated tissue culture plates in a hypoxic incubator (37°C, 5% $O_2$, 10% $CO_2$). Spontaneously differentiated cells were picked off from the edges of the pluripotent colonies with the help of a dissection microscope (Zeiss, Laminar Airflow Workstation). Media was changed daily.

When judged 80-90% confluent (roughly every 4 days), the cells were passaged through enzymatic-free, EDTA-based dissociation and plated onto Matrigel-coated plates at $1/6^{th}$ of the original density. In this method, media was aspirated from confluent pluripotent cultures and the cells were washed twice with -/- phosphate-buffered saline solution (DPBS) [Gibco by Lifetech, Grand Island, NY, USA]. The colonies were subsequently gently treated with EDTA passaging solution (DPBS containing 0.5 µM EDTA [Bio-Rad, Hercules, CA, USA] and 30.8 mM NaCl [Fisher Chemicals, Waltham, MA, USA], pH 8, 340 mOsm osmolarity) for 5 minutes at room temperature. Afterwards, the dissociation solution was removed and 1 ml of E8 was added to the wells. The cells were scraped with the help of a cell lifter, mildly triturated with a wide-mouth

pipette tip to break up the colonies and divided amongst the 6 wells of a new Matrigel-coated 6-well plate containing 2 ml of pre-warmed E8 medium per well.

## ***2.2. Directed Differentiation into the Myogenic Lineage – Optimization of Shelton et al. protocol.***

Prior to the start of the differentiation experiments, extensive optimization was performed in order to determine optimal dissociating agent, plating cell density and CHIR99021 concentration for all the iPSC lines used in this project [185].

### ***2.2.1. Dissociating Agent Selection.***

iPSCs were cultured as described above to achieve 80-90% confluence prior to dissociation (generally achieved every 4-5 days post passaging the pluripotent colonies). At this point, the pluripotent cells were treated with 10 μM Rho-associated kinase inhibitor Y-27632 (ROCK inhibitor [TOCRIS Bioscience, Bristol, UK]) in E8 medium for 1 hour at 37 $^{0}$C (hypoxic incubator). Two dissociating enzymes, TrypLE Express [Gibco by Lifetech, Grand Island, NY, USA] and Accutase [STEMCELL technologies, Vancouver, BC, Canada] were tested for their ability to produce viable single-cell iPSC suspensions.

For TrypLE (original *Shelton et al*. method), the E8 medium was aspirated and the cells were washed once with DPBS. 1 mL of pre-warmed TrypLE per well (6 well TC dish) was applied to the cells, which were then placed in a hypoxic incubator for 5 minutes. After incubation, the cells were vigorously triturated with a 1000 μL pipette to lift and break up the colonies. 1 mL of Neutralizing Medium (5.6 mL KnockOut DMEM [Gibco by Lifetech, Grand Island, NY, USA], 1 mL KnockOut Serum Replacement [Gibco by Lifetech, Grand Island, NY, USA]) was added to the cells which were subsequently transferred into a 15 mL conical

centrifuge tube using a 1000 µL pipette. The cell suspension was centrifuged at 500 G for 3 minutes, after which the supernatant was aspirated. The resulting cell pellet was resuspended with 1 mL E8 medium supplemented with 10 µL ROCK inhibitor. At this point 20 µL of the cell suspension solution was used to perform a cell count using an automated cell counter (Cellometer Auto 2000, Nexcelom Bioscience).

For Accutase (shown to work better on iPSCs), the E8 medium was aspirated and the cells were washed once with DPBS. 1 mL of pre-warmed Accutase per well (6 well TC dish) was applied to the cells, which were then placed in a hypoxic incubator for 10-20 minutes (time for efficient dissociation was found to be dependent on the cell line). During incubation, the cells were periodically taken out of the incubator and monitored under a microscope to judge the degree of dissociation. When the majority of the colonies were detached from the plate, 4 mL of E8 medium was added to the well. The cells were subsequently collected into a 15 mL conical centrifuge tube and centrifuged at 500 G for 3 minutes. The cell pellet was resuspended with 1 mL E8 medium supplemented with 10 µL ROCK inhibitor. Cell count was performed as described above.

Cell stock solution was prepared by pipetting the appropriate amount of cells into desired volume of pre-warmed E8 medium supplemented with 10 µL ROCK inhibitor. The cell stock solution was subsequently used to re-plate the cells into 12 well tissue culture dishes (1 mL per well). Cell viability was assessed 24 hours post seeding under a light microscope by observing the amount of attached viable cells versus floating dead cells. Chapter 3 describes the advantage of using Accutase over TrypLE on the control and HGPS-derived iPSC lines.

### *2.2.2. Plating Cell Density Optimization.*

Following dissociation, the cells were re-plated onto 12 well Matrigel-coated tissue culture dish in E8 medium supplemented with 10 µL ROCK inhibitor. Each of the four cell lines used in this project was plated at densities ranging from $7.5 \times 10^4$ to $4.0 \times 10^5$ cells per well and placed in a hypoxic incubator overnight. The cells were monitored after 24 hours for viability (ratio of attached vs floating cells), amount of cell-to-cell contact (ideally, cell clusters of 10-20 cells in the well) and the subsequent cell response to CHIR (see next section and Chapter 3). For each of the cell lines, a specific density was selected to maximally satisfy the listed parameters.

### *2.2.3. CHIR99021 Concentration Optimization.*

To induce mesoderm, CHIR99021 [TOCRIS Bioscience, Bristol, UK] treatment was administered 24 hours post plating. CHIR99021 was diluted in E6 medium (DMEM/F12 medium supplemented with 64 µg/ml l-ascorbic acid, 543 µg/ml $NaHCO_3$, 19.4 µg/ml insulin, 10.7 µg/ml transferrin, 5 µg/ml gentamicin, 14 ng/ml Sodium Selenite) at concentrations to be tested. In total, 2.5, 5, 7.5 and 10 µM concentrations were tested on each of the iPSC lines used. On day 0 of differentiation (first day post seeding), E8 medium was aspirated and the cells were washed with DPBS. The medium was then changed to E6 containing CHIR99021 and the cells were placed in a normoxic incubator (37ºC, 5% $CO_2$) overnight. After 24 hours the cells were monitored under a light microscope for their response to the treatment. The amount of cell death was noted for each CHIR99021 concentration (see Chapter 3). Subsequently, CHIR99021 treatment in E6 medium was re-administered (keeping each respective concentration constant). After 24 hours, cell morphology was closely monitored using a light microscope, to choose CHIR99021 concentrations that, in concert with the corresponding plating cell density, produced the optimal effect on the cells (see Chapter 3 for explanation).

CHIR99021 concentration and plating cell density optimizations were performed every time prior to the start of major differentiation experiments, as the optimal numbers for each of these parameters were observed to change with time as well as be dependent on the batch and passage number of the cells (Chapter 3).

### ***2.2.4. Directed Differentiation into the Myogenic Lineage – Post Optimization.***

Following two days of CHIR99021 treatment (at the optimized concentration), the old medium was aspirated, the cells were washed with DPBS and cultured in E6 medium without additional supplement until day 12. On day 12, the old E6 medium was removed, the cells were washed with DPBS and the medium was replaced with StemPro-34 complete medium (StemPro-34 SFM [Gibco by Lifetech, Grand Island, NY, USA] containing StemPro-34 supplement [Gibco by Lifetech, Grand Island, NY, USA], 0.45 mM Monothioglycerol [Sigma–Aldrich, St. Louis, MO, USA], 2 mM l-Glutamine [Gibco by Lifetech, Grand Island, NY], 10.7 µg/ml transferrin and 5 µg/ml gentamicin) supplemented with 5 ng/mL recombinant FGF2. Cells were kept in this medium until day 20. All media were changed daily.

### ***2.2.5. Standard Protocol (No Passaging).***

On day 20 of differentiation, StemPro-34 medium was aspirated, the cells were washed with DPBS and placed in E6 medium containing no additional supplements. The cells were cultured in this medium until day 35, at which point, following a DPBS wash, the medium was changed to N2-ITS (DMEM/F12 medium containing 1:100 N2-supplement [Gibco by Lifetech, Grand Island, NY, USA], 1:100 insulin-transferrin-selenium [Gibco by Lifetech, Grand Island, NY, USA] and 5 µg/ml gentamicin). The cells were kept in this medium until day 50 (endpoint of standard differentiation protocol). All media were changed daily.

## *2.3. Modified Protocol (Passaging the Myogenic Cultures).*

Passaging of the myogenic cultures was performed to solve some of the limitations of the standard protocol as well as establish a model of *in vitro* aging (Chapter 3).

### *2.3.1. Selection of Dissociating Agent.*

Prior to dissociation, the myogenic cultures were treated with ROCK inhibitor to increase cell viability by limiting anoikis. On day 20, the old StemPro-34 medium was aspirated and the cells were washed with DPBS. 0.5 mL per well of pre-warmed E6 supplemented with 10 µM ROCK inhibitor was added to the cells which were subsequently placed in a normoxic incubator for 1 hour. Three dissociating agents: TrypLE, EDTA passaging solution and 200 U/mL Collagenase IV [Gibco by Lifetech, Grand Island, NY, USA] were tested for their ability to efficiently dissociate the myogenic aggregates while maintaining their myogenic potential. Prior to dissociation, the medium was aspirated and the cells were washed with DPBS.

For TrypLE, 0.5 mL per well of the agent was added to the cells which were then placed in a normoxic incubator for 7 to 14 minutes (not exceed 15 min). The myogenic cultures were periodically taken out of the incubator and monitored under a light microscope to judge the degree of dissociation. When majority of the cells/aggregates were judged detached, 0.5 mL of Neutralizing solution was added to the cell suspension, which was subsequently transferred into a 15 mL conical centrifuge tube. The cell suspension was repeatedly triturated with a 1000 µL pipette to further break up the aggregates (difficult to achieve) and centrifuged at 500 G for 3 minutes. The resulting cell pellet was resuspended with E6 supplemented with 10 µM ROCK inhibitor and 5 ng/mL FGF2. 20 µL of the cell suspension was used to perform an approximate cell count (note: cell numbers could only be approximated due to incomplete breakup of the cell clumps).

For EDTA passaging solution, 1 mL per well of the solution was applied to the cells which were left at room temperature for 5 minutes. The solution was subsequently carefully aspirated from the cells. 2 mL of E6 was added to the well and the cells were scraped with a cell lifter and collected into a 15 mL conical centrifuge tube. The cell suspension was vigorously triturated with 1000 µL pipette to dissociate the aggregates. The cells were then centrifuged for 3 minutes at 500 G after which the pellet was resuspended with 3 or 4 mL of E6 medium supplemented with 10 µM ROCK inhibitor and 5 ng/mL FGF2.

For Collagenase IV (shown to work best, Chapter 3), 0.5 mL per well of the dissociating agent was added to the cells which were subsequently placed in a normoxic incubator for 15 to 20 minutes. The myogenic cultures were periodically monitored under a light microscope to judge the degree of dissociation. When the majority of the cells/aggregates were judged detached, the cell suspension was transferred into a 15 mL conical centrifuge tube containing 1.5 mL of pre-warmed E6 medium. The suspension was mildly triturated with a 1000 µL pipette and centrifuged at 500 G for 3 minutes. The cell pellet was resuspended with 1 mL of E6 supplemented with 10 µM ROCK inhibitor and 5 ng/mL FGF2. Cell count was performed as described above.

### ***2.3.2. Re-Plating the Cells.***

For EDTA Passaging Solution, re-plating of the cells at either 1:3 or 1:4 of the original density (depending on the volume used to resuspend the pellet after centrifugation) was performed by pipetting 1 mL of the cell suspension into a well of a Matrigel-coated 12 well tissue culture plate. Cell viability was assessed under a light microscope after 24 hours by noting the number dead vs viable cells.

For TrypLE and Collagenase IV, cell stock solution was prepared by pipetting the appropriate amount of cells (approx. estimation) into desired volume of pre-warmed E6 medium supplemented with 10 µL ROCK inhibitor and 5 ng/mL FGF2. The cell stock solution was subsequently used to re-plate the cells into Matrigel-coated 12 well tissue culture dishes (1 mL per well, approx. $2.0 \times 10^5$ cells per well). Cell viability was assessed 24 hours post seeding under a light microscope by observing the amount of viable attached cells versus floating dead cells. Chapter 3 describes the advantage of using Collagenase IV over TrypLE.

24 hours post passaging, the medium was switched to E6 containing 5 ng/ml FGF2. The cells were kept in this medium until they were ready for further passaging. The myogenic cultures were passaged implementing the Collagenase IV method every 15 days. All media were changed daily.

## *2.4. Freezing and Thawing Myogenic Cultures.*

### *2.4.1. Freezing the Cells.*

On days 35, 50 and 65 of differentiation (implementing the modified protocol), the myogenic cells were treated with 10 µM ROCK inhibitor for 1 hour at 37°C. Subsequently, the cultures were washed twice with DPBS and treated with the EDTA dissociation solution for 5 minutes at room temperature. The myogenic cultures were gently lifted from the plate with the help of a cell scraper, collected in mFreSR™ solution [STEMCELL technologies, Vancouver, BC, Canada] and placed in cryogenic vials for freezing. Overall, cells from 1 well of a 12-well tissue culture dish were placed in 1 cryogenic vial containing 500 µL of mFreSR™ solution. The vials were subsequently placed in a programmable Biocooler for gradual freezing until -80°C and, afterwards, transferred into liquid nitrogen for long-term storage.

### *2.4.2. Thawing the Cells.*

The cryogenic vial containing the frozen myogenic cells was placed in a 37ºC water bath for 1.5-2 minutes, after which the cells were transferred into a 15 ml conical centrifuge tube containing 9 ml of E6 medium. Subsequently, the tube was centrifuged at 200G for 5 minutes. The resulting myogenic cell pellet was re-suspended with 1 ml of E6 medium supplemented with 10 μM ROCK inhibitor, 5 ng/ml FGF2 and transferred into a Matrigel-coated well of a 12 well tissue culture plate. The cells were placed in a normoxic incubator. After 24 hours the medium was replaced with E6 supplemented with 5 ng/ml FGF2.

## *2.5. Immunofluorescence Staining.*

### *2.5.1. Brachyury T staining, Pax7/MF20, MyoG/MF20 and Pax7/Ki67 co-staining.*

Immunofluorescence analysis of the indicated proteins was performed on days 2, 35, 50, 65, 80 and 95 of differentiation. Media was aspirated and the cells were washed with DPBS. Next, the cells were fixed with 3.7% formaldehyde solution [Sigma–Aldrich, St. Louis, MO, USA] in PBS for 15 minutes at room temperature. The samples were then washed three times with Stockholm's phosphate buffered saline (sPBS) and permeabilized with sPBS containing 0.5% Triton X-100 [Bio-Rad, Hercules, CA, USA] for 15 min. Afterwards, the cells were washed three times with sPBS and placed to rock in a blocking solution (PBS containing 0.1% bovine serum albumin [Jackson ImmunoResearch Laboratories, West Grove, PA, USA], 10% goat serum [Jackson ImmunoResearch Laboratories, West Grove, PA, USA], 0.1% Triton X-100) for 1 hour at room temperature. The samples were then washed three times with sPBS and stained with antibodies against Brachyury T, Pax7/MyHC, MyoG/MyHC or Pax7/Ki67. All antibodies were diluted in the blocking solution (rabbit-anti-Brachyury T [Abcam, Toronto, ON, Canada]: 1:100, mouse IgG1-anti-Pax7 [DSHB, Iowa City, IA, USA]: 1:10, mouse IgG2B-anti-

MF20 [DSHB, Iowa City, IA, USA]: 1:1, mouse IgG1-anti-MyoG [DSHB, Iowa City, IA, USA]: 1:10, rabbit-anti-Ki67 [Abcam, Toronto, ON, Canada]: 1:100) and applied directly to the samples in the tissue culture plate wells, which were then covered with Parafilm and incubated at 4$^{o}$C overnight. On the next day, Parafilm covers were removed, and samples were washed three times with sPBS. Secondary antibodies were diluted in sPBS (1:100) as appropriate (Cy3-conjugated goat-anti-rabbit, goat-anti-mouse IgG2b, Alexa-488-conjugated goat anti mouse IgG1 [all from Jackson ImmunoResearch Laboratories, West Grove, PA, USA]) and applied to the relevant samples, which were then covered with Parafilm and incubated at room temperature in the dark for 1 hour. After incubation, Parafilm covers were removed and the samples were washed three times with sPBS. 200 µl of mounting media (sPBS:Glycerol, 1:1) containing Hoechst dye [Sigma–Aldrich, St. Louis, MO, USA] was added to the stained samples which were subsequently stored at 4$^{o}$C or visualized under a fluorescent microscope.

### ***2.5.2. Pax7/gH2AX, Pax7/Lamin A/C MyoG/gH2AX, MyoG/Lamin A/C co-staining (Confocal Microscopy).***

Cells were stained as described above with the following modifications. Staining was performed on day 60 of differentiation. For this, day 50 freeze-down cells were thawed as described above and cultured in E6 supplemented with 5 ng/ml FGF2 for 5 days. The myogenic culture was subsequently passaged implementing a method described above and plated onto Matrigel-coated coverslips inside a 12-well tissue culture dish at a rough density of 200K/well. The cells were fixed after further 5 days of culture in E6 medium supplemented with 5 ng/ml FGF2. Primary and secondary antibodies were diluted in solutions described previously (mouse IgG2B-anti-lamin A/C [Abcam, Toronto, ON, Canada]: 1:100, rabbit-anti-γH2AX [Cell Signalling TECHNOLOGY, Whitby, Ontario, Canada]: 1:400). After primary and secondary

staining, the coverslips were retrieved from the tissue culture plates, mounted onto glass slides and sealed with nail polish. The mounting media consisted of sPBS:Glycerol, 1:1 containing 1:100 Hoechst dye. The slides were stored at -20$^0$C.

### ***2.5.3. Quantification of Images.***

ImageJ software was used to quantify the confocal microscopy data. Non confocal immunofluorescence microscopy images were quantified implementing Velocity 8.0 software, where either automated or manual cell counts were performed.

## ***2.6. Quantitative Real Time Polymerase Chain Reaction (q-RTPCR).***

Total RNA was isolated from HGPS and control cells differentiated using the standard and the modified protocols on days -1 (undifferentiated), 0, 2, 8, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 of differentiation. RNA was collected in 350 µl of TRK buffer from wells of a 12-well TC dish and stored at -80$^o$C prior to processing. Subsequently, RNA was purified using the E.Z.N.A. Mini-elute Total RNA kit [Omega, Bio-tek, USA] as per manufacturer's instructions. Nanodrop$^{TM}$ spectrophotometer (ND-1000) (Thermo Fisher Scientific) was used to determine the concentration of the purified total RNA. For reverse transcription of the RNA into cDNA, the Quantitect Reverse Transcription Kit (Qiagen, Gaithersburg, MD, USA) was implemented as per manufacturer's instructions. In total, 500 ng of RNA was used for each reverse transcription reaction and 1/40th of the synthesised cDNA was implemented for qPCR analysis. Amplification and quantification of the template cDNA was achieved through the use of KAPA SYBR FAST Universal qPCR Master Mix (Kapa Biosystems, Woburn, MA, USA). Mastercycler Realplex qPCR machine was used to cycle through the required temperatures for the amplification of the cDNA samples and to collect their respective Ct values. Each cDNA sample was amplified in duplicate, the average of which was

used to assess gene expression. Delta-delta Ct approach was implemented to display gene expression as fold over the undifferentiated. In this method, the Cts of the genes were first normalized to those of GAPDH (housekeeping gene) and subsequently expressed as fold over undifferentiated control cells. Microsoft Excel was used to present the data in a graph format with error bars representing +/- standard error of the mean (SEM) of n=3 independent experiments. Sequences of all the validated primers used for this project are listed in the appendix.

## ***2.7. Statistical Analysis.***

Significance was assessed through two-way Anova and two-tailed Student's t-test, with P-values less than 0.05 considered significant.

# CHAPTER 3 – RESULTS

## ***3.1. Optimization and Modification of ESC skeletal muscle differentiation for iPSC disease modelling.***

The majority of the findings form this section of the Results were published as part of a paper in Methods journal [185] (an extension to the original publication of the directed differentiation protocol by *Shelton et al*.) [179].

A well-established muscle differentiation protocol is required to probe for a possible myogenic defect in HGPS using an iPSC approach. Ideally, this protocol should be robust and straight-forward to implement (as this project required work with several cell lines) as well as be capable of producing sufficient amounts of highly-enriched myogenic cultures for the subsequent analysis. The directed differentiation protocol published by *Shelton et al.,* which was developed using mouse and human ESCs was chosen as a foundation for this purpose. Though the protocol showed very promising results in the H9 ESC line, it required considerable amount of optimization for its application to iPSCs (as well as additional human ESC lines). Modifications to the early steps of the protocol included optimization of the CHIR99021 (CHIR) concentration, the type of dissociating agent used on the pluripotent colonies and initial plating cell density. These changes to the protocol proved to be crucial for a successful myogenic differentiation of the iPSCs (as well as different ESCs). Prior to optimization, differentiation of iPSC cultures produced massive cell death and ultimately resulted in the failure of the viable cells to enter the myogenic program (data not shown).

One of the primary prerequisites of successful myogenic differentiation is to maintain the majority of the pluripotent cell in an undifferentiated state (80-90%) prior to the start of experiments. This generally requires pluripotent cultures to be 70-80% confluent before

dissociation. The development of the feeder cell-free approach for pluripotent cell culture, implementing Matrigel-coated plates and serum-free Essential 8 (E8) medium, greatly improved the consistency of pluripotent cell maintenance [251]. Moreover, implementation of EDTA-based, enzymatic-free dissociation [252], over the conventional collagenase-based approach for passaging produced a more consistent cell proliferation rate, improved survival and reduced spontaneous differentiation. Though the basic principle of any pluripotent cell culture is theoretically the same, in practice, different pluripotent cell lines require varying degree of additional care to achieve optimal conditions prior to start of differentiation. For example, the four HGPS and control-derived iPSC lines, compared to H9 ESCs, were found to undergo spontaneous differentiation at a faster rate than the H9 ESCs, producing irregular, undefined boarders around the pluripotent colonies (data not shown). Thus, these cells required monitoring on a daily basis in order to routinely remove (scrape off) the differentiated areas from the colonies. This was performed implementing a specialized dissection microscope in order to avoid compromising the sterility of the tissue culture environment. Similar to the H9 ESCs, prolonged culture, of the aforementioned cell lines, hindered their differentiation capacity, as a number of high passage cell lines were found to be incapable of entering the myogenic program (data not shown). Thus, it is important to prepare large number of frozen stocks in order to avoid keeping iPSCs in culture for prolonged periods of time prior to the start of experiments.

Efficient breakup of the pluripotent colonies for the subsequent single cell seeding is the first step of this differentiation protocol. Prior to the start of differentiation, single cell seeding allows for better control of plating density which enables consistent amount of crucial cell-to-cell contact in subsequent differentiation experiments. Furthermore, this also allows the molecules, present in the differentiation medium, to act uniformly across all singly seeded cells, ultimately

producing more reproducible results. Thus, the choice of an appropriate dissociating agent that minimizes cell death is of crucial importance. To create a single cell suspension of the H9 ESCs, *Shelton et al.* implemented enzymatic dissociation of the pluripotent colonies using the TryPLE reagent. In this approach H9 ESCs were first treated for one hour with 10 μM ROCK inhibitor (Y-27632) prior to application of the dissociating agent in order to reduce dissociation-associated apoptosis. The TryPLE reagent was then applied to the H9 ESC colonies for no more than 5 minutes, after which its action was stopped by addition of neutralizing medium. This was shown to produce a single cell suspension of H9 ESCs with minimal effect on cell viability. Initially, this approach was used to dissociate the four HGPS and control-derived iPSC colonies used in this project. Application of TryPLE on these cell lines, however, revealed several disadvantages of this reagent. Primarily, after the addition of the enzyme's neutralizing solution, centrifugation and resuspension, the TryPLE treated iPSCs formed cell clumps (**Figure 3.1.1. A**). This, occasionally, led to low cell number yields (**Figure 3.1.1. B**), ultimately requiring more wells per experiment. Furthermore, vigorous tituration, to break up clumps, led to poor cell viability post seeding. In order to tackle these challenges, for the iPSCs used in this project (also tested on a new batch of H9 ESCs), the dissociating agent was changed from TryPLE to Accutase. In this new method, after 1 hour ROCK inhibitor treatment, Accutase was applied to the pluripotent colonies from 10-20 minutes, depending on the cell line. Since this enzyme does not require a neutralizing reagent, Accutase action was stopped by dilution with E8 medium. No clump formation was observed in the cell suspension solution. Consequently, no extensive tituration was performed, resulting in a greater viability of the cells post seeding. Furthermore, this method of dissociation produced greater cell number yields per one well of 6 well TC plate due to more

efficient dissociation (no clumping), eliminating the need for larger scale culture and expansion of the pluripotent cells.

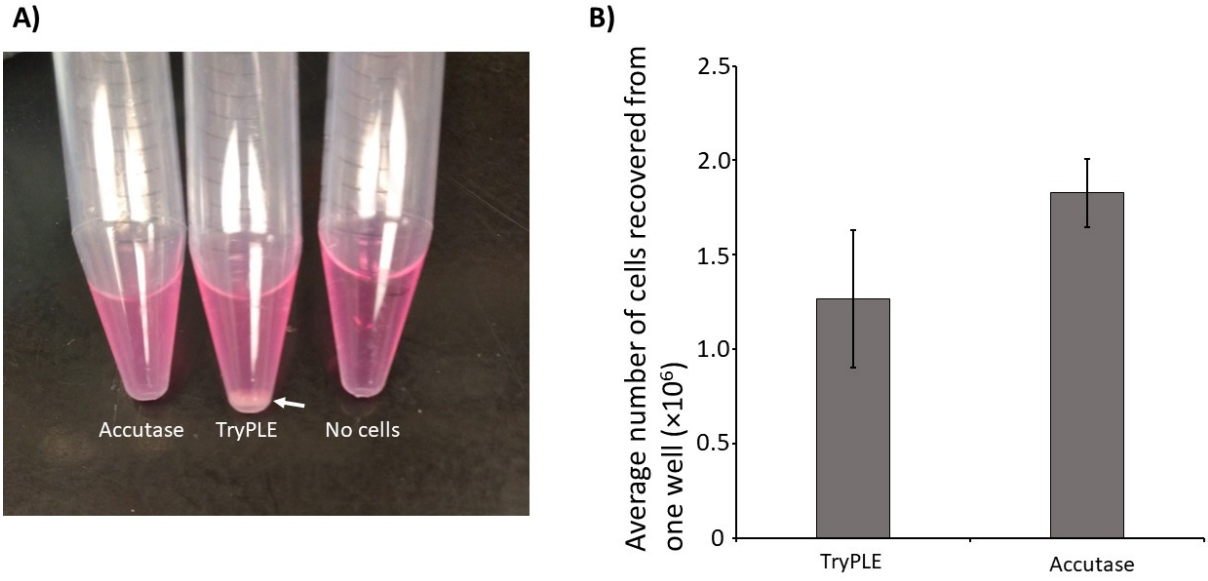


**Figure 3.1.1. Accutase produces uniform single cell suspension for the subsequent myogenic differentiation of the progeria and control-derived iPSCs.** 167-1J HGPS iPSCs were dissociated as described in Chapter 2 using either Accutase or TryPLE. Following centrifugation and resuspension with E8 medium the cells were triturated to create a single cell suspension after which A) images of the conical centrifuge tube were taken to demonstrate cell clumping and B) cell count was performed using an automated cell counter (n=3, error bars represent +/- SEM).

Plating cell density (post initial dissociation) is another determinant of successful differentiation. Primarily, cell density controls the amount of cell-to-cell contact, proper levels of which are essential for pluripotent cell viability. Moreover, cell density is one of the determinants of how much each cell is exposed to the molecules, used in the protocol to guide the pluripotent cell into the appropriate lineage (the other factor is the concentration of the molecule). Dissociation of the pluripotent colonies and the plating of individualized cells takes place one day prior to the start of differentiation. Thus, optimal plating cell density also ensures that the cells do not become over confluent during the subsequent 24 hour incubation. Initially, *Shelton et al.* found the optimal plating density for the H9 ESCs to be $1.5 \times 10^5$ per well of a 12-well tissue culture dish. Subsequently, we demonstrated that this number can vary depending on cell batch and passage. The different iPSC lines used in this project required different plating densities for the protocol as well. These numbers ranged from $7.5 \times 10^4$ to $4.0 \times 10^5$ per well, depending on the cell line used (**Figure 3.1.3**). Interestingly, the optimal plating density was also found to change for the pluripotent cell line following prolonged culture of the cells (data not shown). Thus, for each pluripotent cell line that is to be used for differentiation implementing this protocol, optimization of plating cell density is a necessary prerequisite.

CHIR99021 (CHIR) is a WNT pathway activator which has been shown to induce mesodermal identity in pluripotent cells [253]. This potent GSK3β inhibitor was successfully used by *Shelton et al.* on H9 ESCs to induce mesoderm for subsequent specification of these cells into the skeletal muscle lineage. Following 2 days of treatment at a concentration of 10 μM, *Shelton et al.* demonstrated 90% of the cells displaying mesodermal identity. Initial application of this concentration of CHIR to the iPSC lines, however, resulted in massive cell death and subsequent failure of the remaining cells to recover, demonstrating the variable response of

different cell lines to this molecule and strongly suggesting for the necessity to optimize CHIR concentration for each cell line separately. *Shelton et al.* demonstrated that for H9 ESCs, at optimal CHIR concentration, roughly 30% cell death taking place 24 hours after treatment correlated with efficient mesoderm induction. 48 hours after treatment, a clear change in cell morphology, indicative of epithelial to mesenchymal transition (EMT) is observed and the stressed cells recovered to become highly proliferative. These observations were used to optimize CHIR concentration for the iPSC lines used in this project. Altogether, 2.5, 5, 7.5 and 10 μM concentrations were tested on the iPSC lines plated at different cell densities. It was observed that lowering the CHIR concentration-to-cell density ratio resulted in less cell death, however failed to trigger sufficient cell morphology change, indicative of inefficient mesoderm induction. The opposite was observed for the high CHIR concentration-to-cell density ratio, where massive cell death (>60%) would result in failure of the remaining viable cells to recover after 48 hours (**Figure 3.1.2. A**). Thus, it is important to achieve a CHIR concentration that in combination with optimized plating cell density would result in efficient mesoderm induction while keeping cell death in the range between 25-35% (**Figure 3.1.2. A**). On day 2, following optimized CHIR99021 treatment, 90-100% of the cells should stain positive for early mesodermal marker T (Brachyury T) and have significant upregulation in the expression of *Mesogenin* and *Tbx6* premyogenic mesoderm genes (**Figure 3.1.2. B, C**). **Figure 3.1.3** demonstrates some of the optimized CHIR concentration and plating cell density values for different pluripotent cell lines. Interestingly, however, it was noted that prolonged culture of particular pluripotent cell line often resulted in a change to its response to CHIR (data not shown), requiring periodic concentration re-adjustment.

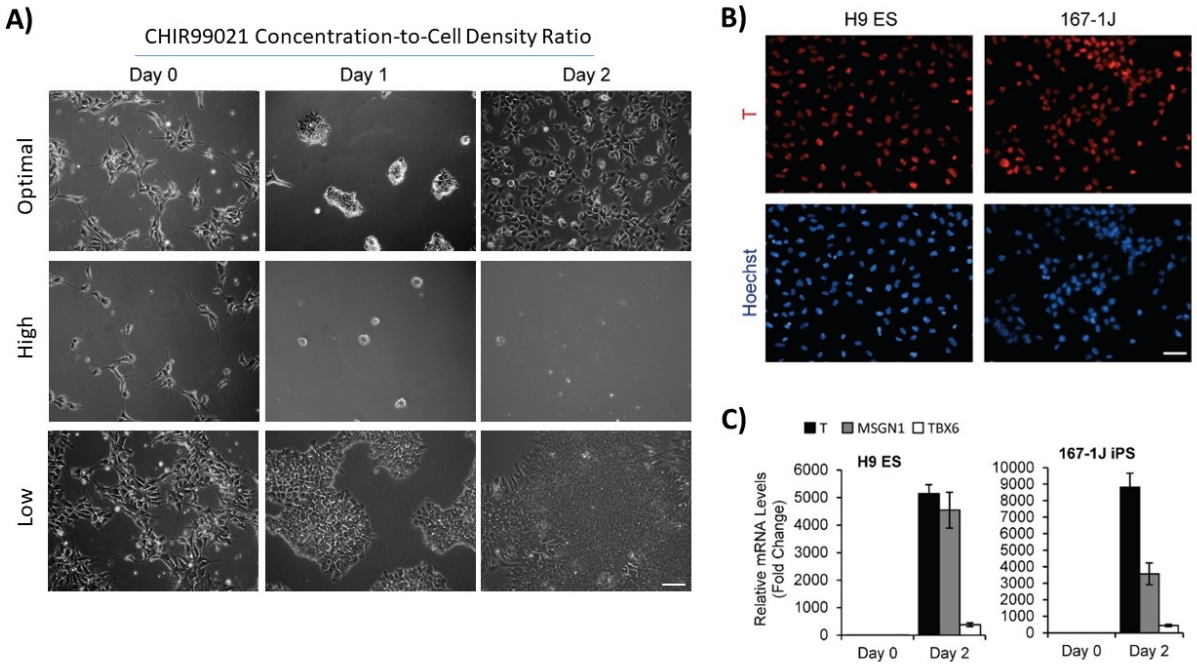


**Figure 3.1.2. General appearance and characteristics of pluripotent cells during the early stages of myogenic differentiation.** Pluripotent cells were differentiated as described above. A) Live imaging of the 167-1J iPSCs undergoing differentiation was performed on the indicated days to demonstrate optimal cell density prior to the start of differentiation and cell response/morphology change following CHIR treatment (Scale bar = 20 µm). B) H9 ESCs and 167-1J iPSCs were fixed and stained with an antibody against T (Brachyury T) on day 2 of differentiation (Scale bar = 20 µm). C) On days 0 and 2 mRNA was collect from the cells and qPCR performed on the indicated early mesodermal genes (n=3, error bars represent +/- SEM). Figure adapted, modified and reproduced from *Shelton, M., **Kocharyan, A.,** Liu, J., Skerjanc, I., Stanford, W. (2016) Robust generation and expansion of skeletal muscle progenitors and myocytes from human pluripotent stem cells. Methods 101:73-84* with permisssion from Elsevier ©.

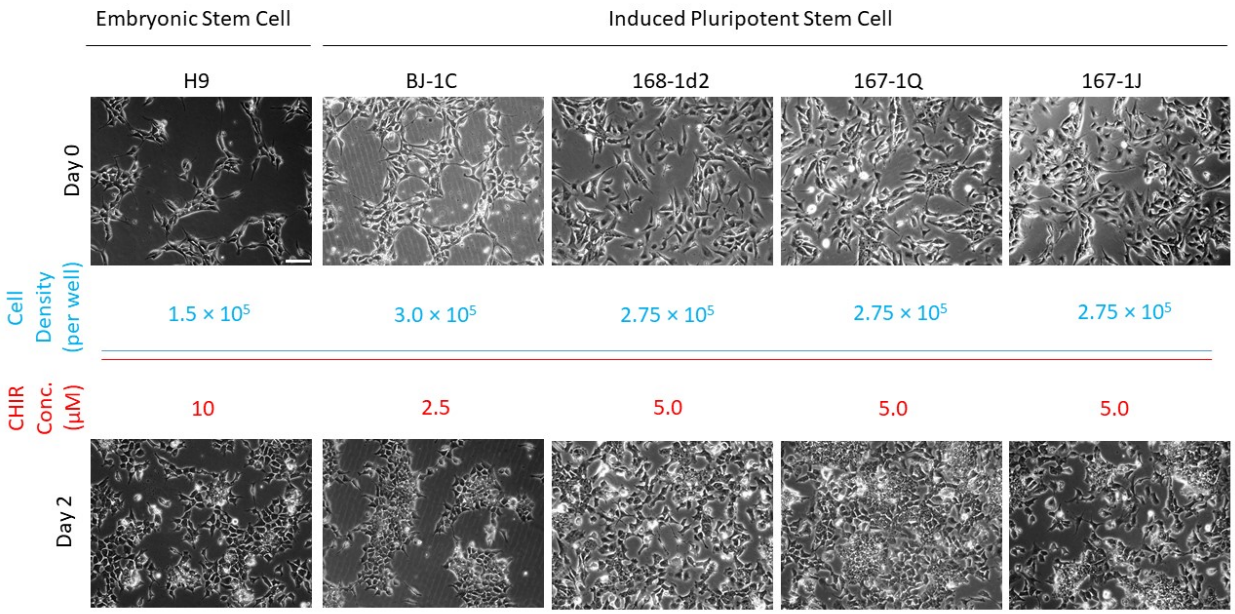


**Figure 3.1.3. Optimal cell plating density and CHIR99021 concentration for various pluripotent cell lines.** Cells were differentiated as described and live imaging was performed on the indicated days to show cell density and response to CHIR treatment (Scale bar = 20 μm). Cells were plated at densities ranging from $7.5 \times 10^4$ to $4.0 \times 10^5$ per well. CHIR was applied at concentrations ranging from 2.5 to 10 μM. Optimal cell density and drug concentration did not remain constant for every differentiation experiment and thus, needed to be periodically re-adjusted for each cell line separately.

After the described initial stages of protocol are optimised, the likelihood of a successful downstream differentiation is greatly increased, though not guaranteed. Following the 2 days of treatment to ensure mesodermal commitment, CHIR99021 is removed from the cultures. As differentiation proceeds, the cells rapidly proliferate, become confluent, generally by day 4, and start to aggregate forming distinct 3-dimentional structures. This normally occurs by day 8 of differentiation (**Figure 3.1.4. red arrows**), when aggregates can be found interspersed uniformly in the tissue culture well. The formation of these structures is a key determinant of a successful differentiation as their failure to develop is accompanied by massive cell death (**Figure 3.1.4.**). From days 12 to 20 the cells are cultured in StemPro-34 complete medium supplemented with 5 ng/ml FGF2, to ensure commitment of the cells to the myogenic lineage and facilitate expansion of the SMPs by supressing premature differentiation. By day 20, 3-dimensional cluster structures increase in size with no signs of cell death occurring in the wells. By this day, a large population of proliferative Pax7 positive SMPs is present in the myogenic cultures. On day 20, FGF2 is removed from the culture to facilitate differentiation of the SMPs (though proliferation is not terminated). Subsequently, starting on day 25 of differentiation, bipolar myocytes can be observed radiating outwards from these 3-dimensional structures (**Figure 3.1.4. yellow arrows**). As differentiation proceeds, however, these 3-dimensional aggregates can continue to increase in size forming large cell overgrowth areas, (**Figure 3.1.4. white arrows**) considerably hindering the visualization of the myocytes and SMPs by immunofluorescence.

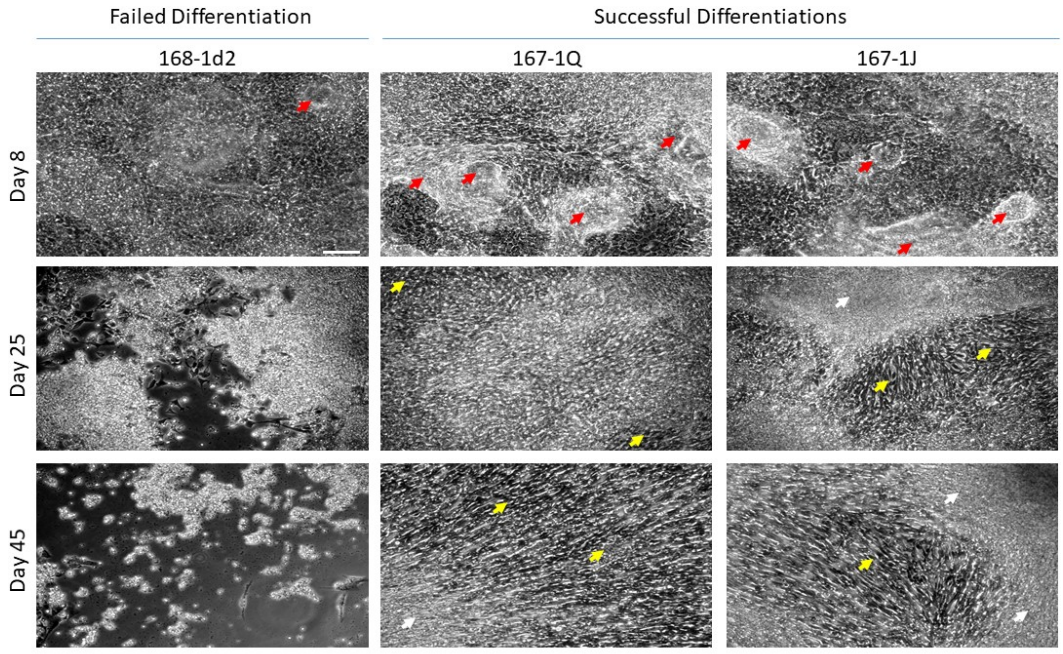


**Figure 3.1.4. Morphology of the iPSCs during the early and later stages of successful and failed directed myogenic differentiation.** Three iPSC lines were subjected to differentiation as discussed above. On the indicated days live imaging was performed (Scale bar = 20 μm). Failed and successful differentiations are demonstrated. The failure to form sufficient numbers of 3-dimesional structures by day 8 results in failed downstream differentiation.

Implementing the aforementioned optimization techniques on the protocol, in total, six HGPS patient and control-derived iPSC were successfully differentiated into the myogenic lineage, validating the findings described by *Shelton et al*. From these cell lines, two of each control (168-1d2, 0901C) and HGPS (167-1J, AG01972B) were ultimately selected for the study of the progeria skeletal muscle phenotype (detailed in Section 3.2). Though very efficient at generating myogenic-rich cultures, the original *Shelton et al.* approach of skeletal muscle differentiation (standard protocol) also revealed several disadvantages, in particular, for the study of the HGPS skeletal muscle phenotype. The primary obstacle was the requirement of a large amount of pluripotent cells at the onset of the experiments to ensure sufficient amount of myogenic cells for analysis at the end time points. Due to the use of 4 iPSC lines for this study, considerable amount of time and material was required to culture, maintain and expand the cells. Secondly, due to the nature of the HGPS skeletal muscle pathology (patients having normal skeletal muscle tone at birth, and atrophy occurring later on during life), HGPS-derived myogenic cell required *in vitro* aging to facilitate the expression of possible muscle pathology. This was demonstrated in HGPS vascular smooth muscle cell (VMSC) pathology modeling implementing the iPSC approach [250]. In this study, differentiated VSMCs from patient-derived iPSC were subjected to serial passaging to introduce replicative stress which resulted in the display of a number of pathology-related phenotypes in these cells. Thus, a technique enabling prolonged culture (*in vitro* aging) of myogenic cells was needed to investigate the skeletal muscle phenotype. Maintaining the myogenic cultures in the wells through feeding (media change, no passaging) would avoid introducing replicative stress. Moreover, this approach often led the 3-dimentional clusters, described earlier, to increase in size, ultimately taking over large areas of the well. This became an increasingly significant hindrance, specifically during

prolonged culture (differentiating up to/past day 50) considerably hindering the analysis of the cells by immunofluorescence.

To tackle these challenges a strategy was developed to maintain the myogenic cultures for a prolonged periods of time through regular passaging [185]. Of central consideration was the choice of the day of differentiation at which point primary passaging of the cells could be successfully performed. Initially, day 20 was selected to be the most appropriate time point to perform the primary splitting, since by this point of differentiation a large population of Pax7 positive SMPs is present in the myogenic cultures, importantly, with no visible terminally differentiated myocytes (data not shown). Subsequently, we have also demonstrated that first-time passaging can also be successfully performed at later stages of the standard differentiation protocol (myogenic cells were successfully passaged on days 35, 40 and 50). The choice of an appropriate dissociating agent which can support myogenesis post dissociation and re-plating was another key point of consideration for this procedure. For this purpose, Collagenese IV, TryPLE and EDTA passaging solution (see Chapter 2) were tested. Prior to dissociation, the myogenic cultures were treated with Y-27632 ROCK inhibitor to maintain cell viability during dissociation. While TryPLE treated myogenic cells (5-10 minutes) dissociated to a certain degree, as was the case with the pluripotent cells, significant cell clumping occurred. Vigorous trituration was implemented to break up the aggregates (create a single-cell suspension) in order to achieve a greater control over the subsequent re-plating density. The majority of TryPLE-treated cells, however, did not re-attach when re-plated onto Matrigel-coated plates. This method was found to be unfeasible for passaging myogenic cultures. While implementing the EDTA passaging solution, non-uniform myogenic cell clumps were produced in the collected cell suspension. Majority of these aggregates attached successfully after re-plating and subsequently

produced myogenic-rich cultures when cultured in E6 supplemented with 5 ng/mL FGF2. Though, as demonstrated, this approach can be used for passaging, re-plating at a desired density is difficult as few single cells are created through this approach. This method, however, was successfully implemented to prepare cells for freezing (discussed later on). We found Collagenase IV to be the most supportive for continued myogenic culture passaging. In this method, the myogenic cultures were treated with the dissociating agent (20-40 min) and followed by gentle tituration to achieve detachment and dissociation of the cells. Collagenase IV treated samples displayed the best viability and myogenic potential after the subsequent re-seeding of the cells. Though implementing this method did not necessarily produce a uniform single cell suspension (small cell clumps/aggregates remained present), a rough cell count could still be obtained using an automated cell counter. Alternatively, this method also allowed for the use of a cell strainer to achieve a more uniform single cell suspension through filtration, thereby permitting for a greater control over re-plating cell density. It was found that for most cell lines used (iPSC as well as ESC) re-plating at densities ranging from $1.5 \times 10^5 – 3.0 \times 10^5$ cells per well (corresponding to roughly 1:6 and 1:3 passaging, respectively) was sufficient to sustain proliferation and differentiation of the passaged myogenic cells. The myogenic cells split in this manner and cultured in E6 media supplemented with FGF2, proliferated and differentiated to produce myocytes and Pax7 positive SMPs at levels observed in the unmodified (standard) protocol (**Figures 3.1.5.C, 3.1.6.**). Moreover, these cells could be maintained in culture for a prolonged periods of time through regular passaging, performed roughly every two weeks (**Figure 3.1.5.C**). Culturing the myogenic cultures in this manner was found to remove the overgrowth areas, allow for faster expansion and more uniform distribution of myogenic cells (**Figure 3.1.5., 3.1.6. white arrows**).

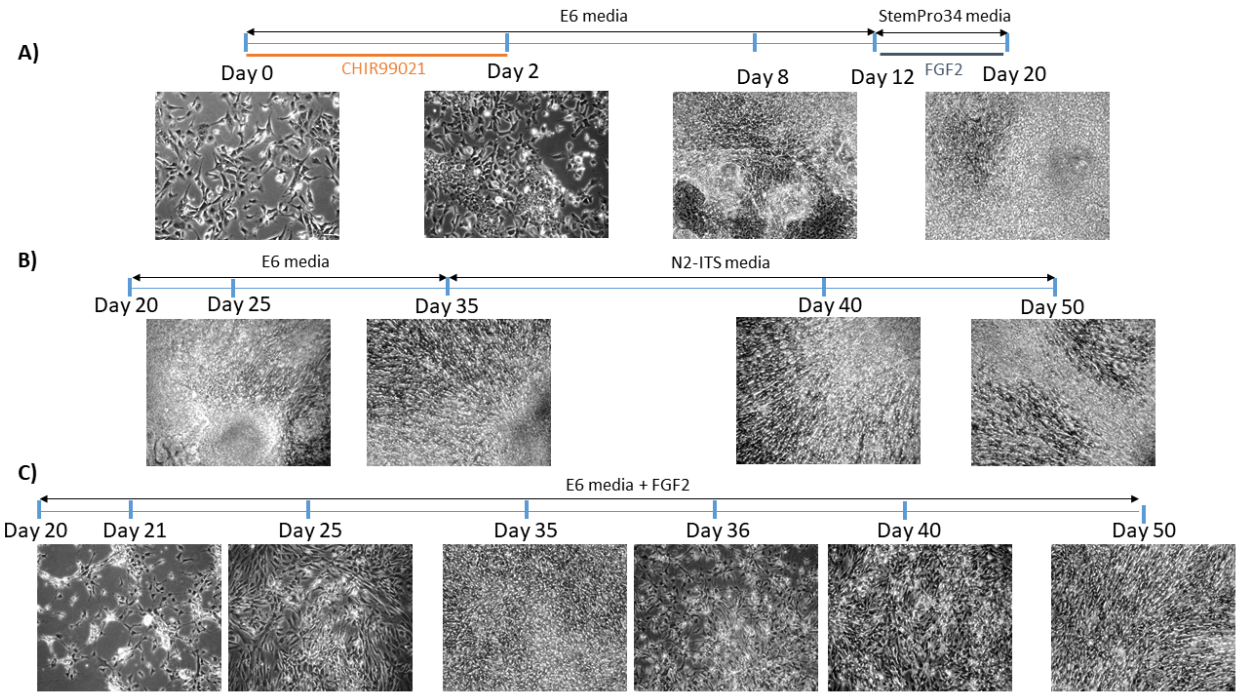


**Figure 3.1.5. Cell morphology of the iPSCs undergoing myogenic differentiation at the key time points of the standard and modified directed differentiation protocols.** 167-1Q HGPS iPSCs were differentiated using the standard and modified differentiation protocol. Live imaging of the cells was performed at the indicated key myogenic differentiation time points. (Scale bar=20 µm). Figure adapted, extensively modified and reproduced from *Shelton, M.,* ***Kocharyan, A.,*** *Liu, J., Skerjanc, I., Stanford, W. (2016) Robust generation and expansion of skeletal muscle progenitors and myocytes from human pluripotent stem cells. Methods 101:73-84* with permisssion from Elsevier ©.

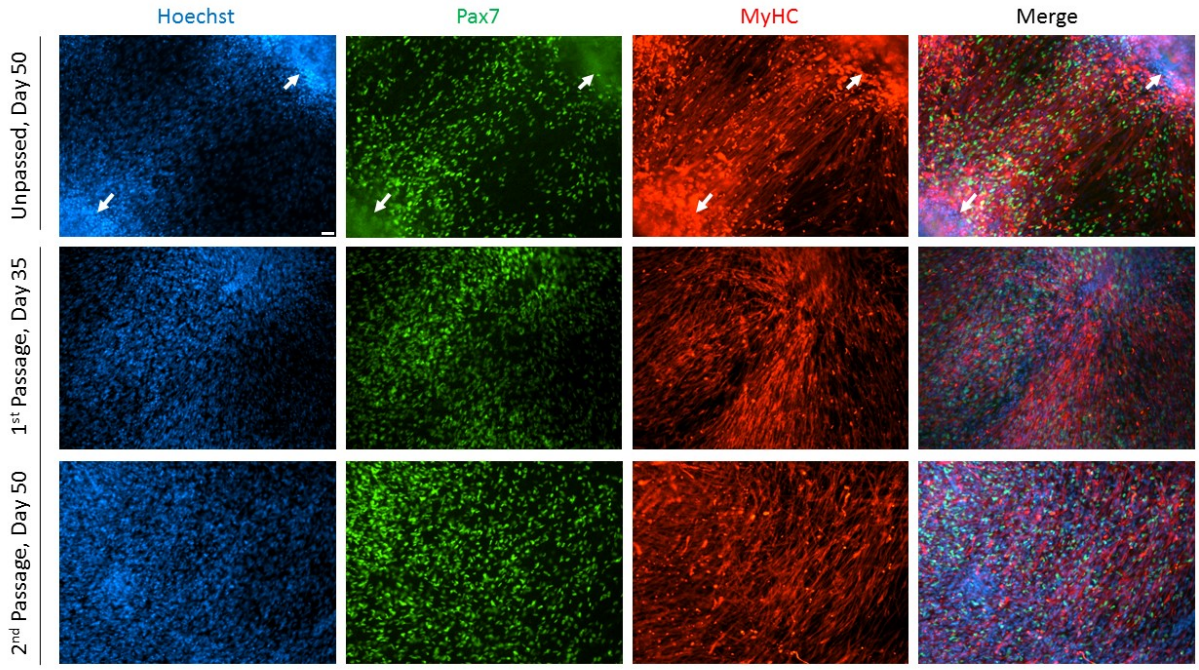


**Figure 3.1.6. Passaging of myogenic cultures results in the production of a more uniform distribution of Pax7 positive SMPs and myocytes.** The 167-1J HGPS iPSCs were differentiated as described until day 20 at which point they were passaged with the use of Collagenase IV. The passaged cells were cultured for 15 days in E6 medium supplemented with FGF2 after which they were re-passaged. On the indicated days of differentiation the cells were fixed and stained with antibodies against Pax7 and MyHC3. (Scale bar=20 μm). White arrows point to the 3D overgrowth areas in the wells of unpassaged cells. Day 50 figures adapted modified and reproduced from *Shelton, M., **Kocharyan, A.,** Liu, J., Skerjanc, I., Stanford, W. (2016) Robust generation and expansion of skeletal muscle progenitors and myocytes from human pluripotent stem cells. Methods 101:73-84* with permisssion from Elsevier ©.

Successful dissociation of the myogenic cultures (while keeping the cells viable) opened the possibility of cryopreservation of the SMPs (**Figure 3.1.7.**). Protocol devised for freezing/thawing human pluripotent cell lines was successfully implemented on the myogenic cultures for this purpose. In this method, EDTA-based dissociation was used to detach the myogenic cultures from the plate while keeping them in large cell clumps (preferentially few single cells). As with freezing pluripotent colonies, this was judged to contribute to the survival of the cells during the subsequent thawing. The myogenic cell clumps were subsequently subjected to gradual freezing (Chapter 2) in mFreSR$^{TM}$ solution. Although a degree of cell death occurred post thaw, myogenic cells were able to recover and expand, producing SMPs and myocytes at levels comparable to those of pre-freeze cultures (**Figure 3.1.7.A,B,C**). The advantages of being able to cryopreserve the myogenic cells are discussed in detail in Chapter 4.

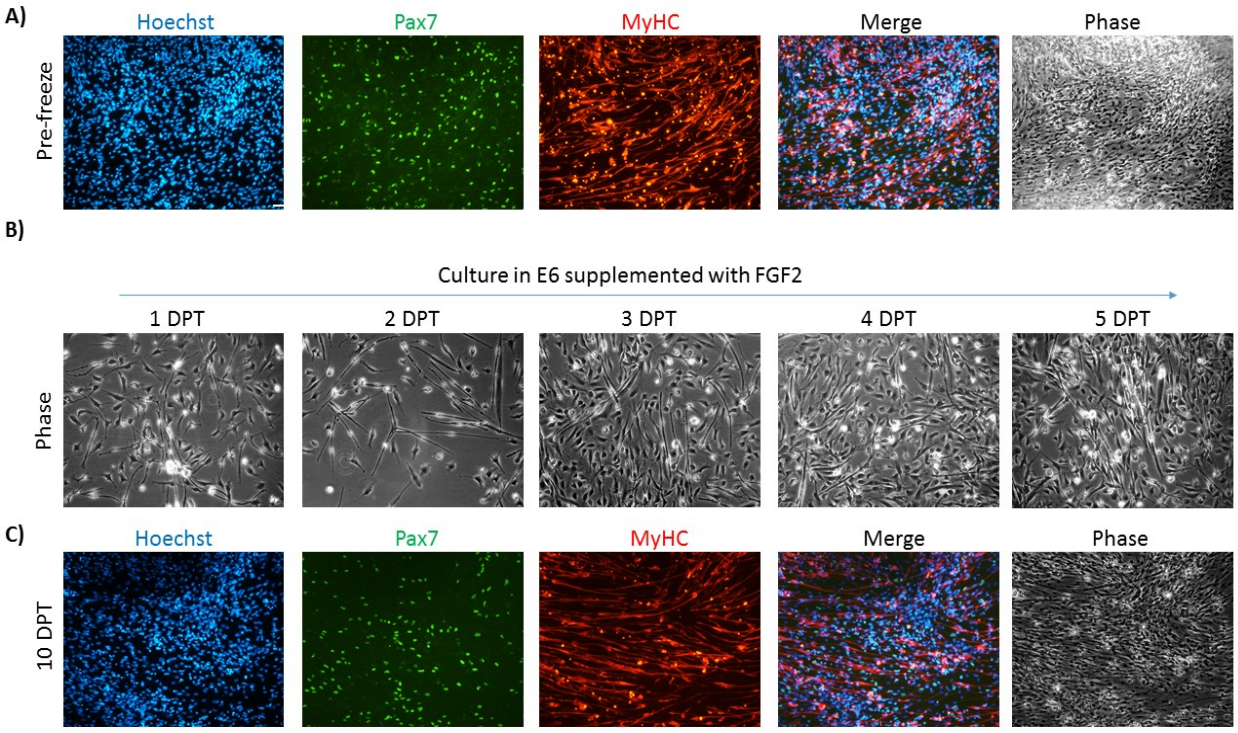


**Figure 3.1.7. The modified directed differentiation protocol allows for successful freezing and thawing of the myogenic cells.** A) Day 65 myogenic cells were fixed and stained with antibodies against Pax7 and MyHC. B) Day 65 cells were frozen and thawed as described in Chapter 2. Live imaging of the cells was performed on each day of post-thaw cell culture (DPT=Days post thaw). C) 10 days after thawing, the cells were fixed and stained with antibodies against Pax7 and MyHC. (Scale bar=20 μm).

***3.2. Characterization of HGPS-derived myogenic cells.***

iPSCs used in this project where derived from dermal fibroblast cells harbouring the classical HGPS mutation. The cell lines used in this study were generated by the Progeria Research Foundation and Dr. William Stanford's Laboratory. In total, two of each, control- (168-1d2, 0901C) and HGPS-derived (167-1J, AG-1B) iPSC lines were used for this project. The 0901C and 168-1d2 control cell lines where derived from the mother and father of the HGPS patient from whom the 167-1J HGPS cell line was generated. The AG01972B (AG-1B) HGPS cell line had no genetic control and was reprogrammed in Dr. Stanford's laboratory.

Though no group has investigated premature aging of the skeletal muscle in HGPS using patient-derived iPSC lines, a successful study performed on the vascular smooth muscle cell (VSMC) HGPS pathology implementing this approach has recently been published by *Chen et al*. [250]. In this study, premature aging in HGPS cells was investigated from the prospective of replicative stress (one of the hallmarks of aging and HGPS). Firstly, this group demonstrated the reversal of the well-documented HGPS fibroblast pathologies (such as abnormal nuclear morphology) upon reprogramming of these cells into iPSCs. These cell lines were also shown to have downregulated *Lamin A* and displaying gene expression signatures similar to those of control pluripotent cells. Secondly, the Stanford lab developed an approach to determine the mechanisms underlying premature aging of HGPS vascular smooth muscle cells (VSMCs) by differentiating iPSCs into VSMCs and serially passaging. In as little as 3-4 passages and a total of 4 weeks in culture, *Chen et al.* found accumulation of PROGERIN protein, replicative stress, and cellular senescence [[250] and unpublished data].

The development of a protocol to maintain the myogenic cultures for prolonged period through regular passaging (described in previous section) served as a method to investigate the

skeletal muscle pathology of HGPS patients, similar to the published HGPS-VSMC strategy. In this method, the proliferation, differentiation and age-associated features of myogenic cells can be investigated during a prolonged tissue culture, introducing replicative stress to the cells with each subsequent passage. As HGPS patients are initially born with normal muscle tone and develop muscle wasting later on during development, this approach of essentially *in vitro* muscle aging was hypothesised to facilitate the expression of a possible phenotypes in the HGPS-derived myogenic cells.

In order to attribute any potential difference(s) between the control and HGPS cells to the putative mutation, while at the same time accounting for the high cell line-to-cell line variability, a special approach was implemented to draw the comparisons. In this method, the two control and HGPS cells were first compared to each other, after which each control was compared to each HGPS cell line. Only in the case, where no significance was detected between the respective controls and HGPS cells and a clear difference was observed during the comparison of each control to each HGPS, a conclusion was drawn that the HGPS mutation is responsible for the difference observed during an experiment.

Based on the initial normal skeletal muscle phenotype of the HGPS patients at birth, the first step of the project was to demonstrate normal skeletal muscle differentiation in HGPS cells. The *Shelton et al.* directed differentiation protocol was optimised and used for this demonstration. As stated in the introduction, high expression of early myogenic markers is a key determinant of a successful downstream differentiation. In order to ensure this, the HGPS and control cells were stained with an antibody against BRACHYURY T, following two days of CHIR99021 treatment. As can be seen in **Figure 3.2.1. A**, the majority of the cells transiently expressed the primitive streak marker, indicating efficient mesoderm induction in all four cell

lines. qPCR analysis also confirmed high expression of *Brachyury T* transcript levels on day 2 compared to day 0 or undifferentiated cells (**Figure 3.2.1.B**). By day 8 of differentiation a significant drop in the expression levels of all early mesodermal markers was observed indicating that the cells have progressed through this stage in pre-myogenic differentiation. No difference in the expression of *Brachyury T* or the other mesodermal markers was observed between the HGPS and control cell lines. Interestingly, there was some variation in the expressions of premyogenic mesoderm markers. Specifically, the AG-1B HGPS cell line showed trends of lower expression in *Mesogenin* and *Tbx6* on day 2 and Meox1 on day 8 compared to the three other cell lines. This, however, can be attributed to cell line-to-cell line variation, as the gene expressions in the other HGPS cell line, 167-1J, did not differ significantly from those of the controls.

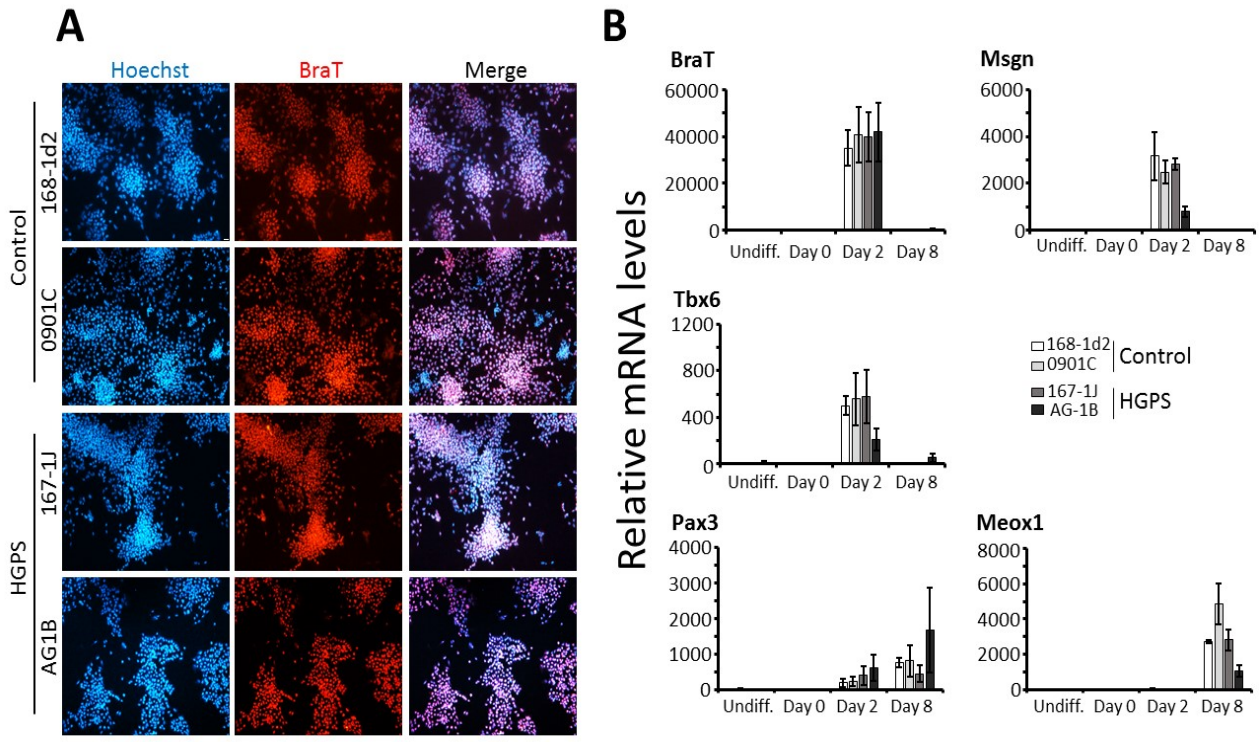


**Figure 3.2.1. HGPS-derived iPSCs do not exhibit early differentiation defects.** The protocol outlined in Figure 1.4 was used to differentiate the control and HGPS-derived iPSCs into skeletal muscle. A) Cells were fixed and stained on day 2 with an antibody against BRACHYURY T. Hoechst dye was used to visualize the nuclei (Scale bar = 20 μm). B) mRNA was collected on the indicated days of differentiation and qPCR analysis was performed on markers involved in early myogenesis (n=3, error bars represent +/- SEM).

Next, gene expression of *Pax7* and late myogenic markers were compared in HGPS versus control cells. The cells were differentiated using the unmodified protocol and total RNA was collected from days 20 to 50 at 5 day increments between the time points (**Figure 3.2.2.**). The expression profiles of these genes showed distinct patterns which were somewhat conserved between the HGPS and control lines. *Pax7* expression showed a trend towards increase from days 20 to days 30-35 followed by gradual decrease towards the end of differentiation on day 50. *Myf5* expression appeared to peak at an earlier time point though the trend of decreasing towards the end of differentiation was similar to that of *Pax7*. Myogenin expression was maintained fairly consistent along the time points, whereas *Myosin Heavy Chain 3 (MyHC3)* expression generally increased as differentiation progressed. Although some variation in the gene expression of *Pax7*, *Myf5*, *Myogenin* and *MyHC3* between the four cell lines was observed, the variation in gene expression was noted across all four cell lines independent of genotype.

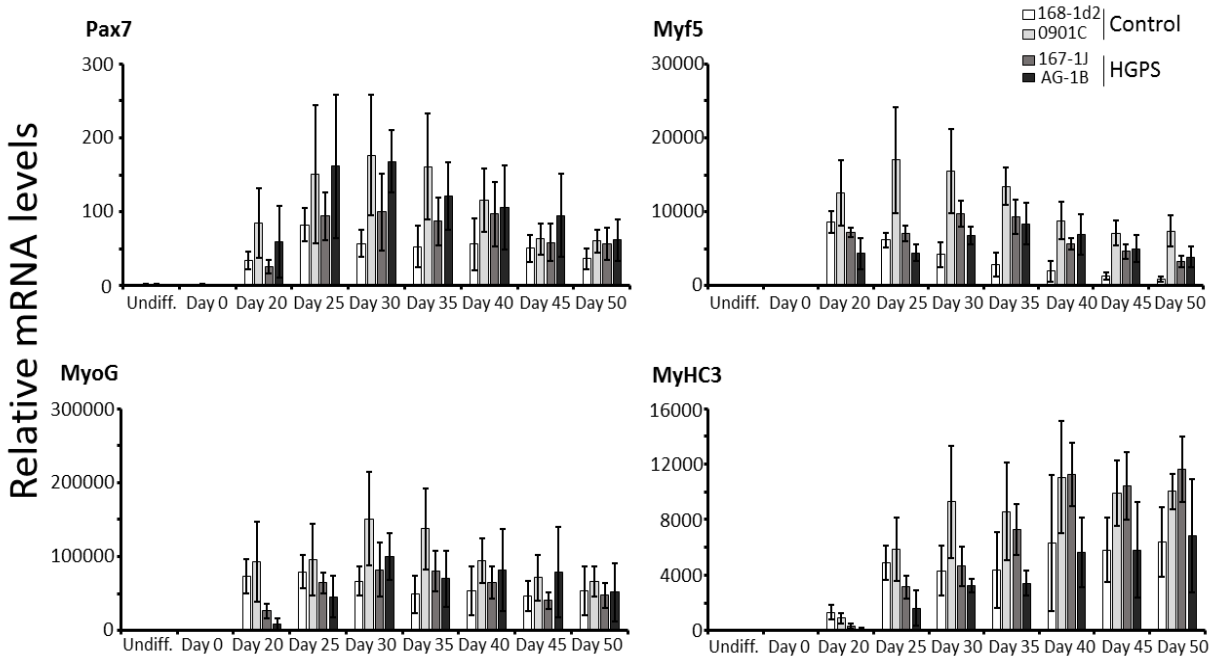


**Figure 3.2.2. HGPS-derived myogenic cells express normal *Pax7* and late myogenic markers.** The cells were differentiated as outlined in Figure 1.4 A) (unmodified protocol) and mRNA was collected on the indicated days of differentiation. qPCR analysis was used to assess the expression of the indicated genes (n=3, error bars represent +/- SEM).

As mentioned earlier, among its advantages, the development of the modified skeletal differentiation protocol enabled long-term culture of myogenic cells to simulate cellular aging through repeated passaging. This strategy was based on the vascular smooth muscle cell (VSMC) aging approach developed by the Stanford lab which effectively modeled HGPS VSMC aging [250]. Hence, to determine whether the HGPS- derived SMPs would demonstrate phenotypes of accelerated aging we implemented the modified skeletal muscle differentiation protocol, which enables longer culturing than the standard 50 day protocol. Starting on day 20 of differentiation all four cell lines were passaged and plated at a lower density. The differentiated cells were subsequently cultured until day 95 in E6 medium supplemented with FGF2. The cells were routinely passaged every 15 days. The expression of various mRNAs was examined by qPCR analysis on time points collected every 5 days, while protein expression was analyzed by immunofluorescence staining every 15 days.

Gene expression analysis showed that the *Pax7* message peaked from days 30 to 55, depending on the cell line, and gradually declined by the endpoint of differentiation (**Figure 3.2.3.**). The late myogenic regulatory factor *MyoG* as well as the terminal differentiation marker *MyHC3* displayed a similar expression trend by peaking at between days 45 and 65 gradually declining towards the end of the culture period. *Myf5* expression (relatively early MRF), on the other hand, peaked early between days 20 and 35. These data suggest the gradual loss of myogenic cells with each subsequent passage. No significant difference was detected in these gene expression profiles between control and HGPS cultures, though, considerable cell line-to-cell line variation was observed.

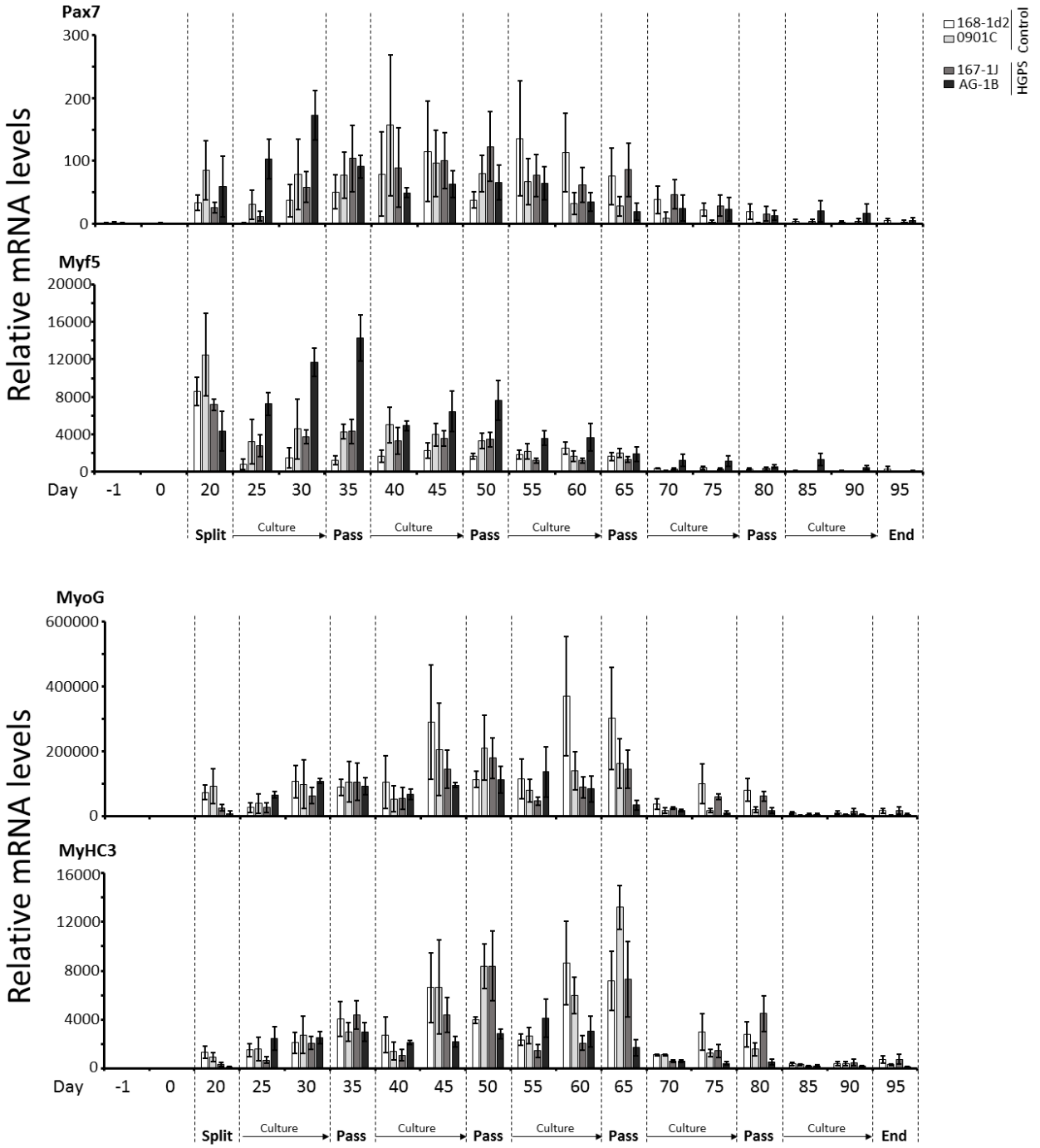


**Figure 3.2.3. Prolonged culture of control and HGPS-derived myogenic cells demonstrates normal myogenesis by HGPS iPSCs.** The cells were differentiated as outlined in Figure 1.4 B) (modified protocol) and mRNA was collected on the indicated days of differentiation. qPCR analysis was used to assess the expression of the indicated genes (n=3, error bars represent +/- SEM).

Immunofluorescence analysis was largely consistent with the transcriptional data, demonstrating the decline of the myogenic population by the endpoint of differentiation (**Figures 3.2.4.A and B**). On day 35 of differentiation, the PAX7 positive SMPs were calculated to comprise roughly 40% of the total population in all four cell lines (**Figure 3.2.4.B**). This percentage gradually decreased with each subsequent passage, approaching numbers close to 0 by day 95. The 0901C control line showed a faster decline in the PAX7 positive SMP population, confirming the presence of cell line-to cell line variation between the controls. HGPS-derived SMP population decline throughout the differentiation time course occurred at a rate similar to that of the 168-1d2 control cell line.

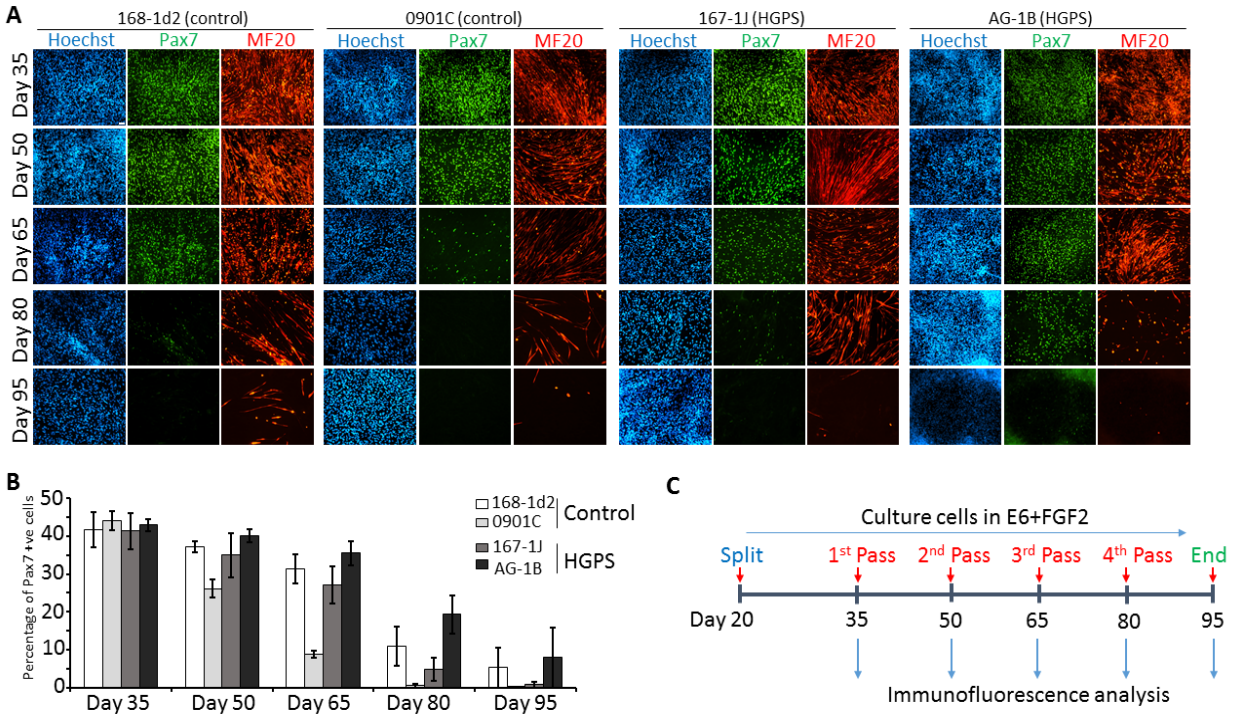


**Figure 3.2.4. HGPS-derived iPSCs develop and maintain normal percentage of Pax7 positive SMPs.** Control and HGPS-derived iPSCs were differentiated as described in Figure 1.4 B). A) On the indicated days, the cultures were stained with antibodies against PAX7 and MyHC. Hoechst dye was used for visualization of nuclei (Scale bar=40 µm). B) Quantification of Pax7 positive cells expressed as a percentage of total cells in the myogenic culture (n=3, error bars represent +/- SEM). C) Schematic overview for the experimental plan used to track the percentage of PAX7 positive cells with each subsequent passage.

The MYOGENIN (MYOG) positive population was quantified to assess the presence of differentiated cells in the cultures (**Figure 3.2.5.**). Day 35 quantification showed 35-40% of total cells expressing MYOGENIN. These numbers were maintained or slightly increased by day 50, depending on the cell line. MYOGENIN positive cell numbers then gradually declined with each subsequent passage (**Figure 3.2.5.B**). No significant difference in percentage numbers was observed when HGPS-derived cells were compared to the controls, suggesting that terminal differentiation is not affected in HGPS-derived skeletal muscle precursors.

Overall, during the extended 95 day culture of control and HGPS-derived myogenic cells no significant differences were observed in terms of maintenance and viability of the cells. Interestingly, however, combining the percentages of Pax7 and Myogenin positive cells gave a rough number of 80% of cells having a myogenic identity by day 35 of differentiation. The decline in the percentages of both Myogenin and Pax7 expressing cells over the course of differentiation suggested the presence of a proliferative, non-myogenic population in both, HGPS and control lines, which took over and perhaps suppressed myogenesis by the day 95 endpoint. The identity of these cells is yet to be elucidated.

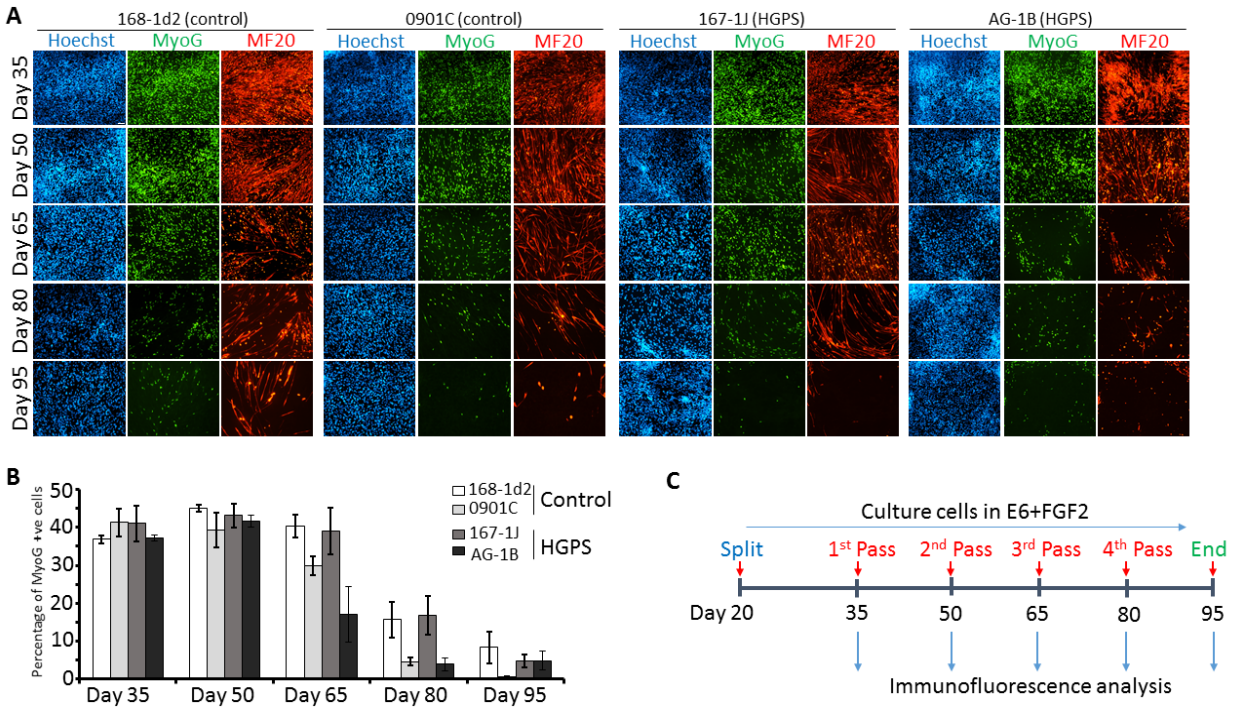


**Figure 3.2.5. HGPS-derived SMPs demonstrate normal differentiation.** Control and HGPS-derived iPSCs were differentiated as described in Figure 1.4 B). A) On the indicated days, the cultures were stained with antibodies against MYOG and MyHC. Hoechst dye was used for visualization of nuclei (Scale bar=40 µm). B) Quantification of MyoG positive cells, expressed as a percentage of total cells in the myogenic culture (n=3, error bars represent +/- SEM). C) Schematic overview for the experimental plan used to track the percentage of MYOG expressing cells with each subsequent passage.

We next analyzed the expression of PROGERIN during myogenic differentiation. A number of experiments were performed to identify PROGERIN positive cells using an anti-PROGERIN antibody; however, only the positive control HGPS VSMCs demonstrated PROGERIN accumulation (data not shown). Thus, qPCR was employed to analyze *Progerin* message (**Figure 3.2.6.A**). Primers designed by *Scaffidi & Misteli,* were implemented for this purpose [254]. Progerin transcripts were detected in the HGPS lines throughout differentiation. Importantly, transcript levels were shown to be at very low levels in the undifferentiated cells, confirming the absence of Lamin A/C expression in pluripotent cells.

As stated earlier, senescence and quiescence have been extensively investigated in aged satellite cells. In particular, cellular senescence has also been widely documented in HGPS-derived dermal fibroblasts [255]. Thus, qPCR was used to probe for markers of quiescence and senescence in the myogenic cultures to determine whether in the HGPS-derived myogenic cells senesce prematurely. Quiescence markers Sprouty1 (Spry1) and P27, did not differ in their expressions in HGPS versus control cell lines (**Figure 3.2.6.B**). Similarly, no difference was observed in the expressions of senescence markers P19 and P21 (**Figure 3.2.6.C**).

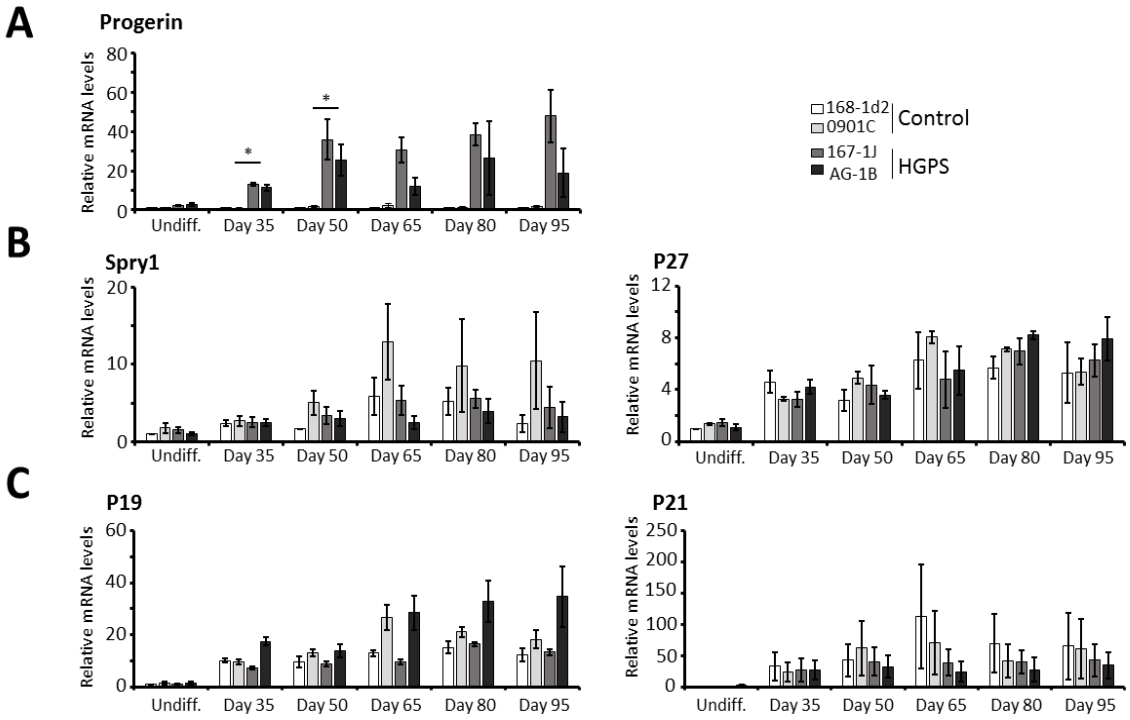


**Figure 3.2.6. Expression of A) Progerin, B) quiescence and C) early senescence markers along the course of differentiation.** Cells were differentiated and cultured as described in Figure 1.4 B). Total RNA was extracted on the indicated days and qPCR analysis was performed to assess the expression of the indicated genes (n=3, error bars represent +/- SEM).

As both, HGPS cells as well as aged satellite cells have been shown to have defects in their proliferative capacity [255][256], we investigated this phenotype in HGPS iPSC-derived myogenic cells. On days 50 and 65 of differentiation, the self-renewal of Pax7 positive SMPs was assessed through Ki67/Pax7 co-staining (**Figure 3.2.7. A and B**). On day 50 roughly 30-35% of Pax7 positive SMPs were Ki67 positive indicating a considerable proliferative population **(Figure 3.2.7.B)**. These numbers did not change significantly on day 65. A comparison between control and HGPS-derived SMPs did not reveal a significant difference in the in the percentage of proliferative cells. These results suggest that HGPS-derived SMPs do not exhibit proliferation defects.

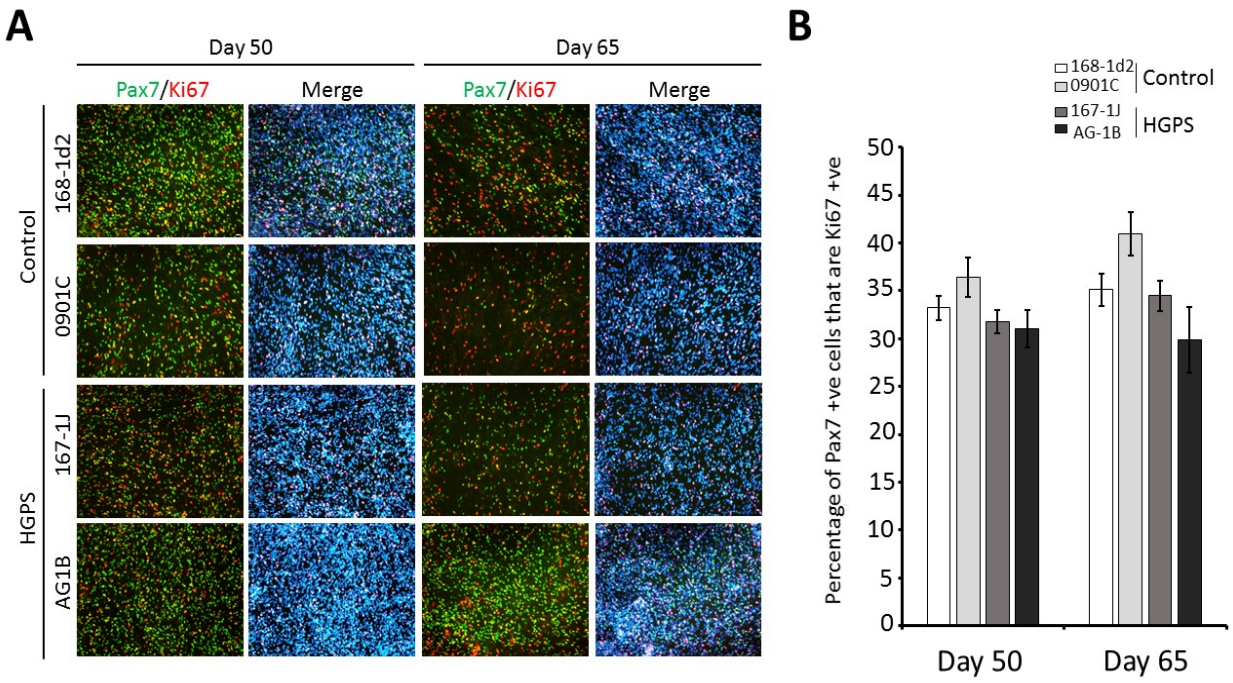


**Figure 3.2.7. PAX7 positive SMP cells demonstrate similar levels of proliferation between control and HGPS cells.** Control and HGPS-derived iPSCs were differentiated as described in Figure 1.4 B). A) On days 50 and 65 cells were fixed and co-stained with antibodies against PAX7 and Ki67. (Scale bar=20 µm) B) Quantification of PAX7/Ki67 double positive cells, expressed as percentage of total PAX7 positive cells (n=3, error bars represent +/- SEM).

Presence of DNA damage has been well documented in HGPS-derived fibroblasts [255][257]. *Chen et al*. subsequently also demonstrated the prevalence of DNA damage in the HGPS-derived VSMCs [250]. Thus, next we assessed the level of DNA damage in the HGPS-derived PAX7 positive SMPs and the more terminally differentiated MYOGENIN positive cells. The cells were co-stained with antibodies against γH2AX and PAX7 or γH2AX and MYOGENIN (**Figure 3.2.8.**). Some level of DNA damage was detected in all of the stained cells (**Figure 3.2.8.A and C**). To count the number of γH2AX foci in the nuclei, an automated ImageJ macro developed by the Stanford lab was implemented. Analysis of this data revealed no significant differences in the number of γH2AX foci between the control and HGPS-derived myogenic cells, suggesting that unlike dermal fibroblasts and iPSC-derived VMSC, HGPS SMPs do not undergo genotoxic stress and DNA damage (**Figure 3.2.8.B and D**).

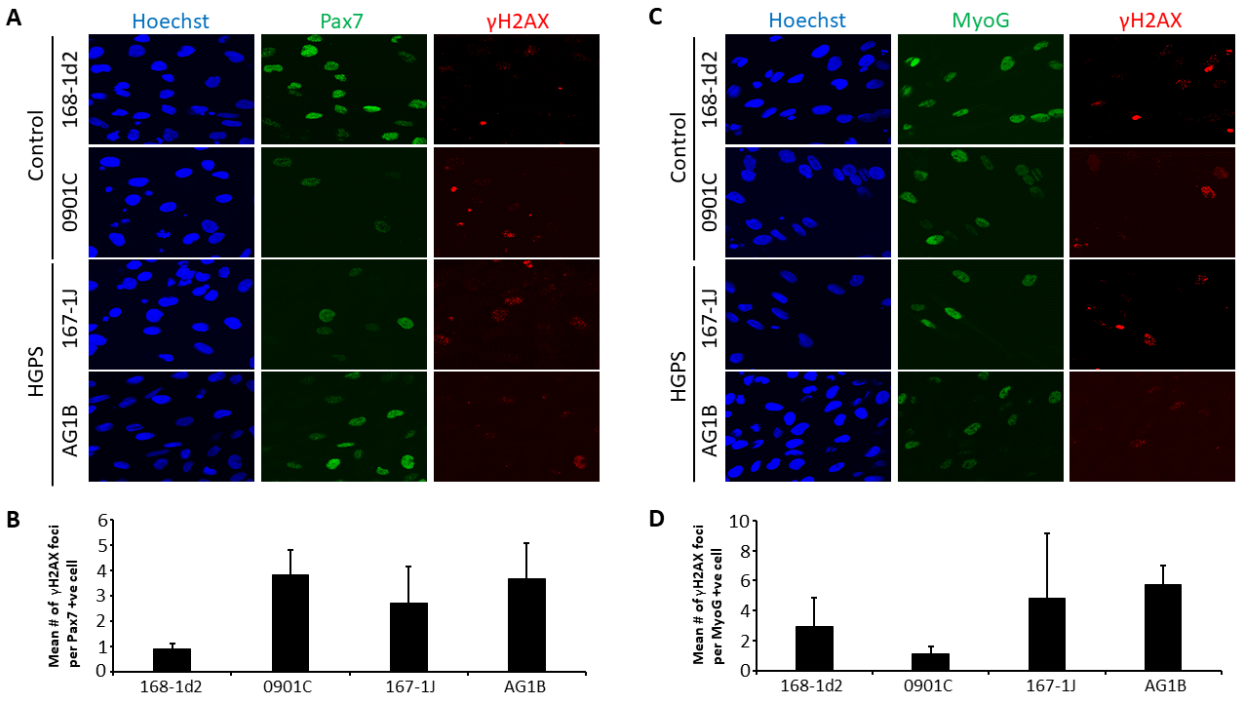


**Figure 3.2.8. Control and HGPS-derived myogenic cells appear to have similar levels of DNA damage response on day 60 of differentiation.** Day 55 cultures were passaged and plated onto Matrigel-coated coverslips to prepare for immunofluorescence analysis. The cultures were stained after 5 additional days of culture in E6 media (FGF2 supplemented) with antibodies against A) PAX7 and γH2AX or C) MYOG and γH2AX. Hoechst dye was used for the visualization of nuclei. A confocal microscope was used to capture high resolution images. Quantification of the number of γH2AX foci in the nuclei of B) PAX7 and D) MYOG positive cells was performed using ImageJ software (n=3, error bars represent +/- SEM).

As nuclear morphology defects have been extensively documented in HGPS-derived fibroblasts and smooth muscle cells, we probed for this phenotype in the skeletal muscle [250][255]. Experiments to assess this were performed on day 60 cultured myogenic cells which were co-stained with antibodies either against PAX7/LAMIN A/C or MYOG/LAMIN A/C. To assess the nuclear shape, high resolution confocal images were generated and analyzed using an ImageJ macro developed by the Stanford laboratory. In this method, an ellipse was fitted into the nuclei of the target cells. The degree of deviation of the nuclear shape from a perfect elliptical shape was quantified for the control and HGPS-derived myogenic cells and presented as score number ranging from 0 to 100 (100 being a perfect ellipse). As **Figures 3.2.9.B and D** demonstrate, no significant differences in nuclear morphology was observed between the control and HGPS-derived Myogenin and Pax7 positive cells.

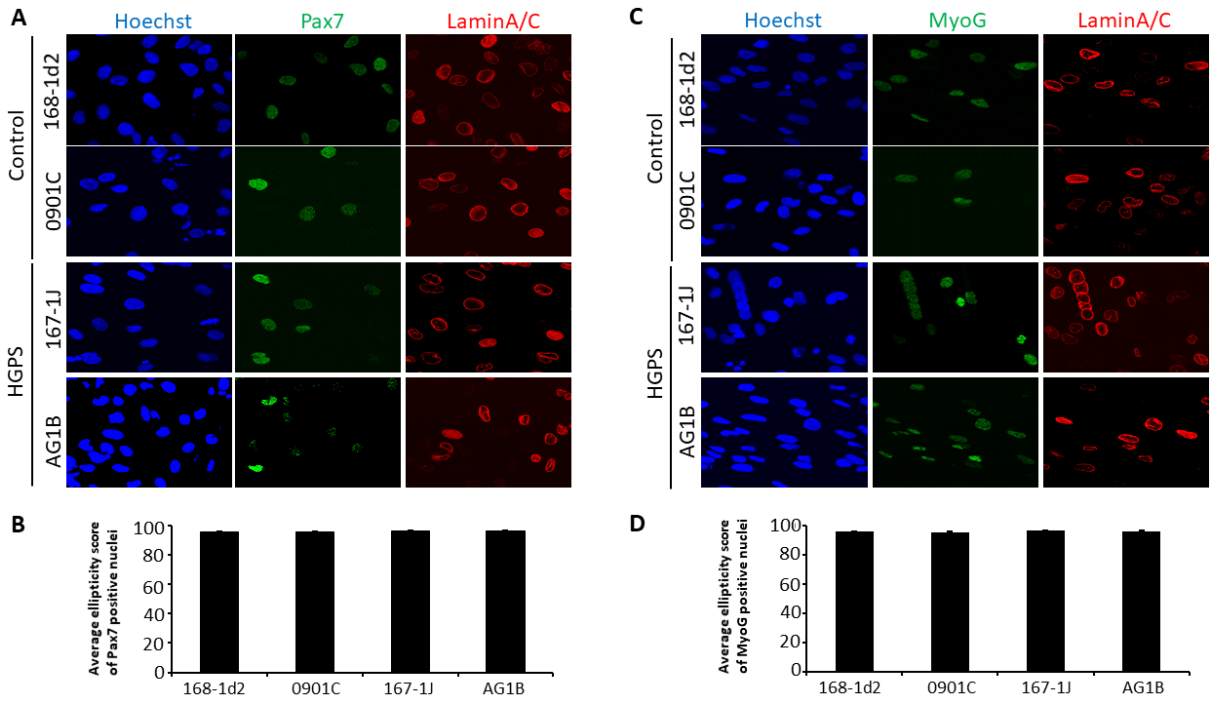


**Figure 3.2.9. HGPS-derived myogenic cells display normal nuclear morphology.** Day 55 cultured cells were passaged and plated onto Matrigel-coated coverslips to prepare for immunofluorescence analysis. The cultures were stained after 5 additional days of culture with antibodies against A) PAX7 and LAMIN A/C or C) MYOG and LAMIN A/C. Hoechst dye was used for the visualization of nuclei. Confocal microscopy was used to capture high resolution images. ImageJ software was implemented to assess and quantify nuclear morphology in B) PAX7 and D) MYOG positive cells (Perfect elliptical shape = score of 100). (n=3, error bars represent +/- SEM).

# CHAPTER 4 – DISCUSSION

Understanding of disease mechanisms ultimately leads to the development of strategies aimed at battling these conditions. These approaches can include halting disease progression, reversing/preventing the disorder or repairing and replacing afflicted damaged tissue. Detailed understanding of HGPS disease mechanisms may also lead to insights about aging in general. Thus, investigation of this disorder can prove to be beneficial to the scientific community in a number of different ways.

## ***4.1. Summary of Experimental Findings.***

The findings in this project can be divided into two general sections, which, together, contain valuable contributions to the stem cell, HGPS, natural aging and skeletal muscle research fields. The first part of the project generated and described the development/optimization of an efficient directed skeletal muscle differentiation protocol. The second part focused on characterization of the HGPS-derived myogenic cells implementing the optimized protocol.

### ***4.1.1. Directed Differentiation Skeletal Muscle Protocols.***

Developing strategies aimed at generating tissue-specific cells from pluripotent cells possesses many advantages, which include disease modeling, tissue engineering and *in vitro* drug screening. In general, human skeletal muscle directed differentiation approaches take advantage of two main strategies to generate myogenic-rich populations. These include guiding the pluripotent cells into the myogenic lineage with defined factors consisting of various molecular supplements [258][259][260] or overexpressing myogenic genes in these cells through gene-manipulation technologies [261][262][263]. Each of these strategies possess inherent advantages and disadvantages. Whereas gene manipulation on the pluripotent cells results in reproducible production of large, enriched populations of myogenic cultures, cells generated through this

approach are less desirable for therapeutic use due to the inherent risk of mutations involving vector integration at random genomic sites. A variety of protocols detailing both approaches have been developed and published during the recent years [reviewed in [264]]. The protocols, used in this project, implement the use of relatively small number of molecules to guide the pluripotent cell into the muscle lineage. Thus, this method avoids the introduction of myogenic transgenes (such as PAX3, PAX7, MYOD) into the cells, thereby avoiding the risk of insertional mutagenesis. This approach, therefore, can be considered more useful for various therapeutic applications. Due to this reason, the protocol published by *Shelton et al.* [179] was used as a foundation to perform the study of the HGPS skeletal muscle pathology. As discussed in the previous chapter, a significant number of optimization experiments were performed on this protocol (which was originally developed using H9 ESCs) for application on patient and control-derived iPSC lines. The successful completion of this stage of the project was a crucial prerequisite for the subsequent study of the HGPS skeletal muscle phenotype. During the process of optimization, (detailed in Chapter 3) we demonstrated that the original protocol could be modified to allow for periodic passaging of the cells starting on day 20 of differentiation [185]. This new method of myogenic cell culture brought about a number of improvements to the original protocol (50 days, no passaging), and proved to be a more applicable approach to perform this study. It is also worth noting that the development of this new and improved protocol, originally designed to probe for possible myogenic phenotype of HGPS, may be implemented for the study of other muscle disorders. These can include a number of other known laminopathies which display a more obvious skeletal muscle pathology (See chapter 1).

As stated, optimization of the base H9 ESC muscle differentiation protocol [179] for implementation on iPSCs, generated a modified method of SMP maintenance (outlined in Figure

1.4) [185]. This protocol allowed for regular passaging of the myogenic cultures starting from day 20 of differentiation. Some of major improvements of the newly developed protocol over the original, described in Chapter 3, included faster expansion of the PAX7 positive SMPs, ability to generate frozen stocks and more promising engraftment results post-injection of these cells into injured mouse skeletal muscle.

Splitting the myogenic cultures and re-plating them at a lower density allowed for a faster expansion of the SMPs and myocytes. Experiments have confirmed that passaging the cultures 1:3 of the original density is sufficient to sustain SMP survival [185]. Passaging in this manner twice (days 20 and 35) in the course of the 50 day protocol leads to a 9-fold expansion of myogenic cells. We have additionally found that for some of the iPSC lines lowering the passaging density to 1:6 and even 1:9 of the original also produced viable, expanding myogenic cultures. The primary advantage of this new method is, the ability to start the differentiation experiments with less pluripotent cells, thus, conserving expensive material in the early stages of the protocol. Moreover, regular passaging allows to keep the myogenic cells in culture for a prolonged period of time. Hence, depending on the density of re-plating they can be cultured for up to 70 days past the initial split on day 20.

The potential to freeze and successfully thaw the myogenic cells is another advantage of the modified differentiation protocol. In addition to giving the researcher more freedom to plan and synchronize differentiation experiments involving multiple iPSC lines, large scale freezing of the myogenic cells can also potentially help create storage banks (much like blood banks) for muscle repair in the future. This would, of course, require positive engraftment results in experiments involving the introduction of SMPs into injured muscle tissue.

Testing of SMP muscle repair capacity is achieved through introduction of these cells into injured skeletal muscle of immune-compromised animals (most commonly NSG mice). In these experiments, SMPs, are injected into the injured muscle tissue to test for their capacity to proliferate, differentiate and fuse into the host's damaged muscle tissue, as well as assess the ability of a subset of these activated SMPs to withdraw from the cell cycle and find the adult muscle stem cell niche. Thus, therapeutically-approved SMPs will essentially have to reconstitute the function of normal satellite cells during muscle injury [140]. Though the original 50 day protocol (no passaging) was very efficient at generating PAX7 positive SMPs, animal experiments involving their introduction into damaged mouse muscle tissue largely failed. Day 10 and day 50 cells largely failed to contribute either to the injured muscle fibers or to the satellite cell pool of mice when transplanted [Shelton, unpublished]. SMPs generated through the modified muscle differentiation protocol, involving the passaging of the cultures, saw improvements in these experiments. Preliminary data showed that day 35 H9 ESC-derived myogenic cells (passaged once on day 20) contributed to the muscle fiber when introduced into injured mouse muscle. However, the SMPs did not seed to the satellite cell pool, unlike bona fide adult muscle stem cells, when placed under similar experimental/physiological conditions [147]. In recent years, several studies have demonstrated the feasibility of using pluripotent stem cell-(PSC)-derived progenitors for muscle repair. Positive engraftment results were obtained from progenitors created through gene manipulation strategies [265][266][267][268][269]. These cells, in addition to contributing abundantly to myofibers, were also shown to seed to the satellite cell compartment [267]. To date, however, no group has generated a skeletal muscle differentiation protocol capable of generating transgene-free Pax7 positive human myogenic progenitors that can fully reconstitute the function of satellite cells *in vivo.*

Though the recent years have seen rapid development of skeletal muscle differentiation protocols using human pluripotent cells, none have demonstrated all of the listed SMP properties/capabilities in one. Thus, altogether, the improvements in directed skeletal muscle generation, achieved during the course of this project, can prove to be of great benefit for the development of strategies aimed at replacing/regenerating damaged muscle tissue through the transgene-free approach.

Though, as discussed above, this skeletal muscle generation protocol possesses many advantages, along the course of this project, various limitations have also been discovered. One primary limitation was the variable response of different iPSC and ESC lines to the differentiation protocol. However, this limitation, in stem cell research field has also been observed by other groups, who have shown different pluripotent cell lines having differential response to various *in vitro* differentiation signals [270][271][272]. The presence of this variability, in turn, tends to increase the variance in the comparison between the control and HGPS iPSC lines, producing a risk of potentially masking a possible HGPS muscle phenotype.

Another, potential disadvantage is the necessity for careful optimization of the early stages of the protocol, as detailed in chapter 3. This aspect, though timely, is crucial for a successful downstream differentiation. The need for optimization is not unique to this protocol but is shared with other tissue generation approaches. Interestingly, however, for this protocol, optimisation did not necessarily guarantee a successful differentiation. Cell death has been noticed to occur on multiple batches of differentiating cells with no feasible explanation of the cause. A weak correlation between high passage number of pluripotent and their subsequent death during differentiation has been established along the course of this project, yet not definitively proven.

This muscle differentiation protocol also fails to generate highly organized muscle structures, such as sarcomeric contractile units, which are absent *in vitro* due to limitations of the 2-dimensional tissue culture setting. Thus, generation of specialized myogenic cells present in the adult organism, such as satellite cells, is very difficult *in vitro*, as these cells reside in a very specific niche in the post-natal muscle. Nevertheless, the development of biomaterials which can mimic various aspects of the 3-dimentional structure of tissues as well as sustain the cells may offer improvements in this area in the foreseeable future [273]. Thus, this protocol could potentially be implemented to provide cell material for the bioengineered muscle scaffolds.

Though high percentage of the cells obtain myogenic identity when subjected to the directed differentiation protocol, there still appeared to exist a population of thus far undefined cells in the cultures. This becomes more evident at later passages, where the percentage of PAX7 positive SMPs and, more terminally differentiated MyoG positive cells gradually decreased (**Figure 3.2.4, 3.2.5**). Eventually, the myogenic cells were lost and the undefined cells took over the cell culture. The identity of these cells is yet to be elucidated and potentially removed from the myogenic cultures (Future Directions).

### ***4.1.2. Investigation of the HGPS Skeletal Muscle Phenotype.***

Although multiple studies have investigated satellite cells aging, this project is the first to analyze HGPS patient-derived skeletal muscle cells. However, the Stanford laboratory recently demonstrated that HGPS patient-derived iPSCs differentiated vascular smooth muscle cells (VSMCs) model the molecular and cellular defects of Progeria, validating the general feasibility to model HGPS using iPSC-derived muscle tissue [250]. In the aforementioned study, patient and control-derived iPSC were differentiated into vascular smooth muscle cells which were subsequently subjected to serial passaging, essentially establishing a model for *in vitro* aging.

After four weeks in culture, Progerin accumulation was observed in the HGPS-derived cells, along with replicative stress and cellular senescence. The use of SMPs, generated from HGPS-derived iPSCs, therefore, can potentially be one novel approach to study skeletal muscle aging in HGPS patients. Moreover, the establishment of the modified protocol, which allows for regular passaging of the myogenic cells and, therefore, *in vitro* aging, can facilitate the expression of age-associated phenotypes. Thus, we hypothesized that the analysis of HGPS-derived SMPs could potentially serve as a model for sarcopenia (loss of muscle mass and function due to age).

The considerable amount of research conducted over the past decade on aged satellite cells (Chapter 1, Rationale) served as a foundation for the formulation of this project's hypothesis. Taking into account this body of work, we predicted that the well characterized malfunctions of aged satellite cells would to a varying degree be translated into the HGPS-derived SMPs. In this model, the HGPS-derived SMPs, which are reminiscent of adult muscle stem cells, would exhibit malfunctions in differentiation and proliferation as their aged satellite cell counterparts.

Studies investigating the various age-associated failures of satellite cells (discussed in Chapter 1) tend to come to two general proposals regarding the mechanistic cause. Though these proposals may not necessarily be antagonistic to each other (i.e. they can both play a role in aged satellite cell decline), they do involve different mechanisms and concepts [reviewed in 274]. In the first category, the cause of the satellite cell malfunction is attributed largely to the cell-extrinsic parameters of the cell which encompasses its immediate niche as well as systemic circulation [275][276][277][278][279]. Naturally, with time, both of these parameters undergo age-associated changes. Thus, in this model, the niche (microenvironment) of the satellite cell, which plays a crucial role in the regulation of satellite cell function [280] is ultimately

responsible for its failure. The aged microenvironment may contribute to the disturbance of the various signals that originate from the niche to regulate the proliferation, differentiation and quiescence of adult muscle stem cells. In support of this view, various studies have demonstrated age-associated changes in signalling pathways that have been shown to be involved in various satellite cell functions. Among others, these include FGF, Notch, TGFβ as well as canonical Wnt signalling [274][reviewed in 281][246]. Systemic circulation, the other important component of the stem cell environment, has also been shown to play a role in muscle stem cell survival and function. In fact, various groups demonstrated the rejuvenation of the aged environment through heterochronic parabiosis experiments in mice. These experiments involved surgically joining the circulatory systems of aged and young animals. As a result, many of the aging effects were found to be reversed by essentially replacing the aged environment with a "youthful" one [275][276]. This particular model would support the largely negative results observed in HGPS-derived SMPs. Indeed, HGPS-derived cells were not found to be defective in either differentiation or proliferation compared to the controls. Analysis of terminal differentiation markers, MYOG and MyHC3 on RNA, as well as protein levels (**Figures 3.2.2, 3.2.3, 3.2.5**) was not significantly different between the HGPS and control myogenic cells. Ki67 staining, performed to assess proliferation of the SMPs, also did not reveal a difference between control and HGPS-derived cells (**Figure 3.2.7**). Provided that the assumption that Pax7 positive SMPs contain cells that are equivalent to satellite cells, the cell-extrinsic model explains the lack of the aged phenotype in HGPS-derived SMPs. As both, HGPS and control cells were cultured in an identical tissue culture setting (i.e. identical microenvironment), the extracellular signals acting on both cells would theoretically be identical. Thus, in this scenario, a common microenvironment would not allow for a phenotype to be revealed in the HGPS cells *ex vivo*.

On the other hand, the cell autonomous model of satellite cell aging attributes the malfunction intrinsic to the muscle stem cells [reviewed in 281][248]. In this model, age related changes in the cells, such as genomic instability, DNA damage, senescence and oxidative stress lead to the described aged-related phenotypes. Orienting on this model, and assuming that SMPs share similarities with satellite cells, defects should have been observed in the cultured HGPS-derived myogenic cells. This, however, did not appear to be the case for this study, as evident by the largely insignificant comparisons between controls and HGPS over the course of prolonged culture. The next step was to probe for the well-documented cell-intrinsic phenotypes found in most aged cells. HGPS-derived SMPs were first assessed for their level of DNA damage, with no difference observed between the control and HGPS cells (**Figure 3.2.8**).The lack of a significant DNA damage (presence of double-stranded breaks in the DNA) phenotype in the HGPS-derived SMPs could potentially be explained by the general resistance of the satellite cells to undergo DNA damage when compared to their more committed progeny [282]. Thus, the SMPs may share a commonality with the bona fide muscle stem cells in exhibiting this particular feature. Contrary to this logic, however, the HGPS-derived MYOG positive cells (essentially the committed SMPs) also had no significant difference in DNA damage. Other cell-intrinsic changes associated primarily with HGPS-derived cells, such as nuclear morphology defects, which have been well documented in patient-derived fibroblast [255] and differentiated smooth muscle cells [250] were absent in the PAX7 positive SMPs and more terminally differentiated MyoG positive myogenic cells (**Figure 3.2.9**). The lack of these phenotypes in a skeletal muscle system is yet to be investigated/explained.

Even though on RNA level *Progerin* was shown to be expressed in HGPS cells (Figure 3.2.6.), immunofluorescence staining performed against this protein on the myogenic cells

consistently failed to produce positive results. Though it cannot be excluded that the antibodies failed to work properly for these experiments (two species of antibodies were used), absence of positive Progerin staining on the HGPS cells raised some interesting prospects. It is possible that in a skeletal muscle system *Progerin* is silenced or that its translated product has a high turnover rate. The skeletal muscle wasting in the patients could then be explained as secondary to the disorders of the surrounding tissues, such as lipodystrophy (part of a cell-extrinsic causal theory). These possibilities would potentially serve as an explanation for the absence of the aged phenotype in an *in vitro* myogenic system. It is important to note that lack of a phenotype(s) for HGPS has been observed in other tissues of the patients, such as the liver, kidneys and the GI tract [283]. One particular group investigating the lack of pathology in HGPS mice brain tissue has discovered the silencing of *PRELAMIN A* RNA through a microRNA mechanism [284]. Specific down-regulation of *LAMIN A* (while sparing *LAMIN C*) in the brain by miR-9 microRNA was established to be the reason for no Progerin protein accumulation in this tissue and, therefore, no neural pathology. Therefore, presence of a similar mechanism in the skeletal muscle is a possibility that needs to be addressed.

Comparison of *in vitro* generated SMPs to satellite cells remains an unsolved challenge in the skeletal muscle field. As discussed above, this notion is central to the hypothesis of this project, which predicts age-associated phenotypes in the HGPS-derived SMPs based on the research conducted on aged satellite cells. It can be stated with confidence that SMPs are not entirely equivalent to the satellite cells, judging from the failure of these cells to fully reconstitute muscle stem cell function in animal experiments. Also discussed, were the limitations of using 2D tissue culture techniques to generate the highly specialized satellite cells, which *in vivo* require a specific niche for development and eventual residence (Chapter 1). On

the other hand, the SMPs are PAX7 positive, which is the universal marker of all quiescent satellite cells. They are also found in a myogenic-rich environment, interspersed between myocytes and their more committed progeny (MYOG, MyHC positive cells). Thus, SMPs appear to share certain characteristics with the satellite cell, while differing in others. Therefore, the failure to observe the aging phenotype and Progerin accumulation in the SMPs can potentially be attributed to the differences between these cells and the bona fide adult muscle stem cells. Ultimately, the degree of similarity between these two cell populations will need a quantitative determination. One approach aimed at achieving this will be discussed in the next section (Future Directions).

Finally, it is also worth noting the considerable cell line-to-cell line variation observed in the experiments. As discussed in the previous section, the variable response of different cell lines to the differentiation hindered the comparisons between HGPS and control cells. An example of this can be observed in **Figure 3.2.7.**, where SMP numbers are monitored prior to each subsequent passage. It can be observed that the Pax7 positive cells are lost at a faster rate for the 0901C control line when compared to 168-1d2. This discrepancy between the control cell lines creates a hindrance in the comparisons between HGPS and control. To counter this, a highly stringent algorithm for determination of significance between HGPS and controls (detailed in Chapter 3) was devised. Thus, it is possible that some age-associated phenotypes were masked by the variable response of the iPSC lines to *in vitro* differentiation ques.

## ***4.2. Future Directions.***

As with the results, the future directions of this project are subdivided into two general sections. The first part focuses on the development of improvements on the directed differentiation protocol and correction of the listed limitations. The second part comprises the

follow up probing into additional possible HGPS muscle phenotypes. For this section, an important objective will be to investigate the reason(s) for the absence of the aged phenotypes in the myogenic cells generated/investigated in this project.

The skeletal muscle generation protocol used in this project, though highly efficient at producing myogenic cells, yields a population of undefined cells, which increase in numbers with each subsequent passage. This can be deduced from experiments monitoring the expression of MYOG and PAX7 positive cells in the myogenic cultures, which combined are less than all the cells. Moreover, during later passages, the percentage of the undefined cells in the myogenic cultures increases. Thus, common with other differentiation assays that do not implement cell sorting, this protocol does not yield cells with exclusively myogenic identity. This particular limitation of the protocol could become a potential hindrance for future therapeutic use of the myogenic cells, as it would be undesirable to introduce undefined/non-myogenic cells into a muscle environment. One way to tackle this issue will be to perform Fluorescence-Activated Cell Sorting (FACS) in order to isolate the PAX7 positive SMPs, thereby purifying the population of myogenic cells. The primary challenge of this approach is the identification of appropriate surface markers that will exclusively mark the PAX7 positive SMPs. As discussed in the introduction, a number of surface proteins mark satellite cells (though not exclusively). Identifying such markers on the SMPs will be crucial for their subsequent sorting. Satellite cell surface proteins will serve as primary candidates for probing SMPs. In fact, currently, mouse satellite cells can be isolated from the muscle to create highly-enriched cell population using a combination of cell surface markers (Integrin alpha/CD34 double positive and CD11b, CD31, CD45 and Sca1 negative cell) [285]. The potential expression of these proteins on the SMPs must be analyzed first. Very recently, a number of surface markers have been identified on

myogenic progenitor cells derived through PAX7 overexpression [286]. This group established the expression of CD54, Integrin α9β1 and SDC2 surface markers on PAX7-induced muscle progenitor cells, allowing for the isolation of these cells through cGMP- and fluorescent-compatible magnetic-based sorting technologies. Moreover, cells isolated through this method were shown to contribute to long-term muscle regeneration *in vivo.* The expression of these markers will need to be assayed in the SMPs generated by the transgene-free approach implemented during the course of this project.

The next step will be to continue the search for molecules (e.g. recombinant proteins, various pathway agonists and inhibitors) that could play a positive role in maintaining and expanding the FACS-sorted SMPs (preventing premature differentiation). In addition to FGF2, our laboratory is currently looking into using a combination of other small molecules in order to further expand the SMP population, minimizing differentiation. One such molecule, a P38 MAPK pathway inhibitor has been demonstrated to expand satellite cells capable of engraftment [162][287]. Another candidate, a Sonic Hedghog (Shh) pathway inhibitor, cyclopamine, has also been reported to promote satellite cell proliferation [288]. These molecules can potentially be added to the differentiation protocol to expand the FACS-sorted SMP pool and, potentially, improve their engraftment capabilities. Currently, the efficacy of using these molecules is being tested on mESC-derived SMPs in our laboratory.

As discussed earlier, a central assumption of this project that remains unverified is the degree of equivalence between the Pax7 positive SMPs and bona fide adult muscle stem cells. An answer to this question will allow for the development of strategies aimed at generating SMPs with better therapeutic potential. One possible approach to achieve this could be through global gene expression comparison conducted between these two cell types. In order to design

this study, firstly, the differentiation protocol will need to be optimized for implementation on mouse iPSCs (due to ease of obtaining satellite cells from these animals). Subsequently, somatic cells from the mouse will be reprogrammed to generate iPSCs which then will be differentiated to generate the Pax7 positive SMPs. From the same animal, satellite cells will be extracted using well-established techniques. This would enable a direct comparison of gene expressions between the generated progenitor cells and adult muscle stem cells through either RNA-seq or microarray experiments. Once differences are discovered and described, a strategy will be developed aimed at bringing the gene expression signature of the SMPs closer to that of satellite cells. Similarly, a comparison between PAX7-induced progenitors to the SMPs generated in this project can suggest alterations to this differentiation protocol that could potentially result in improved subsequent engraftment assays.

The development of future research directions regarding the study of the HGPS muscle phenotype, as stated, is the other main objective of this project. It is important to note, however, that improving the muscle differentiation protocol (i.e. successful FACS-associated sorting and expansion of SMPs) will also lead to improvements in the characterization of the HGPS-derived myogenic cells.

Though characterization of the HGPS-derived myogenic cells, was fairly detailed, as proliferation and differentiation were investigated, there are additional experiments that could be performed to validate these results. Regarding differentiation, there are additional myogenic factors that will need to be examined. For example, MYOD, whose expression in the satellite cells denotes activation was not investigated in the differentiating cultures due to the lack of appropriate primers and antibodies. Additional candidate genes of interest will include other important players of late myogenesis, such as MRF4, Muscle Creatine Kinase (MCK) and

DESMIN. To validate results dealing with cell proliferation, BrdU assays can be performed, though attempts to perform this experiment failed during the course of this project. The availability of many BrdU staining protocols that can be implemented may help solve this problem. Performing these additional experiment will result in a more complete characterization of the HGPS-derived myogenic cells.

A key area that needs to be addressed/validated is the lack of detectable pathology in the HGPS-derived myogenic cells. Indeed, no significance was observed in most of the wide-range comparisons performed between the control and HGPS-derived myogenic cells. Myogenic gene expression, nuclear morphology, DNA damage assay and proliferation were among the experiments performed on the myogenic cultures on various days of differentiation which revealed no significant difference between the control and HGPS cells. As discussed, the absence of these phenotypes can be explained by the lack of Progerin accumulation in the generated myogenic cultures. This model supports the notion of skeletal muscle atrophy in HGPS patients as a secondary effect of tissue pathology surrounding the skeletal muscle (part of cell-extrinsic causal theory of satellite cell malfunction). Several strategies can be implemented to address/validate this possibility. The lack of HGPS pathology in the brain has been discussed earlier, where miR-9 microRNA has been shown to prevent Progerin accumulation in this tissue. Preliminary experiments to test if this mechanism could also be at play in the skeletal muscle were performed by testing miR-9 expression in the HGPS and control- generated myogenic cells implementing qPCR. We predicted that higher expression of miR-9 in the HGPS would suggest a microRNA mechanism of Progerin inhibition in these cells. Unexpectedly, the control cells showed a trend toward higher miR-9 expression than the HGPS cells, though this was not statistically significant. It is important to note, however, that a positive control (e.g. brain tissue)

was not used for this experiment due to unavailability. Thus, it was not possible to determine if miR-9 is significantly upregulated in a myogenic system in general (being it HGPS or control). One future direction will, therefore, be to repeat these experiments with appropriate. Fibroblasts and VSMCs, where HGPS pathology has been well documented, may potentially serve as the negative controls. Establishment of abundant expression of miR-9 in myogenic cells (compared to fibroblasts and VSMCs) may explain the lack of Progerin accumulation in HGPS (even in the case where this expression is not significantly different between the controls and progeria). In addition to miR-9, a bioinformatics approach can be implemented to predict additional potential microRNA candidates that could target Progerin in the myogenic system. These candidates can subsequently be validated and selected for study of the mechanism of action. In order to confirm the cell-extrinsic theory, HGPS and control SMPs can be injected into injured muscle tissue of old or progeric mice. The *in vivo* setting of these animals may then allow for the age-associated phenotypes to resurface in the HGPS-derived SMPs. Once again, for these experiments to proceed, the differentiation protocol will need improvements to allow for efficient engraftment of SMPs into the muscle fiber as well as their contribution to the innate satellite cell population (discussed earlier).

## ***4.3. Conclusions.***

The first part of the project demonstrated the versatility of using the directed differentiation approach published by *Shelton et al.* on multiple pluripotent cell lines. Though significant optimization and a modification was required, this method can serve as a suitable way to study skeletal muscle diseases implementing the iPSC approach. Modification to the standard *Shelton et al.* protocol allowing for serial passaging of the myogenic cells starting on day 20 resulted in several improvements, including faster expansion and more uniform distribution of

the SMPs in the TC wells. Furthermore, the modified protocol allowed for the generation of SMP frozen stocks at different time points of differentiation.

In summary, the results from the second part of the project demonstrated, the HGPS-derived myogenic cultures did not appear to be defective in formation, differentiation or proliferation. Also, no difference between HGPS and controls was detected in the expression of senescence and quiescence markers. In addition, no DNA damage or nuclear morphology defects were detected in the HGPS cells.

The lack of phenotypes in the HGPS-derived myogenic cells can be attributed to the absence of Progerin accumulation in SMPs due to silencing/degradation mechanisms. In this case the noted muscle decline in HGPS patients could be attributed to niche factors. Therefore, skeletal muscle atrophy can be explained as secondary to the pathology of the surrounding tissue, such as lipodystrophy. This would offer support to the discussed cell-extrinsic model of aged satellite cell malfunction.

Another potential explanation could lay in the nature of SMPs generated through our directed differentiation protocol and how these cells compare to true satellite cells. Though the SMPs have satellite-cell-like features (such as being Pax7 positive), these two cell populations are not equivalent. Therefore, Progerin might be expressed in the satellite cells of HGPS patients but not the iPSC-generated SMPs. Thus, phenotypes observed in the aged/progeric satellite cells may not necessarily translate into the skeletal muscle precursors generated in this project.

Finally, there was substantial cell line-to-cell variability throughout the experiments. These variations resulted due to the inherently variable response of the different pluripotent cell

lines to the directed differentiation protocol and might have contributed to the masking of certain age-associated phenotypes in the HGPS myogenic cells.

# APPENDICES

## ***Appendix A***

Table 1 – Human forward and reverse primers used for the qPCR experiments.

| GENES | FORWARD PRIMER (5'-3') | REVERSE PRIMER (5'-3') |
|---|---|---|
| GAPDH | TGGTGCTGAGTATGTCGTGGAGT | AGTCTTCTGAGTGGCAGTGATGG |
| Brachyury T | TTCATAGCGGTGACTGCTTATCA | CACCCCCATTGGGAGTACC |
| Mesogenin | AACCTGCGCGAGACTTTCC | ACAGCTGGACAGGGAGAAGA |
| Tbx6 | CATCCACGAGAATTGTACCCG | AGCAATCCAGTTTAGGGGTGT |
| Pax3 | CTCACCTCAGGTAATGGGACT | CGTGGTGGTAGGTTCCAGAC |
| Meox1 | GCAGGGGGTTCCAAGGAAAT | GTCAGGTAGTTATGATGGGCAAA |
| Pax7 | CCCCCGCACGGGATT | TATCTTGTGGCGGATGTGGTTA |
| Myf5 | AATTTGGGGACGAGTTTGTG | CATGGTGGTGGACTTCCTCT |
| MyoG | GCTGTATGAGACATCCCCCTA | CGACTTCCTCTTACACACCTTAC |
| MyHC3 | TTGATGCCAAGACGTATTGCT | GGGGGTTCATGGCGTACAC |
| Progerin | GCGTCAGGAGCCCTGAGC | GACGCAGGAAGCCTCCAC |
| Spry1 | GCCTTCTTTGGATAGCCGTCAG | TCATTGCTGCCTCTTATGGCC |
| P27 | AACGTGCGAGTGTCTAACGG | CCCTCTAGGGGTTTGTGATTCT |
| P19 | CGCTGCAGGTCATGATGTTT | GGGTGTCCAGGAATCCAGTG |
| P21 | TGTCCGTCAGAACCCATGC | AAAGTCGAAGTTCCATCGCTC |

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## Curriculum Vitae

**Personal:**

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Citizenship: Canadian

**Academic Background:**

2014 - 2018 Master of Science, Biochemistry
Faculty of Medicine, University of Ottawa
Ottawa, Ontario, Canada

2009 - 2014 Bachelor of Science, Specialization in Biochemistry
University of Ottawa
Ottawa, Ontario, Canada

**Awards (Selected):**

2017 Member of Parliament Volunteer Recognition

2016 The Sovereign's Medal for Volunteers

2015 Volunteer Long Service Award

2015 Letter of Appreciation for Volunteering from Ottawa City Council

2014 Ontario Graduate Student Scholarship

2014 University of Ottawa Excellence Scholarship

2014 University of Ottawa Admissions Scholarship – Master's

2013 Dean's Honour List

2012 Dean's Honour List

2010 Dean's Honour List

2009 University of Ottawa Admissions Scholarship (+ renewals) – Undergraduate

2009 Queen Elizabeth II Scholarship – Undergraduate

2009 Top Academic Award, Governor General's Academic Medal – High School

**Work Experience**

2013 (fall) Student Researcher, Laboratory of Dr. Ilona Skerjanc
University of Ottawa
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2013 (summer) Student Researcher, Laboratory of Dr. Ilona Skerjanc
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2012 (summer) Student Researcher, Laboratory of Dr. Ilona Skerjanc
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2011 (summer) Junior Research Assistant at Pharm*Ideas*
Ottawa, Ontario

2010 Tutoring
Ottawa, Ontario

**Volunteer Activities**

2012- Volunteer Team Leader at Cancer Center
Ottawa General Hospital, Cancer Center
Ottawa, Ontario

2010- Volunteer, Cancer Center, Patient Ambassador
Ottawa General Hospital, Cancer Center
Ottawa, Ontario

2010- Volunteer, Information Desk
Ottawa General Hospital, Cancer Center
Ottawa, Ontario

2010- Volunteer, Cancer Center, Clinics (Physician/RN assistance)
Ottawa General Hospital, Cancer Center

Ottawa, Ontario

2009 (summer) STRIDE Wheelchairs plus

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**Abstracts/Posters**

Ritso, M., Dore, C., **Kocharyan, A.,** Brun, C., Stanford, W., Rudnicki, M. Cell Division Mechanisms in Human Dystrophic Satellite-like Cells. OHRI Research day, 2016.

Ritso, M., Dore, C., **Kocharyan, A.,** Stanford, W., Rudnicki, M. Modelling Human Satellite Cell Homeostasis in Mouse Skeletal Muscle. CNMD, 2017

**Publications**

Shelton, M., **Kocharyan, A.,** Liu, J., Skerjanc, I., Stanford, W. Robust generation and expansion of skeletal muscle progenitors and myocytes from human pluripotent stem cells. *Methods* 101:73-84 (2016).

**Conferences**

**Ottawa International Conference on Neuromuscular Disease and Biology.**
September, 2015.

**Languages**

- English
- Russian
- Armenian

**Other Interests**

- Chess
- Literature