CALIFORNIA STATE UNIVERSITY, NORTHRIDGE

Directing Skeletal Muscle Progenitor Cell Fate from Human Pluripotent Stem Cells

A thesis in partial fulfillment of the requirements

For the degree of Master of Science in Biology

By

Richard Swan

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Thesis of Richard Swan is approved by:

_____	_____
Dr. Cindy Malone	date
_____	_____
Dr. April Pyle	date
_____	_____
Dr. Virginia Vandergon, chair	date

California State University, Northridge

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Thomas O. Dial[b], Zheng Luo[c], Shuang Hou[c], Jinliang Peng[c], Rebecca E. Andersen[b], Tuan Tran[d], Sean P. Sherman[e], Cindy Malone[a], Hsian-Rong Tseng[c] and April D. Pyle[b,e,f]

[a]California State University Northridge, [b]Department of Microbiology, Immunology, & Molecular Genetics (UCLA); [c]Department of Molecular and Medical Pharmacology (UCLA), [d]Department of Molecular, Cell, & Developmental Biology (UCLA), [e]Molecular Biology Institute (UCLA) ; [f]Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research (UCLA), Jonsson Comprehensive Cancer Center; University of California, Los Angeles

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Dedication

I would like to thank my family, friends, professors and colleagues for the various forms of support they have provided me durring the course this project. You know who you are.

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ABSTRACT

Directing Skeletal Muscle Progenitor Cell Fate from Human Pluripotent Stem Cells

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Master of Science in Biology

Duchenne Muscular Dystrophy (DMD) is a devastating and fatal muscle wasting disease with no known cure, caused by mutations in the dystrophin gene. Stem cell based regenerative treatments hold promise to potentially restore healthy muscle to DMD patients. However, the differentiation of pluripotent stem cells must be learned to be precisely controlled for any such treatment to be usable, as uncontrolled differentiation can lead to tumor formation. Skeletal muscle progenitor cells (SMPCs) are a muscle-specific stem cell type which generate only muscle cells, and so would be an ideal cell type to test for use in treatment. Development of an efficient protocol for differentiating human pluripotent stem cells (hPSCs) to a skeletal muscle progenitor cell (SMPC) would open up the possibility of a cell based treatment for Duchenne Muscular Dystrophy (DMD) and other muscle wasting diseases. Any such treatment would have to be used in combination with one of many gene based therapies currently being studied to repair the mutations in the dytrophin gene responsible for causing DMD. In this study we test two different experimental methods for their ability to differentiate hPSCs to SMPCs: directed differentiation with developmentally relevant growth factors, and direct reprogramming via overexpression of master regulator transcription factors via a novel

nanotechnology system. While not yet successful in deriving the SMPC cell type from hPSCs, this study describes significant progress towards that goal.

Duchenne Muscular Dystrophy

One research interest of the Pyle lab is focused on developing a potential treatment for Duchenne Muscular Dystrophy (DMD). DMD is a currently incurable progressive muscle wasting disease that results from an X-linked recessive mutation in the dystrophin gene, and effects 1 in 3,500 boys worldwide. X-linked diseases such as DMD effect males, but can be passed on by female carriers, though one third of DMD cases are due to a spontaneous mutation in the fetus (Emery et al, 1991). DMD is generally diagnosed in early childhood, with boys displaying marked and distinctive lack of muscle tone and pseudohypertrophy (swelling) leading to difficulty in normal activities (Yanagisawa et al, 2008). The disease can be diagnosed by observed symptoms or a genetic test. The disease progresses from proximal to distal regions in the patient's body, and can debilitate cardio-respiratory function in addition to skeletal muscle function. DMD patients generally lose the ability to walk sometime in their childhood or teenage years. As the disease progresses, patients acquire increased skeletal and muscular deformities, further complicating their prognosis. Most patients die of respiratory or heart failure in their twenties (Poysky et al., 2007; Bushby et al. 2010). DMD patients totally lack dystrophin protein, a structural protein in muscle cells, the absense of which leads to the disease phenotype. The absence of dystrophin pre-disposes muscle tissue to chronic injury throughout the lifetime of the patient. Damaged muscle cells are attacked by the immune system. DMD patients have an abnormally large amount of connective tissue as well as fatty deposits between the muscle fibers (O'Brien et al, 2006). DMD also effects cardiac muscle and smooth muscle. Diagnosis used to be based solely on symptoms, but can now be confirmed by testing for frame shift mutations of the dystrophin gene (O'Brien et al, 2006). There is a hypothesis that lack of dystrophin causes excessive

stretching in the cell membrane which in turn opens stretch-activated ion channels, resulting in excess calcium in the cytoplasm. It is thought that this contributes to cytosolic oxidative stress, which damages and eventually kills the cell (Allen et al, 2010). Clinicians observe a continual and chronic degeneration and regeneration cycle in the skeletal muscle. Necrotic muscle cells are replaced with fibrotic and connective tissue as the muscle stem cell pool is exhausted, gradually decreasing strength in all skeletal muscle (O'Brien et al, 2001; Muntoni et al, 2003). [Figure 1] . The defects in dystophin seen in muscular dystophy patients sometimes result in mental retardation due to damage to the dystrophin produced in these patient's brains (Yanagisawa et al, 2008).

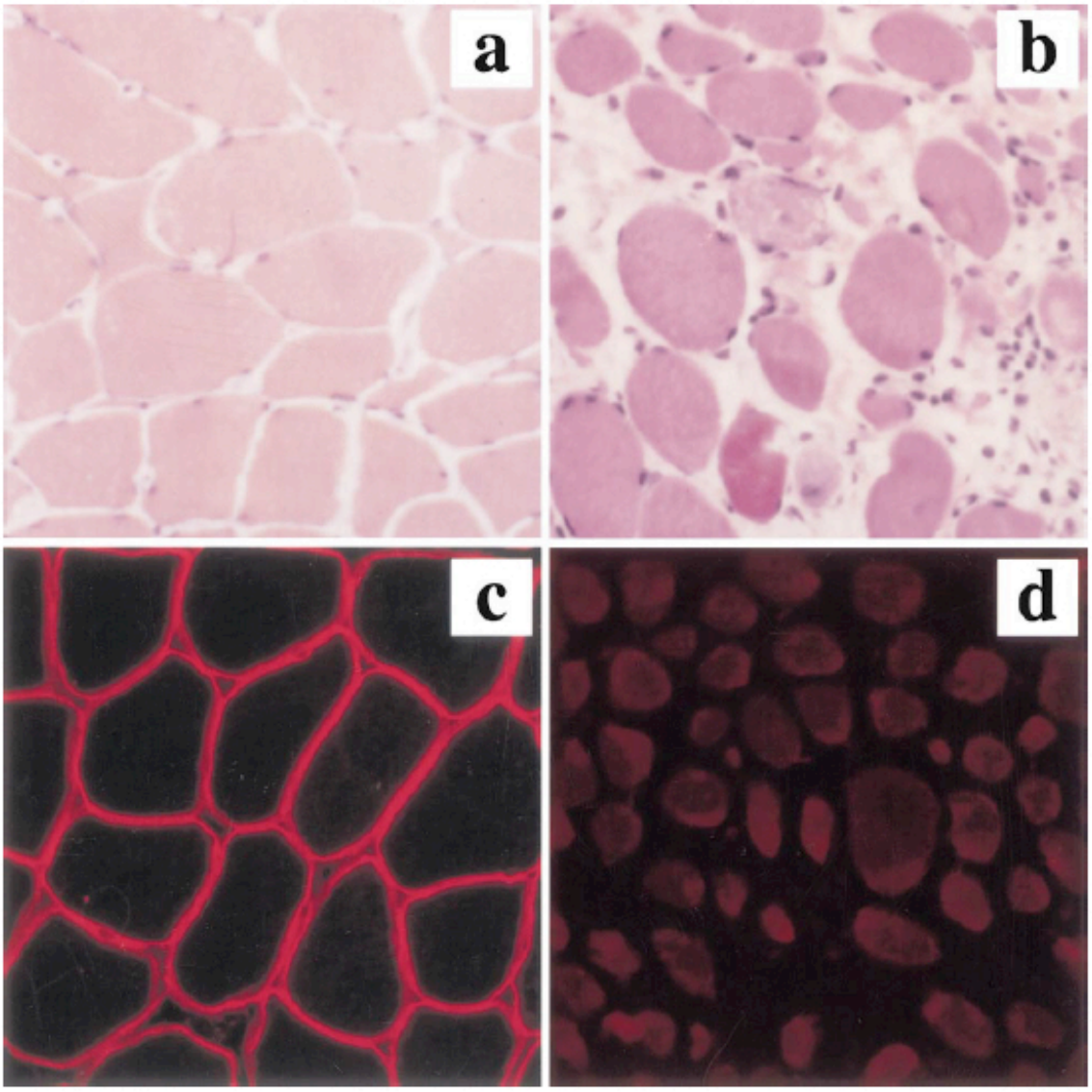


Figure 1 Histology of DMD muscle. Hematoxylin and eosin staining of control (a) and DMD patient (b) biopsies show abnormal variation in fiber size, degenerating and regenerating fibers, immune cell infiltration, and increased fibrosis in DMD. Immunofluorescence analysis of dystrophin in control (c) and young DMD patient (d) biopsies illustrates the loss of sarcolemmal staining in DMD. (O'Brien et al, 2001)

Dystrophin

Dystrophin is the largest gene in the human genome spanning 2.6 million base pairs on the X chromosome, consisting of 86 exons and seven tissue-specific promoter regions. After processing, the dystrophin transcript is 14,000 base pairs long, and the subsequently translated protein (Dp427) contains 3,685 amino acids and weighs 427 kilodaltons (kDa) (Hoffman, et al, 1987). Dystrophin is expressed in multiple tissues including skeletal muscle, smooth muscle and cardiac muscle, as well as the brain. Each tissue-specific promoter is associated with a tissue-specific first exon, which is then spliced to the remainder of the transcript. When expressed in skeletal muscle, the dystrophin protein normally binds the actin filaments in muscle cells to the transmembrane dystrophin/glycan complex spanning the cell membrane, or sarcolemma, which in turn is bound to the extracellular matrix. This skeletal muscle dystrophin protein is composed of four domains: the C-terminal domain and a domain with a dense concentration of cysteine both of which bind to various proteins of the transmembrane Dystroglycan complex, a rod domain, and the N-terminal actin binding domain (Figures 2 & 3)(O'Brien et al, 2001; Muntoni, et al, 2003). Dystrophin is an important structural protein in the muscle cell, which acts to protect the muscle cell membrane from the forces exerted by contracting actomyosin myofibrils (Figure 4) (O'Brien et al, 2001; Muntoni et al, 2003). In patients with DMD, there are frame shift mutations in the dystrophin gene (located at locus Xp21) which introduce premature stop codons, causing an absence of dystrophin and a severe disease phenotype. As dystrophin is the largest human gene, it follows that it is a large "target" for spontaneous mutations. There are a diversity among Dystrophy-inducing mutation types, including duplications, point mutations, and deletions. Most DMD inducing mutations are found within the "hotspot" region of exons

44 and 55. This is the region of the dystrophin gene encoding the rod domain of the protein composed of spectrin repeats, which are thought to have characterisitics analogous to a shock-absorber; imparting structural stability as well as flexibility and cushioning. (Nelson et al, 2009).

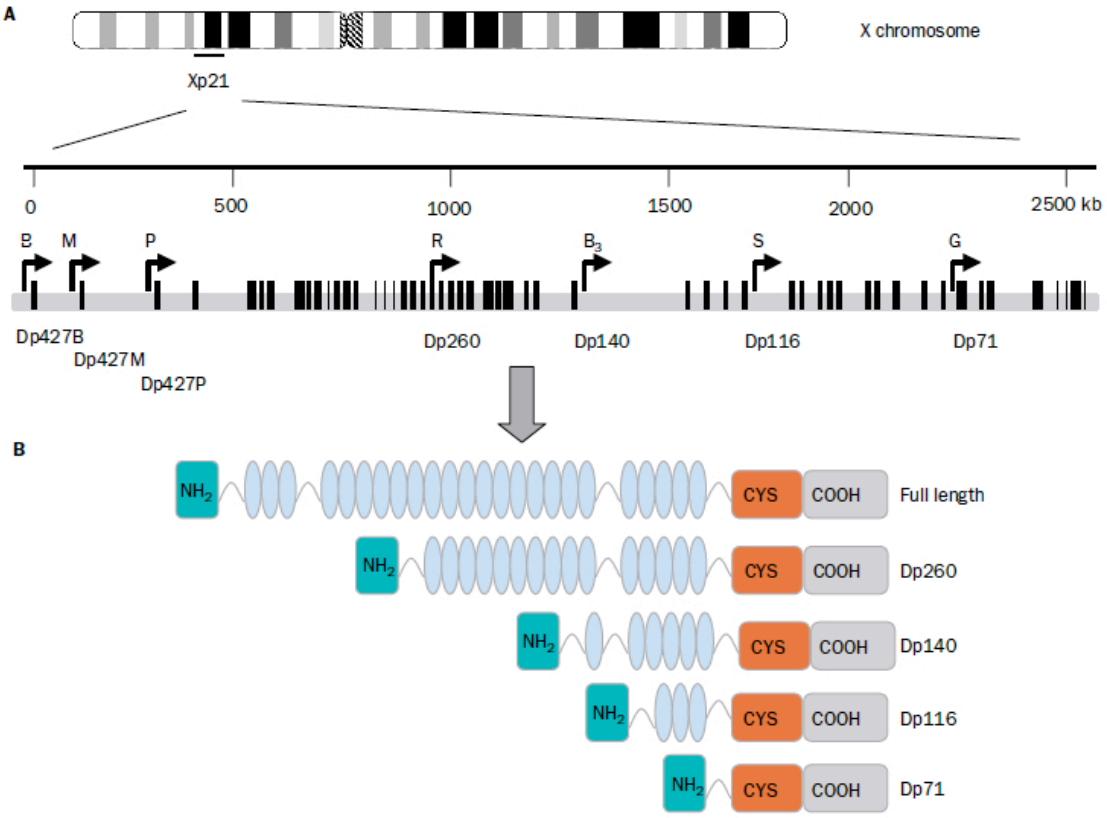


Figure 2. A: Genomic organization of the dystrophin gene, located in Xp21. The black vertical lines represent the 79 exons of the dystrophin gene distributed over about 2·5 million bases. The arrows indicate the various promoters: in particular are brain (B), muscle (M), and Purkinje (P) promoters; R, B3, S, and G represent the Dp260 (retinal), Dp140 (brain3), Dp116 (Schwann cells), and Dp71 (general) promoters. B: The domain composition of the various dystrophin proteins is indicated. The amino-terminal domain is followed by the spectrin like domain, the cysteine rich, and the carboxy-terminal domain. (Muntoni et al, 2003)

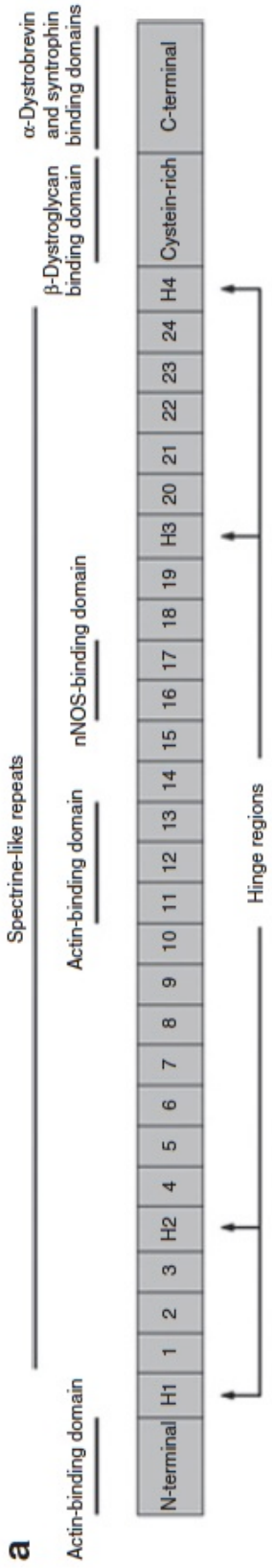


Figure 3. Schema representing the four main domains of dystrophin: the N-terminal part, central rod domain (containing 24 spectrin-like repeats and four hinge domains), cystein-rich region and the C-terminal part. The protein binding domains are also indicated. (Pichavant et al, 2011)

Figure 3. Schema representing the four main domains of dystrophin

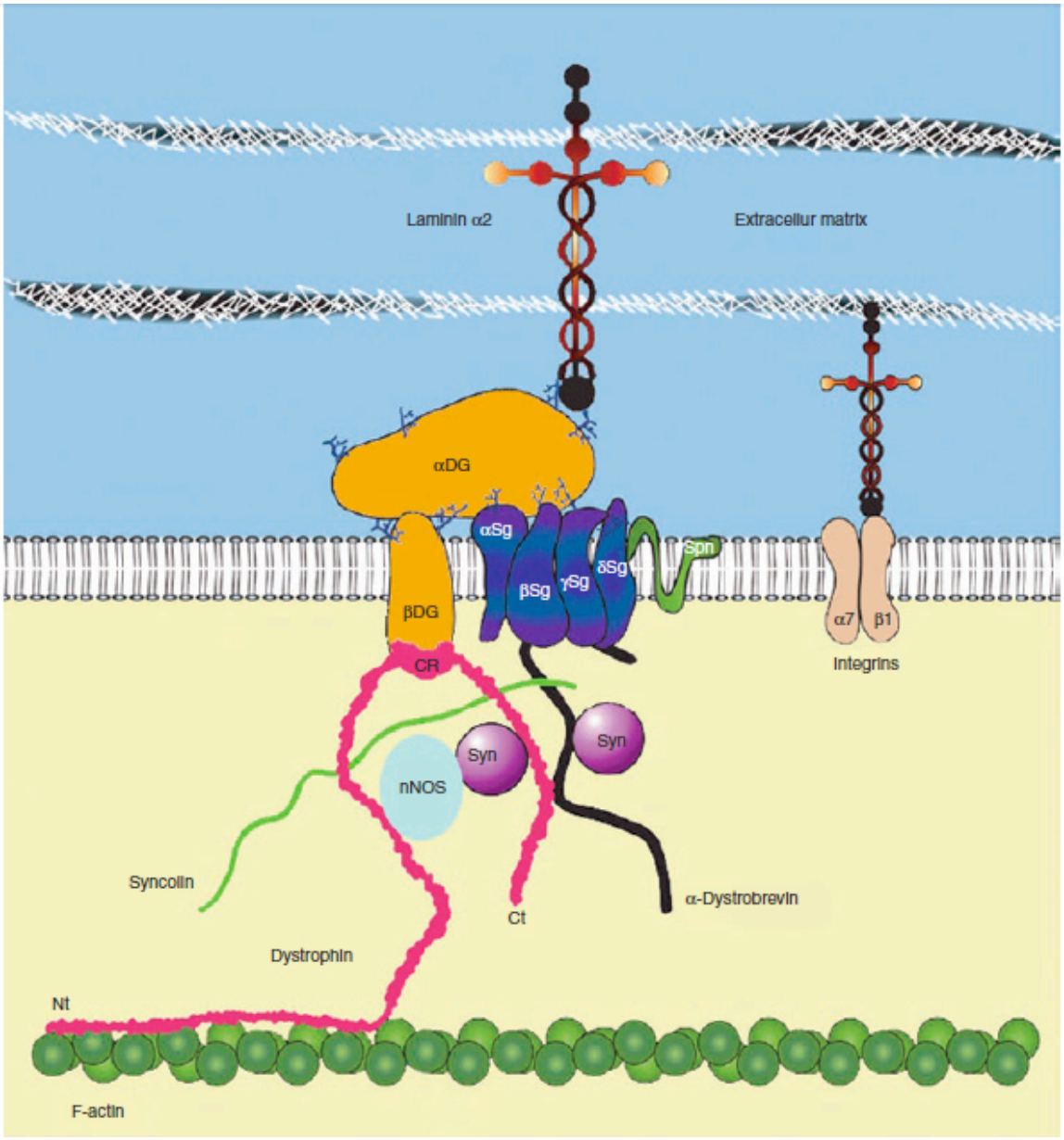


Figure 4. Diagram of the dystrophin-associated glycoprotein complex (DGC). This complex includes dystrophin with its C-terminal (Ct), cysteine-rich (CR), and N-terminal (Nt) regions as well as proteins associated in this complex. DG, dystroglycan; nNOS, neuronal nitric oxide synthase; Sg, sarcoglycan; Syn, syntrophin. Modified from Odom et al. (Pichavant et al, 2011

Becker's Muscular Dystrophy suggests possible treatments for DMD

There are other dystrophies, of which the most directly correlated to DMD is Becker's Muscular Dystrophy (BMD). BMD is a X-linked dystrophy caused by in-frame deletions within the dystrophin gene, resulting in an abbreviated but functional dystrophin protein (Becker et al, 1955; Malhotra et al, 1988). BMD is much less common than DMD, occuring in 1 in 30,000 live male births (Shapiro et al, 1993). The severity of disease phenotype varies in BMD based on where in the dystrophin protein the mutations occur, with the most severe symptoms correlating with the various binding regions of the protein, but in general most mutations occur in the rod-like spectrin repeat region and result in a phenotype much less severe than DMD patients. BMD patients can live past their fifth decade and generally retain the ability to walk (Manzur et al, 2008; Beggs et al, 1991).

Various drugs are currently at various levels of pre-clinical and clinical testing to determine their efficacy and safety in treating dystrophy. Several classes of these drugs attempt to restore the dystrophin in DMD patient's cells to resemble a more BMD-like form of the protein. One such class are stop codon read-through drugs including but not limited to gentamycin and ataluren. These drugs interact with ribosomes to introduce an amino acid into the transcript when it encounters a premature stop codon, allowing protein translation to continue (Kaufman, 1999; Aurinoet al, 2006). These drugs have performed well in mice, but yielded less convincing results in human trails, and are accompanied by undesireable side effects (Barton-Davis et al, 1999; Wagner et al, 2001; Politano et al, 2003, Malik et al, 2010). Furthermore stop codon read through drugs would only be useful for 10 to 15% of DMD patients.

A second class are exon skipping drugs consisting of antisense oligonucleotides (AO) composed of small lengths of RNA or DNA designed and sythesized to bind to specific sites on pre-mRNA to eliminate specific exons from becoming part of the translated protein. By designing these AOs to eliminate the region containing the DMD pateint's premature stop codon from the mRNA it is possible to restore the reading frame resulting in a shorter but functional dystophin protein (Stein et al, 1993; Monia et al; 1997). (Figure 5) Such drugs have been proven effective in producing cells with dystrophin in trials with mice and dogs, and in clinical trials with DMD patients, with no toxicity or side effects observed (Lu et al, 2003; Lu et al 2006, van Deutekom et al 2007). Thus far exon skipping drugs have been found to be more safe and effective than stop codon read-through drugs in DMD, though more testing needs to be done to confirm this finding.

Alternatively, gene therapies involving various methods of introducing a functional dytrophin protein have been tested. Adeno-associated viral (AAV) vectors are potential therapeutic gene delivery vectors, as they do not cause major immune response and can integrate their genomes into dividing and non-dividing host cells. AAV vectors containing dystrophin genes with internal deletions in the rod-like spectrin repeat region (in order to fit in the viral genome) have been tested in mice, dogs and primates (Wang et al, 2000, Wang et al, 2008, Wang et al, 2007, Ohshima et al 2009, Rodino-Klapac et al 2010). Dystophin containing muscles were increased by up to 80% in mice and primates, however immune response to the viral capsid was seen in tested dogs and primates, decreasing efficiency of dystrophin expression. Immune system recognition of the viral capsid was also seen in a small human DMD patient study (Mendell et al, 2010).

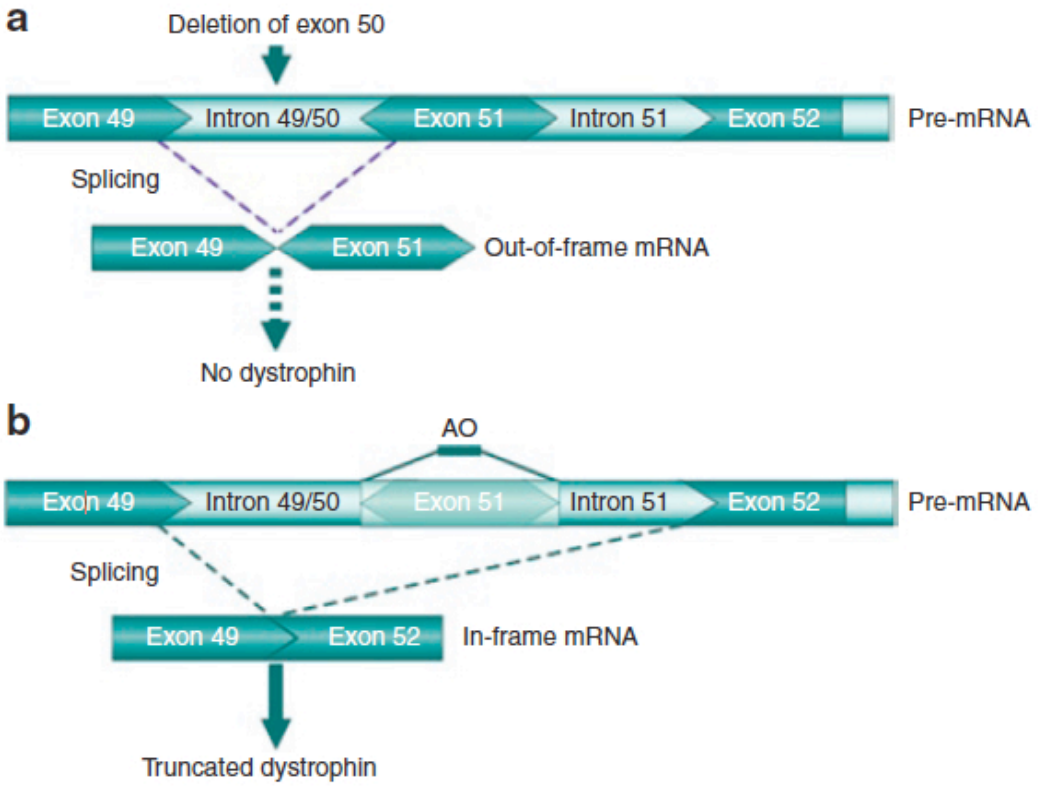


Figure 5. Example of exon skipping in Duchenne muscular dystrophy (DMD) patient who has a deletion of exon 50. (a) The absence of exon 50 in the dystrophin gene leads to an out-of-frame mRNA creating a premature stop codon in exon 51, thus aborting dystrophin synthesis during translation. (b) Using an antisense oligonucleotides (AO) targeting exon 51, this exon is skipped during splicing. This restores the open reading frame of the transcript and allows the synthesis of an internally deleted dystrophin. Modified from Van Deutekom et al. (Pichavant et al, 2011)

Lentiviral vectors can integrate into non-dividing host cells, and have also been used to deliver abbreviated dystropin genes in gene therapy studies in mice, with some success though no larger subjects were tested (Kobinger et al 2003; Kimura et al 2010). Use of lentivirus risks random integrations of the viral genome into the host genome, and thereby introduces the risk of interrupting vital genes or the risk of causing tumors. Another method of using lentivirus to introduce dystrophin was also attempted. In this *ex vivo* study, cells grown in tissue culture were transformed with a lentivirus containing an

abbreviated dystrophin gene, and then these cells were engrafted in test animals. This resulted in very low dystrophin expression in mice, and higher dystrophin expression in dogs, though some of the dogs died of pneumonia mid experiment (Bachrach et al, 2004, Bachrach et al, 2006, Sampaolesi et al, 2006).

One final method of gene therapy does not use viruses at all, but instead simply used plasmid overexpression vectors containing an abbreviated dytophin insert and another containing a full length dystrophin insert. This method was used in an experiment in mice and a clinical trial with DMD patients, but each resulted in dystrophin expression at a clinically irrelevant level (Ascadi et al, 1991; Romero er al, 2004).

The benefits of these plasmid treatments are limited to a small area around the injection site. In order to be truly therapeutic or curative of the disease, the dystrophin inducing effect would need to be able to spread far from the injection site to benefit large muscle tissues. Several methods of increasing the distance of effective engraftment from an injection site have been tested. One such method is the use of hydrodynamic pressure via a tournequet, which has proven safe and effective in increasing dystrophin expression when treated with dystrophin containing plasmids (Budker et al, 1998; Hagstrom et al, 2004; Hegge et al, 2010; Zhang et al, 2010). However this method is only useful on limbs. For hydrodynamic pressure to be effective by itself, methods of applying it to the torso and head would need to be devised. Another method is electroporation, which can increase plasmid uptake (Aihara et al 1998; Mir eat al 1999), yet can also inadvertently allow calcium to cross the cell and activate proteases which are both deleterious to cells (Gissel et al, 2001). In order to diminish the negative effects of electroporation, careful control of the voltage is a must. Most relevant to this paper, one study showed that

electroporation could transfect satellite cells (the muscle-specific stem-like cell type most likely to be useful in cellular treatments of DMD) into muscle cells, though follow up on this study is needed (Peng et al, 2005). Electroporation of mice and dogs treated with dystrophin containing plasmids showed increased dystrophin expression, though the dogs also showed an adverse immune response (Chapdelaine et al, 2000; Vilquin et al, 2001; Pichavant et al, 2010)

The best way to use these gene therapies in a treatment has yet to be determined. It may be most effective to administer the gene therapies directly to patients, where they will effect the patient's own muscle cells. Alternatively, it may be more effective to combine gene therapy and cell therapy. The focus of this study is to differentiate a cell type appropriate for use in combination with gene therapies for treatment of DMD.

Animal model of dystrophy

As with many human diseases, researchers studying DMD use animal models in their experiments. An animal model of a human disease consists of a genetic line of animals with a trait similar to the human disease. Until recently X chromosome-linked muscular dystrophy (mdx) mice have been the standard, though imperfect, model for DMD. Mdx mice have a nonsense mutation in exon 23 of their dystrophin gene, rather than a premature stop codon brought on by an out-of-frame mutation as in human DMD patients. Although they do not express dystrophin, the disease in mdx mice is phenotypically much less severe than human DMD patients, and is marked by the absence of fibrotic lesions and the ability to regenerate skeletal muscle as well as upregulation of compensatory proteins (Bulfield et al, 1984). The mice do show damage and degeneration to muscle upon exercise, and problems in the diaphragm muscle. There has been a need for a better animal model of DMD in mice since mdx mice were characterised (O'Brien et al, 2001).

In cell therapy studies, various experimental cell types are tested for their ability to engraft in the DMD mouse model, as well as other characteristics. This involves injecting the experimental cells into one leg muscle of the mice, and a control cell line or saline into the adjacent leg. When human derived cell lines are used, an immunocompromised mouse model is necessary to avoid immune system rejection of the human cells. This is done by crossing the DMD model mouse line with an immunocompromised mouse cell line to generate a genetic line of mice with both DMD model characteristics and an impaired immune system.

The CMAH/mdx mouse represents an improved version of the DMD mouse model. During the course of our evolution, humans aquired a mutation which rendered

the CMAH gene inactive, while mice retain the active form of the gene. CMAH codes for N-glycolylneuraminic acid, which codes for an enzyme involved in determining the structure of sialic acids attached to glycoproteins and glycolipids in the plasma membrane of muscle cells. By replicating the mutation in the human form of CMAH in mdx mice, the CMAH/mdx mice have disease phenotypes closer to those of human DMD patients (Chandrasekharan et al, 2010).

Another improvement of the mdx mouse model is the mdx/mTR mouse lineage, in which the Terc gene has been knocked out. Terc codes for the essential RNA component of the repair enzyme telomerase, which repairs chromosome telomeres. Mdx/mTR mice display phenotypes much closer to human DMD patients than the standard mdx mice (Sacco et al, 2010).

Similarly, mdx mice in which the utophin gene has been knocked out (called dko mice: double knock out) also display phenotypes markedly similar to human DMD patients. Utrophin is another structural protein in the dystroglycan complex thought to function in a complementary fashion along with dystrophin (Deconinck et al, 1997).

In addition to developing a stem cell based treatment for DMD, these engraftment experiments could have other experimental benefits as well. Any muscle cells derived from a DMD patient engrafted in a DMD mouse model would lack the dystrophin gene, and thus could serve as an *in vivo* platform for testing candidate drugs for DMD treatment on human cells.

Human Pluripotent Stem cells

Pluripotency is a term used to describe the ability to develop into any cell type, and is a characteristic unique to stem cells. The process of development into specific cell types is called differentiation. Pluripotent stem cells are cells with the ability to continuously divide (called self renewal), and retain their pluripotency while doing so. Because of their unique properties, pluripotent stem cells have great potential for use in regenerative medicine. A new and rapidly developing scientific field, regenerative medicine is the replacement of damaged or diseased patient tissue.

In order to establish a human embryonic stem cell line, the inner cell mass of a human blastocyst at four or five days after fertilization is removed and cultured using cell culture practices in media formulated to help maintain pluripotency (Figure 6). The source of these embryos are fertility clinics, from institutional review board (IRB) approved and consenting donors (Chiu et al, 2003; Lanza et al, 2006). Once such a cell line is established, it can be grown and divided seemingly indefinitely while retaining it's pluripotency.

Pluripotent embryonic stem cells were first isolated from mice embryos in 1981. These were shown to be capable of long term culturing and differentiation (Evans et al, 1981; Martin 1981). In 1994, an invitro fertilization (IVF) specialist culturing surplus human embryos grew human stem-like cells for two passages. In doing so, he noted their morphological similarity to mouse embryonic stem cells, a normal karyotype (a method of detecting gross chomosomal abnormalities), the expression of alkaline phosphatase, and their ability to differentiate to fibroblasts (Bongoso et al, 1994).

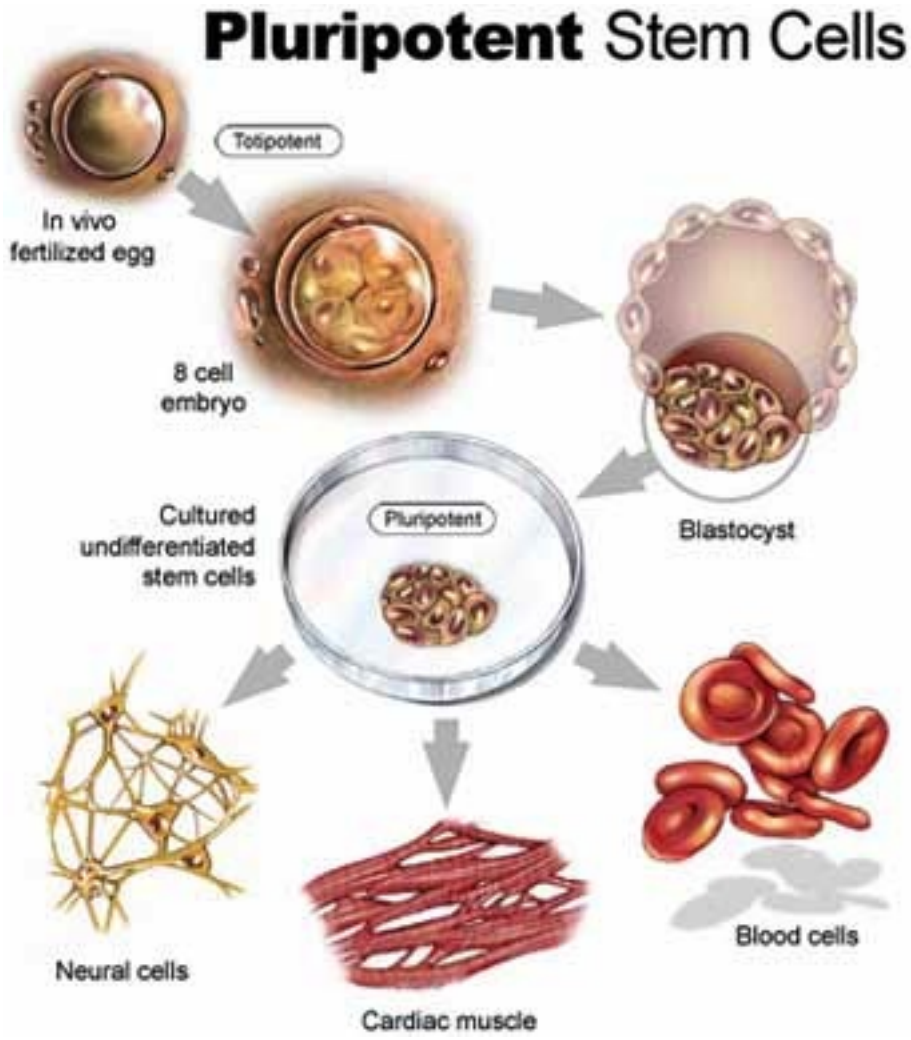


Figure 6. Pluripotent Stem Cells. A totipotent fertilized egg, to an embryo, to a blastocyst. The inner cell mass of the blastocyst is removed and plated in a cell culture dish in media designed to maintain the plutipotent hPSC state. Various differentiation conditions can be applied to the hPSCs to differentiate them to cells from any of the three germ layers: endoderm, ectoderm or mesoderm. HPSCs have the potential to develop into any cell type in the body, if the proper differentiation conditions can be identified.
(image: http://www.hyscience.com/archives/2006/03/stem_cell_innov.php)

The H9 stem cell line I use in the experiments described in this study were originally created by Dr. Jamie Thompson in the course of the key human embryonic stem cell paper he published in 1998. As part of this paper, Dr. Thompson provided a proof of hESC's potential to form any cell types and tissues of the human body. In this

experiment hESCs were first cultured *in vitro* then injected into the leg muscles of immunocompromised mice, where they formed tumors called teratomas. The teratomas were composed of numerous cell types derived from all three embryonic germ layers (endoderm, mesoderm, and ectoderm) (Thompson et al, 1998).

These three germ layers are present in early embryos, and subsequently develop into specific groups of tissues. Endoderm is the innermost layer and forms the gastrointestinal tract, respiratory tract, endocrine glands and organs, auditory system, and urinary system. Mesoderm is the middle layer which forms organs, muscle, and bone. Ectoderm is the outermost layer and forms the nervous system and the epidermis. Every organ in the human body can be assigned to one of these three layers as it's source of origination in embryonic development. As multiple tissue types known to be derived from each of these three embryonic germ layers were detected in the hESC derived teratomas, Dr. Thompson concluded that hESCs retain the potential to develop into any cell type in the human body. This conclusion has been supported by extensive testing in the stem cell research field.

This study also characterised various markers associated with the pluripotent state including TRA-1-60, and confirmed the expression of alkaline phosphatase and telomerase, the former being an enzyme which repairs the telomere region of chromosomes which otherwise degrade over successive rounds of cell division. Dr. Thompson's paper was revolutionary in proving the scientific potential of hESCs. In addition to their potential for use in regenerative medicine, hESCs can be used to discover the previously hidden phases of early human tissue development *in vitro*.

Nuclear transfer techniques can allow the *in vitro* study of genetic diseases when the transferred nucleus is derived from a patient.

While Dr. Thompson's paper proved that hESCs could be cultured and retain their pluripotency, it also introduced a new problem. Pluripotent stem cells introduced into the body are potentially tumorigenic. In order for regenerative treatments using pluripotent stem cells to be safe and effective, researchers must learn to control the cell's development. The goal of any regenerative medicine project is to start with hPSCs then differentiate them to the desired progenitor cell type appropriate for regeneration of a specific cell type or tissue *in vitro* prior to intoduction to a patient. In preparing cells for such a treatment, one must also eliminate any remaining hPSCs prior to use in regenerative medicine, so as to avoid introducing teratomas into the patient. After Dr. Thompson's paper was published it was thought hESC derived cells would be the cells used to treat patients in regenerative medicine treatments, and that large scale collection and destruction of embryos would be necessary for these treatments to advance.

While hESCs hold tantalizing promise for regenerative cell-based therapies, they also come with inherent ethical and technical problems. Great political, religious, and general moral contoversy surrounds the ethics of destruction of human embryos from which hESC lines are derived, and the federal funding of stem cell research involving embryo destruction. The ongoing ethics debate surrounding stem cell research has rightly or wrongly been intertwined with the highly contentious issues of abortion and human cloning, and hence with partisan politics. Laws and policies in the US governing federal funding of fetal and stem cell research have changed several times over the course of the stem cell ethics debate, with other countries adopting their own laws and policies at times

out of step with ours. Use of federal funding has been and continues to be prohibited in the direct destruction of human embryos, though no limits on private funding have been enacted nor have any laws prohibiting the act of embryo destruction been passed (Stem cell research timeline, 2014). Prior to 2009 US law limited federal funding of research on hESC lines to the 21 lines created with private funds before August 2001. As of 2009, federal funding of research on hESC lines created with private funds after August 2001 is permitted. Individual US states have varying laws and policies governing stem cell research, with some embracing it, some severely limiting it, and others taking various nuanced approaches (Stem Cell Research, 2014). The study described in this paper was funded in part by the California Institute for Regenerative Medicine (CIRM), a state agency founded to promote and support stem cell research.

In addition to the ethical and political issues, there is also the practical problem of patient immunorejection of hESC derived cells. This necessitates a lifelong course of immunosupressant drugs for any patient treated with hESCs, and the attendant side effects and risk of infection for any taking such a course of drugs.

In 2006, Dr. Shinya Yamanaka published a paper which greatly advanced the science of stem cells for regenerative medicine, and simultaneously directly addressed the ethical and practical problems with using hESCs for regenerative medicine. The transformative discovery outlined in this paper was the ability to reprogram mouse adult somatic cells to a pluripotent state. Cells reprogrammed in this way are called induced pluripotent stem cells (Takahashi and Yamanka, 2006). A follow up paper was published the following year successfully replicating the same methods in human cells (Takahashi et al, 2007; Okita et al, 2007). Human induced pluripotent stem cells (hIPSCs) are

derived from terminaly differentiated human somatic cells typically obtained by a skin punch biopsy which are subsequently reprogrammed to a pluripotent state with a specific set of transcription factors. Takahashi and Yamanaka used the transcription factors OCT4, KLF4, C-MYC and SOX2 to reprogram mouse and human somatic cells to IPSCs, having arrived at this combination after a clever process of elimination after starting with a collection of 24 factors known to be involved in the pluripotency of embryonic stem cells, embryonic carcinoma cells, and germ line cells. Dr. Thompson's lab published a paper on inducing the hIPSC cell fate from adult somatic cells using an alternate combination of transcription factors consisting of OCT4, SOX2, NANOG, and LIN28 (Yu et al, 2007). These transcription factors were integrated into the human adult cells genome via a retrovirus vector.

One major advantage of using hiPSCs rather than hESCs for cell based therapy is the ability to create a patient-specific pluripotent cell line, and so use a patient's own cells to treat their disease, thereby avoiding rejection by the immune system or the use of immunosuppressive agents. Furthermore, they can be used to study diseases in the lab. IPSC lines have been created from patients with diseases as diverse as Parkinson disease, type I diabetes, Gaucher disease, Down syndrome, ALS, and have been used to study these disease *in vitro* (Park et al, 2008; Dimos et al, 2008).

hIPSCs also eliminate the need for collection and destruction of human embryos for use in stem cell based treatment of patients. Many of the groups vehemently critical of the embryo destruction inherent in hESC line creation and research actually embrace the study and use of hIPSCs. However, hESC lines are still necessary for study of development and differentiation.

HIPSCs are not without their own inherent problems. The adult somatic cells from which they are made have undergone many cell divisions durring which mutations can occur before they reached their terminally differentiated state. Furthermore they may have been exposed to environmental factors such as sunlight or other potential mutagens. The use of retrovirus to integrate the key iPSC inducing transcription factors into the host genome can also potentially induce tumors, depending exactly where they integrate. Retroviruses preferentially target integration at active gene sites, so the potential for disruption of critical genes is significant. Furthermore, some of the pluripotency inducing transcription factors themselves have oncogenic potential. While hIPSCs induced with retrovirus can be used to study diseases *in vitro*, alternative methods of delivering the pluripotency inducing transcription factors are needed for cells to be used in regenerative medicine. Various non-integrating methods of gene expression including episomal vectors, nonintegrating viral vectors, transient DNA transfection, transposons, and protein transduction have been tested. These yield positive results but at low efficiency (Yee, 2010).

Despite these issues, hIPSCs are widely embraced as the best available pluripotent cell type for use in regenerative medicine. However hESCs are still considered the "gold standard" for pluripotent stem cell research, and are used by researchers to develop protocols for the differentiation of pluripotent stem cells to a desired cell type (Okano et al, 2013). While Yamanaka's breakthrough did not end the ethical debate surrounding stem cell research, it did go a long way toward convincing many researchers, politicians, religious groups and concerned lay people that stem cell based regenerative medicine and

research is technically feasable, can be conducted in a manner ethically acceptable to many people, and could have wideranging medical benefits to human society as a whole.

Skeletal Muscle Progenitor Cells

Progenitor cells are closely related descendents of stem cells, which can self replicate and form one or more cell types. Progenitor cells have limited potential compared to pluripotent stem cells in terms of what cell types they can generate, with each progenitor cell lineage being specificly capable of generating only certain terminally differentiated cell types. Progenitor cells are therefore an ideal cell type to use in regenerative medicine, as their specific regenerative potential could avoid the formation of teratomas seen in engrafted pluripotent cells. A major goal of many researchers is to determine the steps necessary to differentiate pluripotent stem cells to the appropriate progenitor cell type relevant to the disease they are focusing on.

Satellite cells are one of several potential skeletal muscle progenitor cells (SMPCs), are adult skeletal-muscle-specific, and occupy a niche between muscle fiber sarcolemma (cell membrane) and the basal membrane (Figure 7). Satellite cells were first described and their function guessed over 50 years ago (Mauro, 1961). Since that time, it has been shown that satellite cells are the primary cells responsible for the regeneration of skeletal muscle upon damage while also maintaining the ability to self-renew (Wagers et al, 2007; Zammit et al, 2006). The satellite cells in healthy resting muscle tissue are normally in a quiescent state, with no mitotic divisions (Schultz et al, 1978; Seale et al, 2000). Activation occurs in response to mitogens originating from damaged skeletal muscle fibers (Bischoff, 1986). There are at least two subpopulations of satellite cells, one of which responds rapidly to mitogenic signals released by the damaged muscle by entering the cell cycle to mitotically divide and repair damaged muscle, and makes up ~80% of the total satellite cell population. The remaining ~20% of the satellite cells are

thought to only enter the cell cycle in response to the need for extensive muscle repair, and function as a satellite cell reserve (Schultz, 1996).

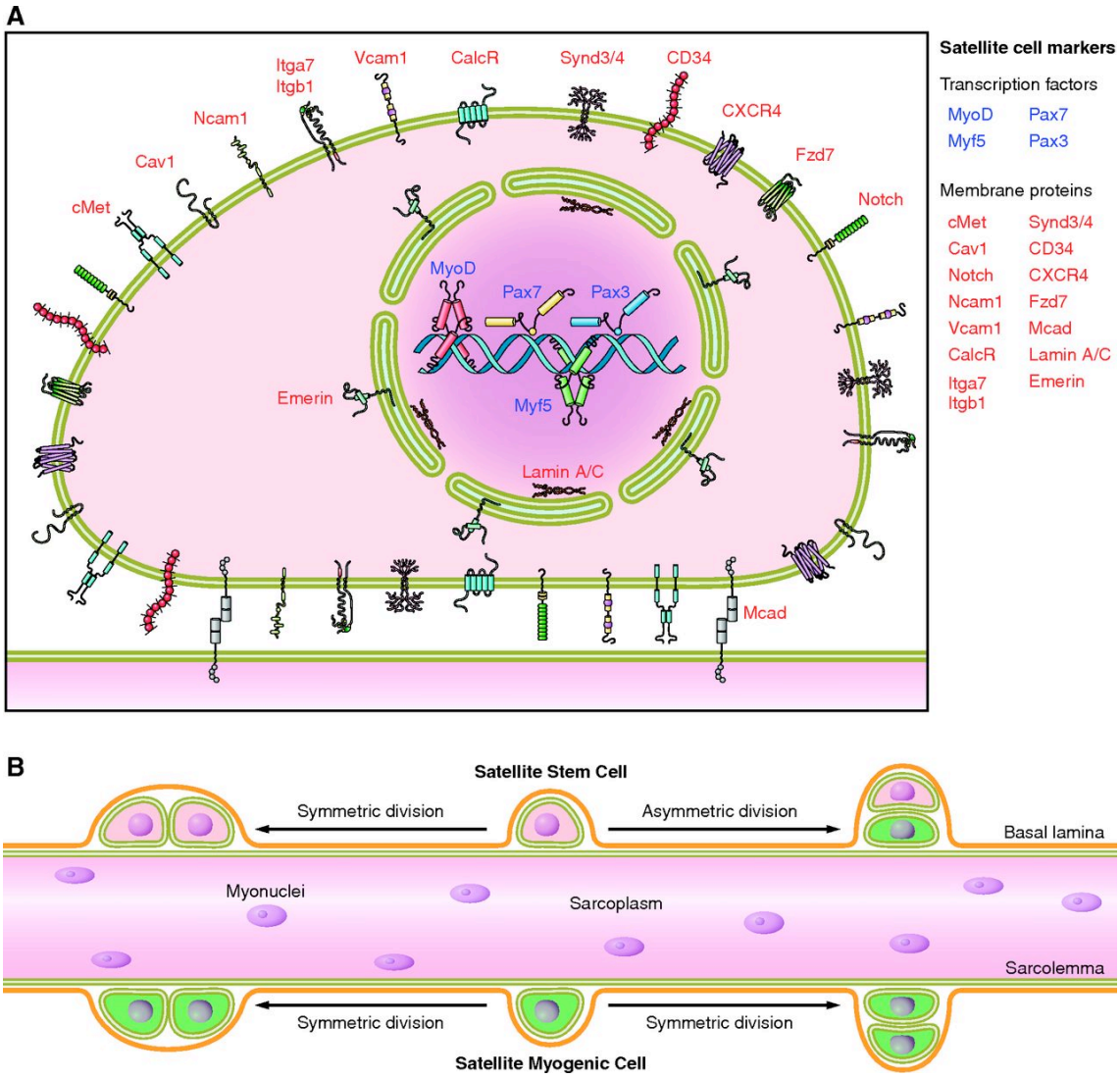


Figure 7. Characteristics of the satellite cell. *A*: numerous proteins are expressed in satellite cells and have been used as markers to distinguish between surrounding cell types within skeletal muscle. Due to heterogeneity in satellite cell populations, it is unlikely that all of these markers are expressed in a given satellite cell at a specific time. Nevertheless, this panel summarizes the cellular location of markers used to identify satellite cells. *B*: the satellite cell population is heterogeneous and can be classified in a hierarchical manner based on function and gene expression. Evidence from lineage tracing experiments identified a subpopulation of satellite cells having never expressed the myogenic transcription factor Myf5 (satellite stem cells) are placed hierarchically above satellite cells that have expressed Myf5 at some point during development (satellite myogenic cells). Satellite stem cells, upon asymmetric division (typically in a apical-basal orientation), will give rise to two daughter cells, only one of which has activated Myf5. Functional differences in regenerative potential exist between satellite stem cells and satellite myogenic cells. Following transplantation, satellite stem cells preferentially repopulate the satellite cell niche and contribute to long-term muscle regeneration. In contrast, satellite myogenic cells preferentially differentiate upon transplantation in vivo. (Yin et al, 2013)

A complex process of differentiation leads to the satellite cell fate (Figure 8). The developmental origins of satellite cells have been traced back to pre-somitic paraxial mesoderm, which is adjacent to and follows the anterior-posterior orientation of the notochord. Paraxial mesoderm divides into epithelial somites at day 8 in mice (Aulehla et al, 2006), and these somites develop into cartilage, endothelial cells, tendon, connective tissue, dermis, and skeletal muscle. The formation of somites is controlled by the Fibroblast Growth Factor (FGF), Wnt, and retinoic acid pathways (Gossler et al, 1997; Kalcheim et al, 2005). Developmental studies in mice, chick and quail have shown that satellite cells are descended from the somites of developing embryos (Ben-Yair and Kalcheim, 2005; Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005; Schienda et al., 2006), however, other sources of satellite cells have not been excluded. The dorsal portion of these somites is called the dermomyotome, which has an epithelial identity in early stages, and later transitions to mesenchyme. The dermomyotome is surrounded by several tissues, each of which send signals to the cells of the dermomyotome influencing their development in a concentration dependent manner. The overlying ectoderm secretes Wnt7a and Wnt6, the neural tube secretes Shh and Wnt1, the notochord secretes Shh, and the lateral mesoderm secretes BMP4 (Boryckia et al, 2000; Münsterberg et al, 1995; Pourquié et al, 1996; Tajbakhsh et al, 1998). This leads to a regionalized dermomyotome, which is the developmental source of head, trunk and limb muscle, and is where PAX3+/PAX7+ satellite cells first develop. Some of these cells migrate to the myotome located underneath the dermomyotome, where they first divide and differentiate to myocytes which later form the first embtryonic trunk. (Ben-Yair et al, 2005; Gross et al, 2005; Kassar-Duchossoy et al, 2005; Relaix et al, 2005). Another

population of PAX3+/PAX7- satellite cells from the ventral lip of those somites designated for limb development migrates to form primordial limb muscle (Buckingham et al, 2003; Tajbakhsh et al, 2003). Limb somites produce PAX3+ muscle progenitors and Vascular Endothelial Growth Factor Receptor2 (VEGFR2+) expressing endothelial progenitors, and it cannot be ruled out that these endothelial progenitors may contribute to satellite cell populations (Tozer et al, 2007; Kardo et al, 2002). This thesis focuses on satellite cells of limb muscle, but satellite cells of the trunk and head muscles would also be important for a cell based treatment of DMD.

Once they have been diferentiated, Satellite cells are regulated by a complex set of transcription factors designating several downstream cell types with varying capabilities (Figures 8b & 9). Satellite cells are known to express paired box transcription factors Pax3 and Pax7, neural cell adhesion molecule (NCAM), muscle cell adhesion protein M-Cadherin, and tyrosine receptor kinase c-Met among other markers. Pax7 is considered the definitive marker and master regulator of the adult skeletal muscle progenitor cell (Barberi et al, 2007, O'Brien et al, 2002; Yin et al, 2013). Pax3 regulates embryonic muscle development and is highly expressed during those stages, but is expressed at much lower levels by the time satellite stem cells are specified and committed. Pax7 is highly expressed during satellite stem cell specification and commitment (Maqbool and Jagla 2007). One study showed Pax7 overrexpression induced stem cell quiesence and prevented activation (Olguin et al, 2004), but other studies indicated it did not (Relaix et al, 2006; Zammit et al, 2006). Muscle cells contain Hepatocyte growth factor/Scatter factor (HGF/SF), which is thought to release upon

muscle damage and to be among the mitogenic signals detected by satellite cells inducing them to the activated state (Allen et al, 1995; Anastasi et al, 1997; Gal-Levi et al, 1998).

Activated Pax7 expressing satellite cells divide asymetrically into varying cell populations, some of which repopulate the satellite cell niche and others which further divide and differentiate into myoblasts, which in turn give rise to terminally commited myocytes which fuse and form myotubes. Studies show that Pax7 regulates Myoblast determination protein (MyoD) (Relaix et al, 2006; Zammit etal, 2006; Olgoin et al, 2007). MyoD regulates the transition to the myoblast stage, while the downregulation of Pax7 and upregulation of myogenin transforms the myoblasts to myocytes (Kuang et al, 2008; Zhang et al, 2010; Rudnicki et al, 2008).

There is a variable expression of the Myf5 marker among asymetrically divided satellite cells. Satellite cells which do not express Myf5 are able to repopulate the satellite cell niche and successfully regenerate muscle over the course of several rounds of successive damage and repair. Activated satellite cells which do express Myf5 differentiate to muscle without regenerating satellite cells. Satellite cells with Pax7+/Myf5- expression were shown to divide asymmetrically, giving rise to both Pax7+/Myf5- and Pax7+/Myf5+ daughter cells. However, Pax7+/Myf5+ expressing satellite cells could not regenerate Pax7+/Myf5- expressing cells. Myf5 is also expressed by the myoblast (Conboy et al, 2002; Conboy at al, 2007; Shinin et al, 2006; Kuang et al, 2007).

Myogenesis is known to be influenced by various developmental regulatory pathways. Bone morphogenic protein 4 (BMP4) and Fibroblast growth factor (FGF) together with an phosphoinosidtide kinase 3 inhibitor (LY294002) have been shown to

induce mesoderm lineage cells (Bernardo et al, 2011).

BMPs are part of the Transformation growth factor β superfamily. BMPs act as a ligand simultaneously binding two otherwise unassociated receptors. In the canonical BMP pathway, one of the receptors phosphorylates two intracellular rSMAD molecules, which forma complex with coSMADS. The SMAD complex enters the nucleus and regulates gene expression. The non-canonical BMP pathway is SMAD independent, involving signaling through TAK1 and MAP kinases (Figure 11) (Derynck and Zhang, 2003).

Fibroblast growth factor signaling is initiated by the FGF ligand binding with and dimerizing two FGFR receptors that together form a complex with heparin sulfate proteoglycans (Beenken & Mohammadi, 2009). Subsequently, a number of intracellular tyrosines may be phosphorylated, notable FGFR substrate 2 which forms a complex with the Grb2 adapter protein and son of sevenless (SOS), a nucleotide exchange factor (Kouhara et al, 1997; Ong et al, 2000). This sets off a chain of phosphorylation events involving first Ras and then a MAP kinase, which in turn phosphorylates transcription factors and effects gene expression (Randi et al, 2009; Nentwich et al, 2009). Alternatively, the FGF pathway can activate the phosphoinositide kinase 3 pathway via Grb2 (Figure 12) (Nicholson & Anderson, 2002)

The Wnt pathway plays a role in various stages of the myogenic development process, including several Wnt signals mentioned previously influencing the fate of the dermomyotome. Additionaly, Wnt7a has been shown to be expressed in satellite cell expansion, and may be involved in regulating satellite cell division (LeGrand et al, 2009). Wnt signaling is highly conserved, and consists of canonical and non-canonical pathways

(Figure 10). Wnt7a a non-canonical Wnt ligand, operating through the calcium dependent protein kinase C pathway in somites (Kuhl et al, 2000; Cossu et al, 1999), and through the planar cell polarity pathway in satellite cells (Legrand et al, 2009).

The canonical pathway is β-catenin dependent. In the absence of a Wnt signal, β-catenin is phosphorylated by the serine/threonine kinases, GSK3β and Casein Kinase and is subsequently degraded by a proteasome. If a Wnt ligand binds to it's Frizzled receptor, the kinases are inhibited and β-catenin accumulates in the cell, and is transported to the nucleus where it transforms the T-cell factor/Lymphoid enhanced factor from a repressor to an activator of gene expression (Logan et al, 2004).

Non-canonical Wnt signaling is β-catenin independent and varies greatly in its action, though all begin with a Wnt ligand binding to a Frizzled receptor. One such pathway releases calcium ions into the cytosol, activating various calcium dependent enzymes, which in turn dephosphorylate and thereby activate a transcription factor (Kohn et al, 2005). Another non-canonical Wnt signaling pathway is the planar cell polarity pathway, which activates JNK and Rho family GTPase to direct asymmetrical reorganization of the cytoskeleton and cellular polarization oriented to the plane of epithelial sheets (Fanto et al, 2004; Strutt et al, 2008; Katoh et al, 2005).

Satellite cells have been isolated from muscle tissues, but they do not expand in culture well, and the amount of satellite cells safely extractable from patients would be insufficient to use in combination drug/cell therapies. Hence the need to differentiate satellite-like cells from pluripotent cells. The goal of this study is to differentiate hPSCs to a satellite-like cell fate. We refer to the satellite-like cells we intend to differentiate as skeletal muscle progenitor cells (SMPCs), as they may not be identical in every way to

the satellite cells native to muscle tissue, and this potential distinction should be noted. If the functional characteristics of satellite cells can be matched by our SMPC cells, our goal will have been met.

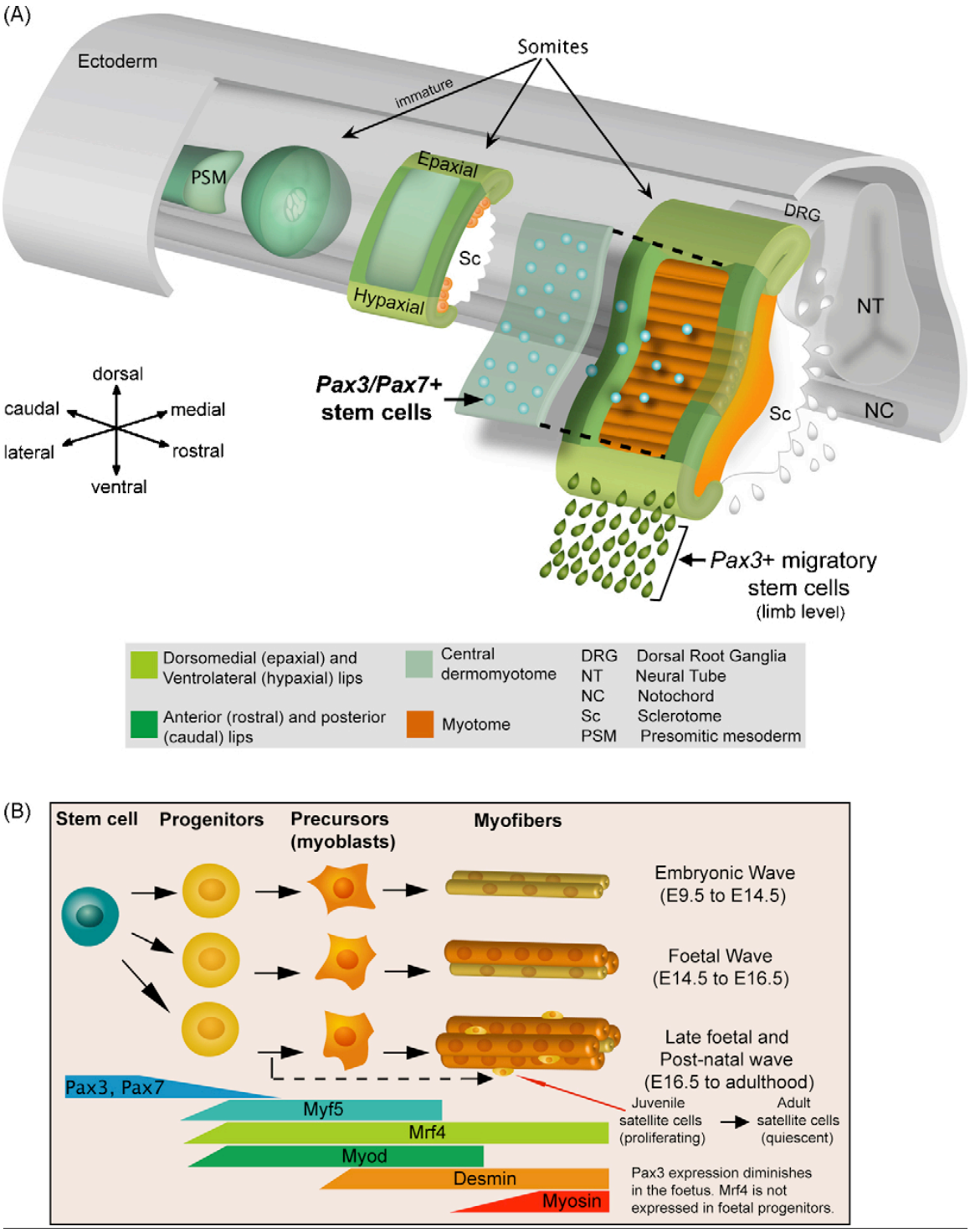


Figure 8. A model of skeletal muscle formation from stem and progenitor cells in the mouse embryo. (legend next page)

Figure 8. A model of skeletal muscle formation from stem and progenitor cells in the mouse embryo (see previous page). (A) Presomitic (paraxial) mesoderm (PSM) segments into epithelial somites. Dorsal portion of somites—dermomyotome (DM) harbours muscle stem/progenitor cells. The progenitors in the dorsomedial lip of the DM are the first to commit to myogenesis. They undergo an epithelial to mesenchymal transition, migrate underneath the DM to form the myotome where they differentiate into mononucleated myocytes that are attached to the anterior (rostral) and posterior (caudal) edges of the somite. The progenitors from the other three lips follow suit and contribute to the growth of myotome. Pax3/Pax7 expressing stem/progenitors from central portion of the DM (represented as an overlying layer, displaced) "parachute" into the underlying differentiated myotome to assure muscle growth. Myotomes are referred to here as the anlagen of trunk muscles. Progenitors from the ventrolateral lip of limb level somites migrate to establish limb muscles. In the mouse, these express Pax3 but not Pax7, and Pax3 null mutants are deficient in limb (as well as diaphragm and tongue) muscles. Note that not all DRGs are indicated; only representative somites along the rostral-caudal axis are illustrated; the nascent spherical epithelial somite buds from the mesenchymal PSM located more caudally; the myotome and sclerotome extend the full width of each somite; once the somites dissociate, myofibres fuse along the rostral-caudal axis across previous somite borders. (B) Illustration of lineage progression and the multiple waves of developmental myogenesis. The expression patterns indicated at the bottom represent primarily the onset during the embryonic wave. Pax3 is not expressed in head muscle progenitors and in the body its expression declines in the foetus. Mrf4 is not expressed in head and foetal progenitors. Desmin is an intermediate filament protein expressed in muscle and Myosin is a component of the contractile apparatus. Myogenin, which is required for muscle differentiation from myoblasts, is not indicated here. The lineage relationship between the stem cell from the dermomyotome and the progenitors within each wave of myogenesis is yet to be resolved. Around E16.5, proliferating, Pax7+ cells appear in a satellite cell position. A subset of these cells will become the future adult quiescent satellite cells. (Sambasivan & Tajbakhsh, 2007)

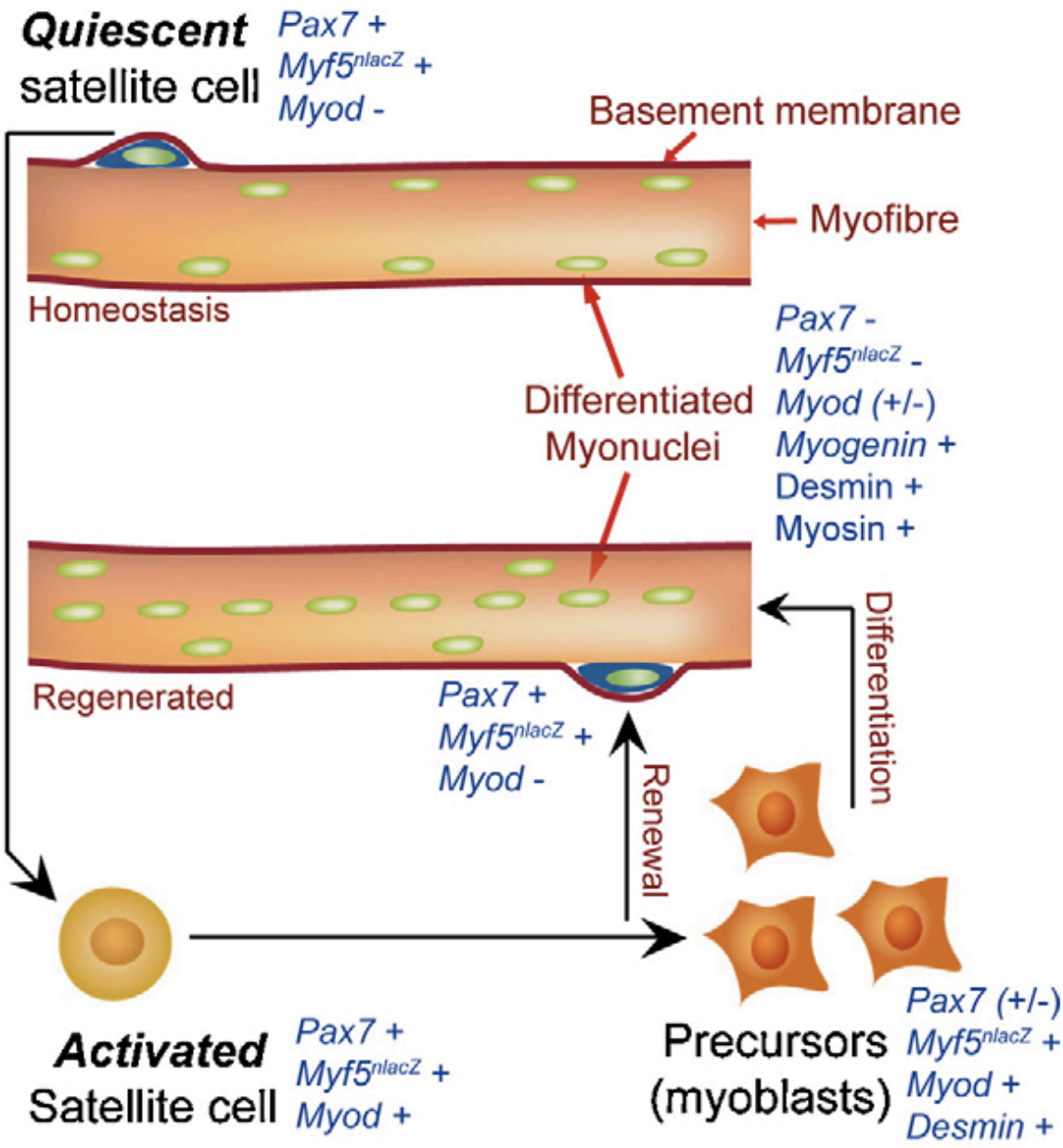


Figure 9. Satellite cell activation and self-renewal in the adult. Plasmalemma of the host myofibre and its basement membrane are components of the satellite cell niche. Quiescent satellite cells in adult muscles are Pax7+. Activation of satellite cells, upon injury, is accompanied by induction of Myod expression. Once activated, they enter cell cycle, proliferate and differentiate to accomplish regeneration. A subset of cells downregulates Myod but retains Pax7 expression and these cells are thought to renew the satellite cell pool (Sambasivan & Tajbakhsh, 2007).

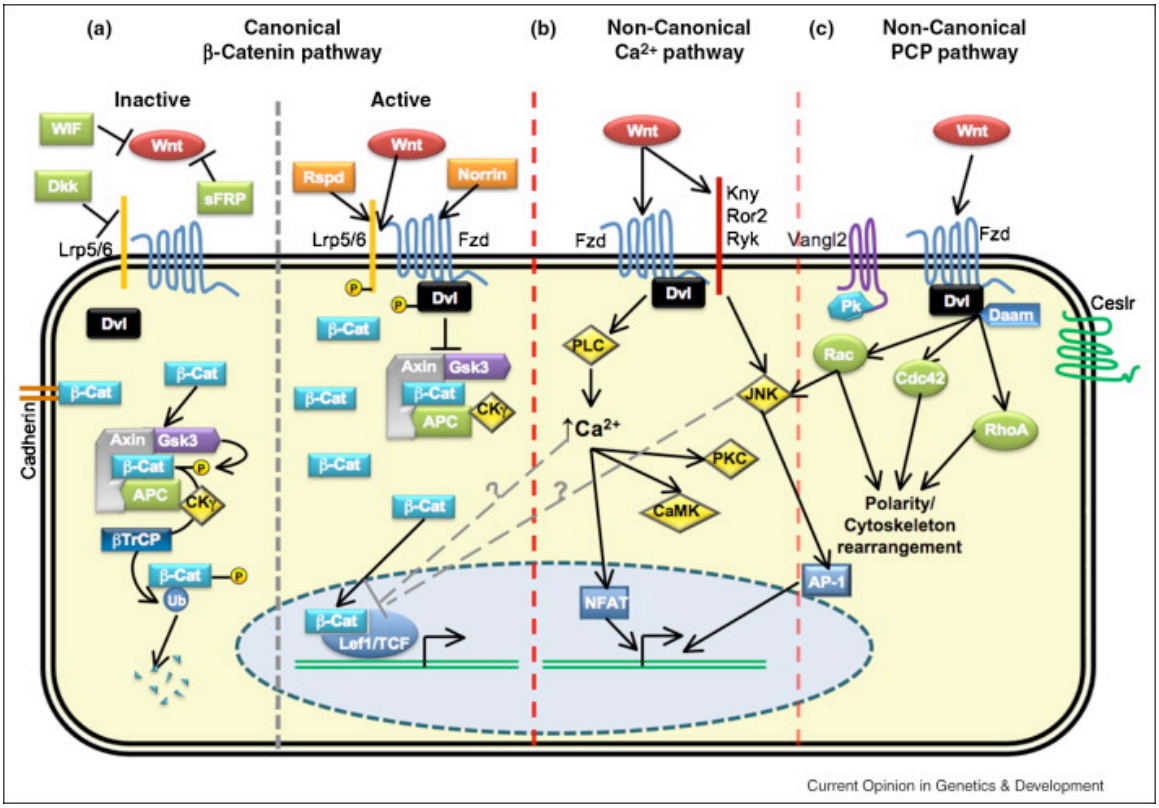


Figure 10. Three Wnt-dependent pathways have been proposed: (a) canonical Wnt/β-catenin pathway and (b and c) non-canonical Wnt/PCP and Wnt/Ca2+ pathways. Canonical and non-canonical pathways possess clear different signaling events; however, the distinction between Wnt/PCP and Wnt/Ca2+ pathways is less obvious and common events occur on those pathways. (Dashed lines illustrate the idea that no clear boundaries exist between the different Wnt pathways.) (a) Canonical Wnt/β-catenin pathway. In cells, β-catenin is normally associated with adherens junctions and can also be free in cytoplasm. In cells non-stimulated by Wnt ligands (which can additionally be inhibited by WIF, sFRPs and Dkk protein family members) cytosolic β-catenin is targeted to proteolytic degradation through phosphorylation by the APC–Axin–GSK3 β–CK1γ complex and further ubiquitination through action of βTrCP-dependent E3 ubiquitin ligase complex. On stimulation by Wnt ligands though binding to Fzd receptors and its co-receptors Lrp5/6, Fzd recruits Dvl. Dvl will inhibit APC–Axin–GSK3β–CK1γ complex formation by the recruitment and inhibition of GSK3β, CK1γ and Axin to the cytoplasmic membrane. Consequently, β-catenin can accumulate in the cytoplasm and enter the nucleus, activating transcription of target genes through association with Lef1/TCF transcription factor family. (b) Noncanonical Wnt/Ca2+ pathway. Interaction of Wnt ligands with Fzd receptors can lead to an increase in intracellular calcium level, through possibly the activation of PLC. Intracellular calcium will subsequently activate CAMKII and PKC in cells, as well as the transcription factor NFAT. This pathway is particularly important for convergent-extension movements during gastrulation. Additionally, Fzd receptors in association with Kny, Ror2 or Ryk receptors can also activate JNK promoting expression of specific genes through activation of AP-1. (c) Non-canonical Wnt/PCP pathway.

This pathway is characterized by an asymmetric distribution of Fzd, CELSR, Pk and VANGL2, resulting in the polarization of the cell. Also, Wnt-signaling activates Rho GTPases Cdc42, RhoA and Rac1 leading to cytoskeleton rearrangement, with the participation of Daam1. Rac1 can also activate JNK, activationspecific gene transcription through modulation of AP-1 protein complex. (From Franco et al. Current Opinion in Genetics & Development 2009, 19:476–483)

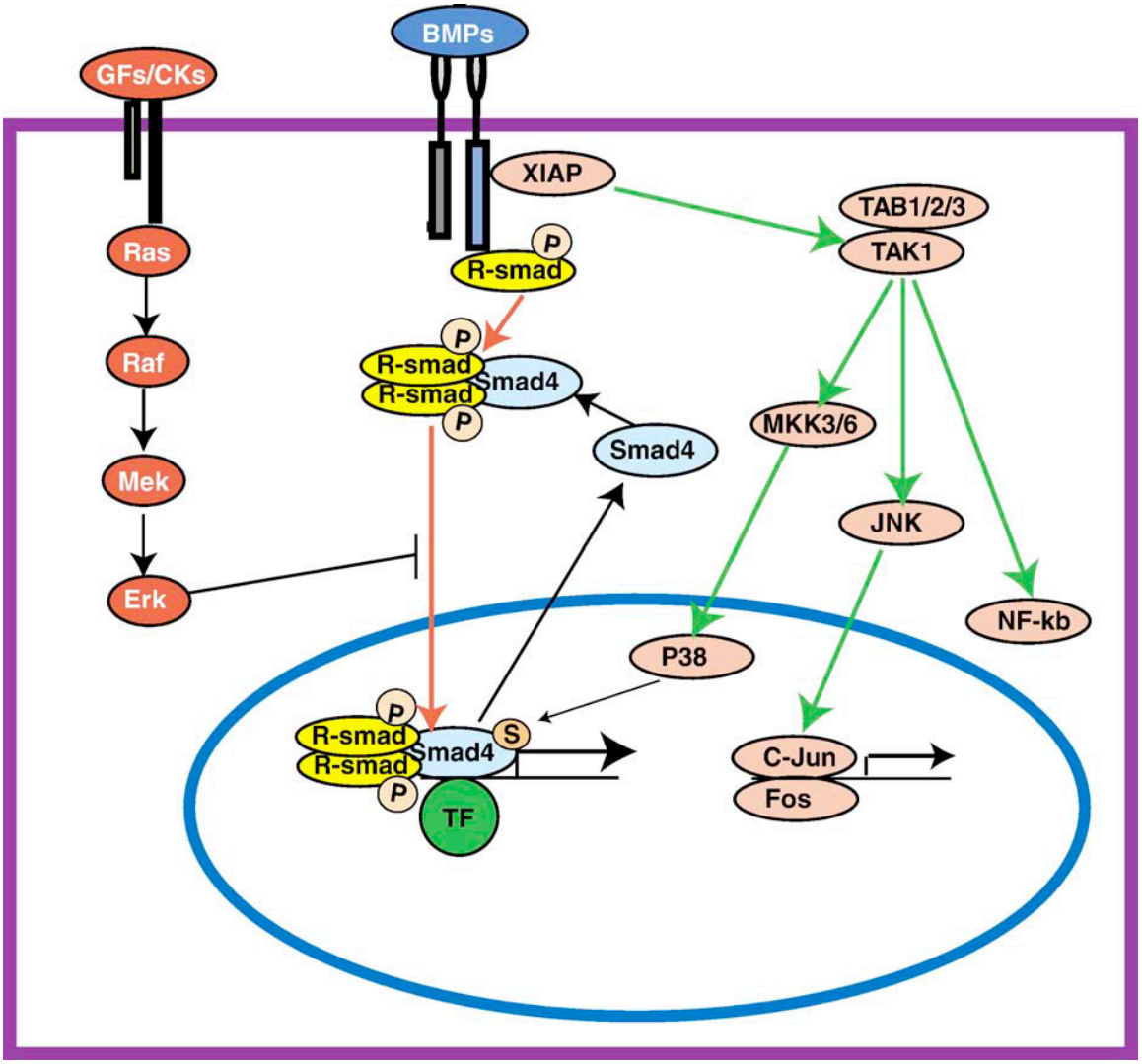


Figure 11. BMP signal transduction pathways. Upon ligand binding, BMP type II receptor recruits type I receptor to form a complex and mediates type I receptor phosphorylation. There are at least two signaling pathways involved in BMP receptor-mediated signal transduction: Smad and TAK1/MAPK. The canonical Smad pathway is mediated by receptor-regulated R-Smad (Smad1/5/8) phosphorylation and R-Smad/Co-Smad (Smad4) complex formation. After the R-Smad/Co-Smad complex is formed, it transfers to the nucleus where it regulates target gene expression by cooperating with other transcription factors. The BMPregulated MAPK pathway is mediated by TAK1, a MAPKKK tyrosine kinase which has multiple substrates. The mechanism of receptor-mediated TAK1 activation is still unknown. It has been reported that XIAP links the receptor to TAK1 to engage in TAK1 activation. TABs are required for fully mediated TAK1 activation. GFs/CKs induce activation of the Ras/Raf/Mek/Erk cascade. Activated Erk inhibits the Smad signal by phosphorylation of its link region and blocks Smad nuclear transfer (Zhang & L1, 2005).

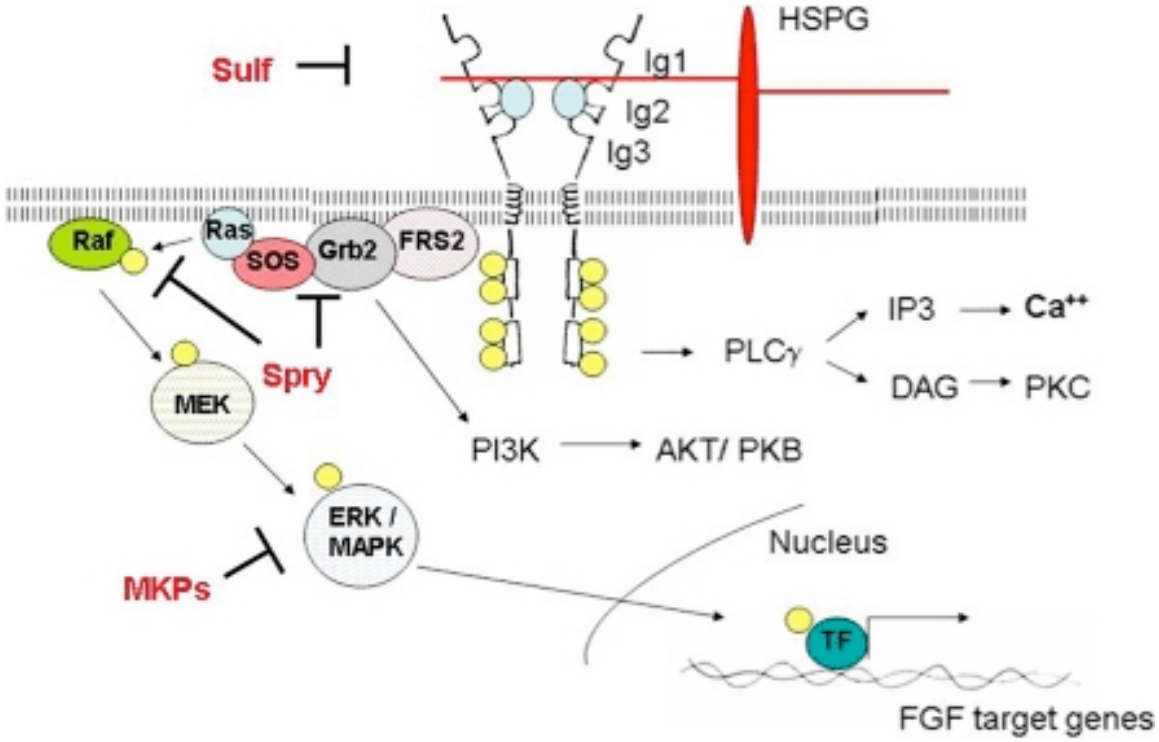


Figure 12. FGF signal transduction. Formation of the FGF:FGFR:HS signalling complex causes the activation of the intracellular kinase domains and the cross-phosphorylation of tyrosines on the FGFRs. FRS2 interacts with the phosphorylated tyrosines and is phosphorylated itself. FRS2 then activates the adaptor protein Grb2 that associates with SOS, a nucleotide exchange factor which activates Ras. Ras is a small GTP binding protein that activates Raf, which activates MEK which activates MAPK (ERK). FRS2 activity also activates phosphoinositide-3 kinase, which activates AKT/PKB. PLCγ binds the actvated FGFR by its SH2 domain and then generates inositol-1,4,5-trisphosphate and DAG from phosphotidylinositol-4,5-diphosphate resulting in the activation of protein kinase C and the release of intracellular calcium. The FGF pathway is negatively regulated by Sulf, Spry, and MAPK phosphatases (Pownal et al, 2010)

Proposed treatment of DMD with patient derived Skeletal Muscle Progenitor Cells

The combined drug/cell DMD treatment model our lab proposes is as follows. First, a skin punch biopsy would be taken from a DMD patient (Figure 13). The patient's cells would then be reprogrammed to an induced pluripotent stem cell state via the human induced pluripotent stem cell reprogramming transcription factors. Next, these induced pluripotent stem cells would be differentiated to the skeletal muscle progenitor cell stage by means we intend to discover in this study, and expanded via cell culture. Then the progenitor cells would be treated with one of several potentially effective gene therapies to restore functional dystrophin. Finally, these hiPSC derived progenitor cells with functional dystrophin would be introduced back into the patient, where they would repopulate the patient's muscle tissues with muscle cells containing Becker-like functional dystrophin, greatly improving the patient's disease phenotype (Figure 14). Our lab has successfully generated DMD patient derived hiPSCs and are now concentrating on differentiating the hESCs and hIPSCs to skeletal muscle progenitor cells capable of expansion through cell culture and differentiating to fused myotubes. Even if we succeed in differentiating hIPSCs to skeletal muscle progenitor cells, and dystrophin correcting gene therapies are tested and proven safe, there will still be major technical hurdles to overcome before our proposed treatment could be effective. Engraftment of stem cells in patient muscle will still present major challenges, as muscle satellite cells do not migrate on their own towards damaged muscle, but stay localized to the injection site. Additional protocols will be required to engage the skeletal muscle progenitor cells to migrate systemically to skeletal muscle.

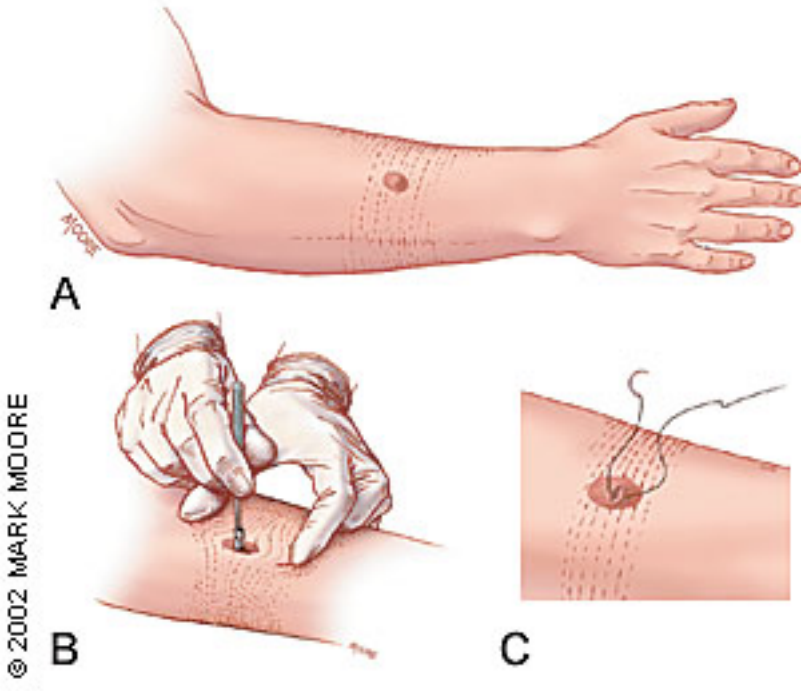


Figure 13. Skin punch biopsy (Zuber, 2002)

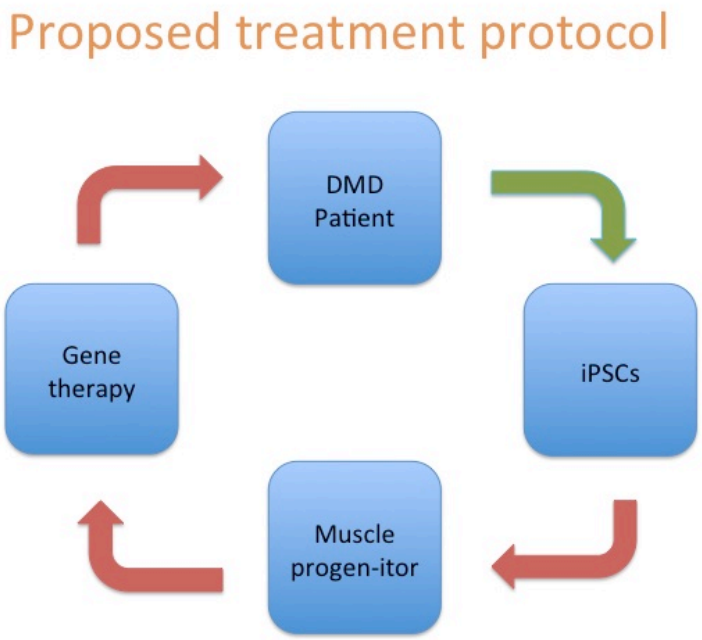


Figure 14. DMD treatment protocol proposed by the Pyle lab. Somatic cells isolated from DMD patients are used to derive a line of induced pluripotent stem cells (hiPSCS) capable of generating any cell type in the body. These hiPSCs would then be differentiated to skeletal muscle progenitor cells (SMPC) capable of expansion, self-regeneration, and repair of damaged miscle tissues [this step is the focus of this paper]. These patient derived SMPCs would then be treated with an as yet undetermined gene therapy to repair the mutation in the dystophin gene that causes DMD. Lastly, these cells would then be reintroduced to the original patient, where they would repair damaged muscle using cells with functional dystrophin. [green arrow indicates step with known protocol, red arrows indicate steps with unknown protocols].

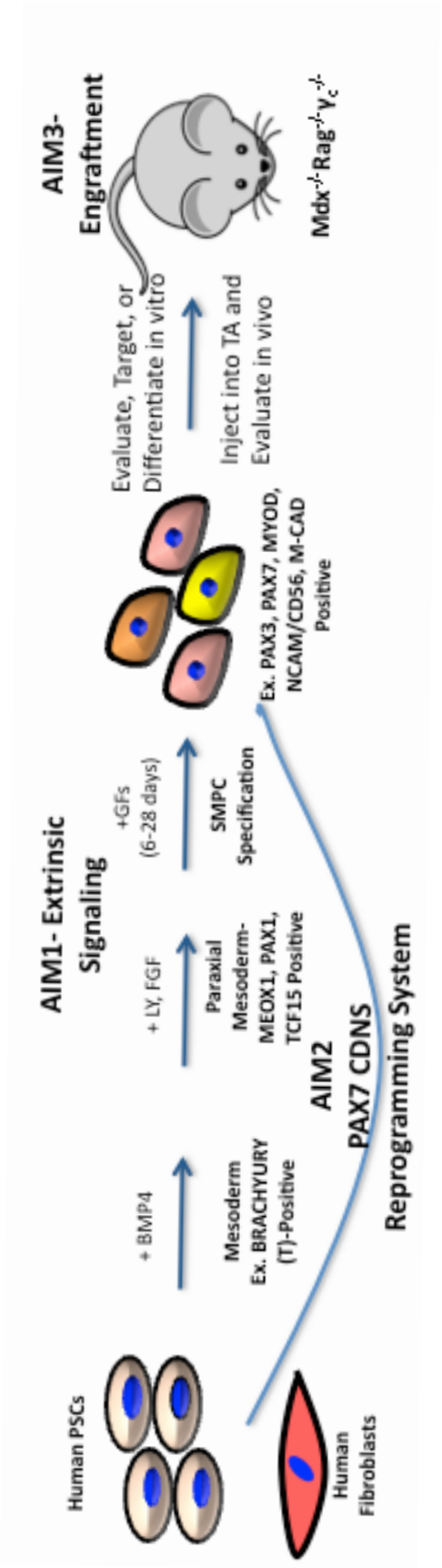


Figure 15. Comparison of *Directed Differentiation* [in this slide: Extrinsic signaling] and *Direct Reprogramming* [in this slide: CDNS Reprogramming] methods conducted in the lab of Dr. April Pyle. Each method begins with hPSCs differentiated to mesoderm lineage cells by exposure to BMP4. The directed diferentiation method further exposes the cells to growth factors FGF and Ly in order to specify paraxial mesoderm, and subsequently tests various growth factors to differentiate to SMPC cells. These growth factors include Wnt7a, LiCl, Bio & Noggin, which are tested individually and in combination. The direct reprogramming method attempts to bypass the intermediate mesoderm and paraxial mesoderm stages by overexpression of Pax3 and/or Pax7 paired box transcription factors which have been shown to be master regulators of the myogenic program. Non integrating overexpression is achieved by the Catalytic Delivery NanoSubstrates developed by the Tseng lab at the California NanoSystems Institute, longstanding partner of the Pyle lab. Successful differentiations will be tested by engraftment in immunosuppressed DMD mouse models, represented by the *mdx* mouse in this figure. Notable markers of each relevant stage of development are listed.

Figure 13. Comparison of *Directed Differentiation* and *Direct Reprogramming*

Directed differentiation

Our lab has compared two approaches to generate skeletal muscle progenitor cells (SMPCs) from hPSCs. The first approach, directed differentiation, attempts to mimic the molecular signals a hPSC cell would be exposed to over the course of normal *in vivo* development to be differentiated to a SMPC (Figure 15). My colleague Tom Dial has conducted most of the research testing this directed differentiation method. In order to differentiate hPSCs to SMPCs, we need to understand the development of SMPCs in normal human development, and then attempt to mimic the timing and concentration of the various growth factors associated with each stage of that development. Somites of the paraxial mesoderm were determined to be the origin of SMPCs in chimeric quail-chic studies (Armand et al, 1983). Paraxial and lateral plate mesoderm differentiate along a gradient of Bone Morphogenic Protein 4 (BMP4) present in the primitive streak during embryogenesis (Dosch et al, 1997). In order to differentiate hPSCs to SMPCs we tested various growth factors known to induce muscle lineage cells from paraxial mesoderm.

The Pedersen group has recently developed a method for differentiating mesoderm lineage cells from hPSCs by exposure to specific growth factors (Cheung et al. 2012). They use Fibroblast Growth Factor 2 (FGF2), Phosphoinositide 3-kinase inhibitor (Ly), and Bone Morphogenic Protein 4 (BMP4). They confirmed this by the presence of Brachyury (T), a marker of early stage mesoderm. Then they further differentiated the mesoderm cells to paraxial mesoderm lineage cells by further exposure to FGF2 and LY or lateral mesoderm lineage cells by further exposure to FGF2 and BMP4. Paraxial mesoderm can be verified by the presence of Meox1, Pax1 and TCF15 proteins.

The Rudnicki group has shown that the signaling protein Wnt7a stimulates the Wnt receptor Fzd7 on adult satellite stem cells, leading to growth and expansion of the

satellite stem cells and muscle fibers. To test the strength of the resultant muscles, specific force was measured on the muscles of mdx mice treated with Wnt7a, which outperformed those of the control by a factor of 1.2. This work showed that treatment with Wnt7a is an effective treatment for dystrophy in mdx mice, and should be considered for clinical trials in humans as well (von Maltzahm et al. 2012). Unmodified Mdx mice were used as they do provide a good DMD animal model of increased damage due to contraction and exaggeration of degeneration and regeneration (Petrof, etal, 1993; Anderson etal, 1988; DiMario et al, 1991). Wnt7a, via the Fzd7 receptor, has been shown to activate MyoD, a known marker of muscle lineage cells (Pownall et al. 2002), through a noncanonical protein Kinase C signaling mechanism (Borello et al, 2006; Brunelli et al, 2007).

The Nakayama group took a different approach to paraxial mesoderm differentiation. They reasoned that because Wnt signaling works through the canonical β-catenin-mediated transcription pathway, they could activate the same pathway by inhibiting the glycogen synthase kinase (GSK)3β known to break down β-catenin. They tested the ability of the small molecules BIO and Noggin to inhibit the enzyme, and found that they can induce the canonical β-catenin-mediated transcription pathway and successfuly induce paraxial mesoderm (Umeda et al, 2012).

Direct Reprogramming

The second approach we tested, direct reprogramming, is an attempt to bypass the complex order, timing and concentration of molecular signals inherent in the directed differentiation approach by using transcription factors rather than molecular signals. By overexpressing transcription factors known to be master regulators of our desired cell type, the SMPC, we expect to simplify the differentiation process (Figure 15). In these experiments we overexpress the PAX7 and/or PAX3 paired box transcription factors, which have been shown to be master regulators of the human skeletal muscle satellite cell genetic program (Bentzinger et al, 2012). PAX3 is expressed in embryonic muscle, while PAX7 is expressed in adult muscle (Seale et al. 2000; Hutcheson et al. 2009). Our goal is to directly reprogram hiPSCs or hESCs to SMPCs by overexpressing PAX3 and/or PAX7 using a novel nanoparticle based delivery system that avoids the need for lentivirus or genetic manipulation.

The Darabi lab has had remarkable success at directly reprogramming non-dystrophic hESC and hiPSC cells to the muscle progenitor cell type via lentiviral integration of the PAX7 gene under the control of a doxycycline (dox) inducible promoter. This work provided proof of principal that PAX7 overexpression could differentiate hESCs and hiPSCs to muscle progenitor cells which were subsequently able to expand via cell culture, and differentiate into functional myotubes (Darabi et al. 2012). However the use of lentiviruses raises the risk of oncogenesis due to the inherent random gene integrations associated with this viral transformation. This has the potential to inactivate vital genes, or induce tumor growth if a host oncogene is expressed by a strong donor promoter. The risk of oncogenesis in virally transformed pluripotent or progenitor cells has hampered the gene therapy field. There is a need for effective and efficient

methods of gene overexpression techniques which avoid the integrations into the host genome inherent in viral techniques.

Non-Integrating Reprogramming

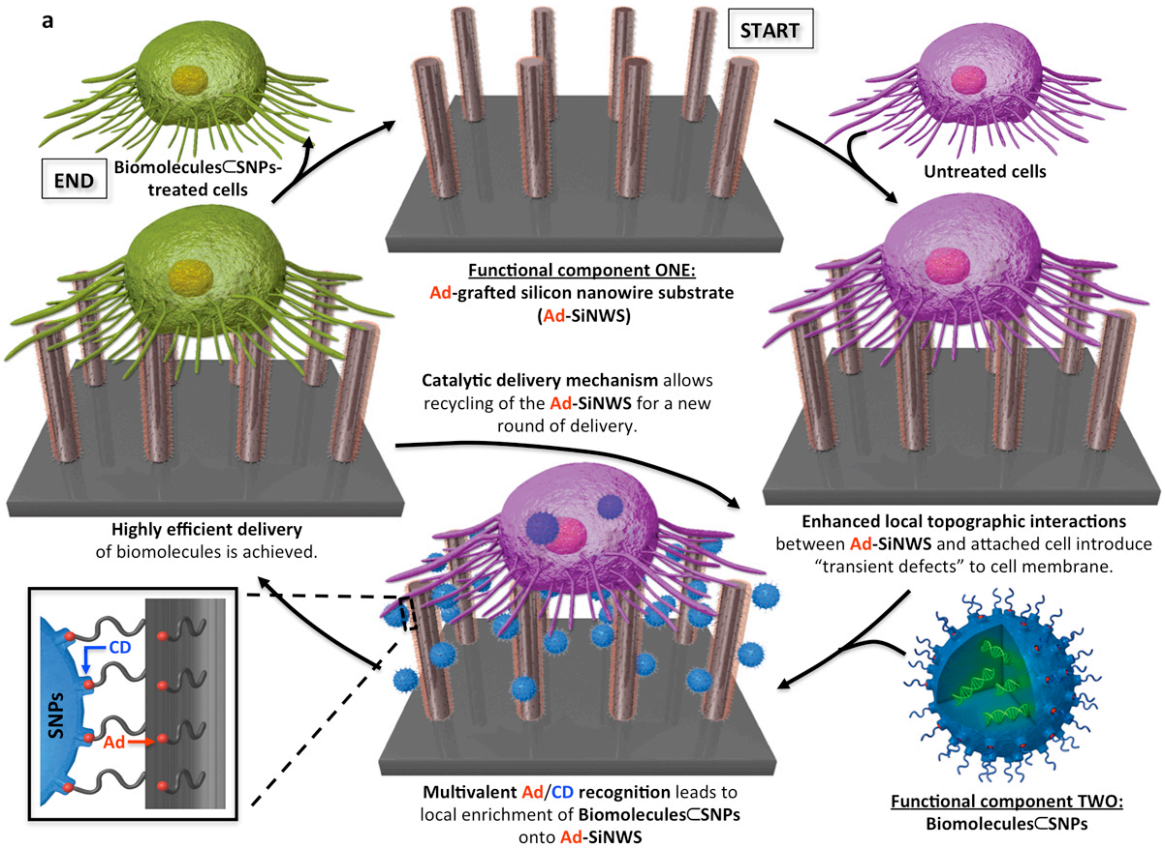
As described above, we are also developing a direct reprogramming approach using PAX7 overexpression without the need for viral mediated overexpression or genetic manipulation. In order to achieve this we have used a novel transient overexpression system developed by the Tseng lab, our longstanding collaborator at the California Nanosystems Institute (CNSI), to overexpress Pax7 in somatic cells or hPSC derivatives. The Tseng lab has developed a Catalytic Delivery NanoSubstrate (CDNS) (Figures 16 & 17) for consistent and efficient delivery of biomolecules (Hou et al, 2012). This system consists of an adamantane-grafted silicon nanowire substrate (Ad-SiNWS), and a supramolecular nanoparticle (SNP) vector for encapsulation of biomolecular payloads. The adamantine groups on the nanowires bind to the cyclodextrin groups on the nanoparticles, creating a high concentration of SNPs localized around the nanowire substrate. The cells sit on the surface of the Ad-SiNWS nanowire substrate, which we commonly refer to as "chips", and create transient defects in the cell's membranes sufficient to allow the SNPs to pass into the cell's cytoplasm. Once inside the cell, the SNPs can open and deliver their payload of biomolecules. This platform was developed in order to increase the efficiency of transfection by non-viral vectors while avoiding the problem of damage to the cell membrane associated with other physical methods of biomolecule delivery such as gene guns, electroporation, or microinjection. This platform also has the advantage of allowing for the continual administration of biomolecules over an extended time span, allowing for the extended exposures needed for reprogramming. In a preliminary study the Tseng group proved their platform was 95% efficient at transfecting the human U87 glioblastoma cells using vectors containing GFP, greatly

exceeding the transfection efficiencies of the industry-standard lipofectamine and RGD-jet-PEI transfection reagents. The Tseng lab in collaboration with Dr. Pyle's lab currently has a paper under review for publication showing they were able to differentiate mouse embryonic fibroblasts, human dermal fibroblasts, and human foreskin fibroblast cells to neural cells with fully formed dendrites and axons at 50-60% efficiency and neural stem cells at an efficiency of 10% by overexpressing the four neuron-specific transcription factors Ascl1, Brn2, Myt1l and NeuroD1. These cells retained their identities after they were removed from the CDNS and SNP system and grown in neural cell media (Hou et al, 2012). We have chosen to use the SNPs to deliver plasmid vectors designed for overexpressing genes in mammalian cells. Human specific PAX7 and PAX3 genes have been cloned into these vectors. Once inside the cell, the human cytomegalovirus promoter and enhancers induce the transcription of the PAX7 and PAX3 genes. The PAX7 and PAX3 transcription factors are expressed in the cytosol, and are transported by the cell's machinery to the nucleus. There they should upregulate the cell's myogenic program and induce the SMPC cell fate.

In this study we compare two different methods of differentiation to specify the skeletal muscle progenitor cell type. The first method we call Directed Differentiation, which is a stepwise administration of growth factors meant to mimic *in vivo* development of skeletal muscle progenitors from pluripotency to mesoderm to paraxial mesoderm to the skeletal muscle progenitor cell type. The second method we call Direct Reprogramming, which is the overexpression of transcription factors known to be master regulators of the myogenic program (Figure 15). My work has focused on the direct reprogramming method, in which I have utilized a novel nanotechnology based

overexpression system developed with collaborators. Each of these methods are tested for skeletal muscle progenitor markers, and I have also conducted some preliminary *in vitro* functional tests. This thesis will compare the abilities of directed differentiation and direct reprogramming protocols to induce human pluripotent stem cells to differentiate into skeletal muscle progenitor cells capable of generating fused muscle fibers.

Figure 16


a
START
END
Biomolecules⊂SNPs-treated cells
Untreated cells
Functional component ONE:
Ad-grafted silicon nanowire substrate
(Ad-SiNWS)
Catalytic delivery mechanism allows recycling of the Ad-SiNWS for a new round of delivery.
Highly efficient delivery of biomolecules is achieved.
Enhanced local topographic interactions between Ad-SiNWS and attached cell introduce "transient defects" to cell membrane.
CD
Ad
SNPs
Multivalent Ad/CD recognition leads to local enrichment of Biomolecules⊂SNPs onto Ad-SiNWS
Functional component TWO:
Biomolecules⊂SNPs

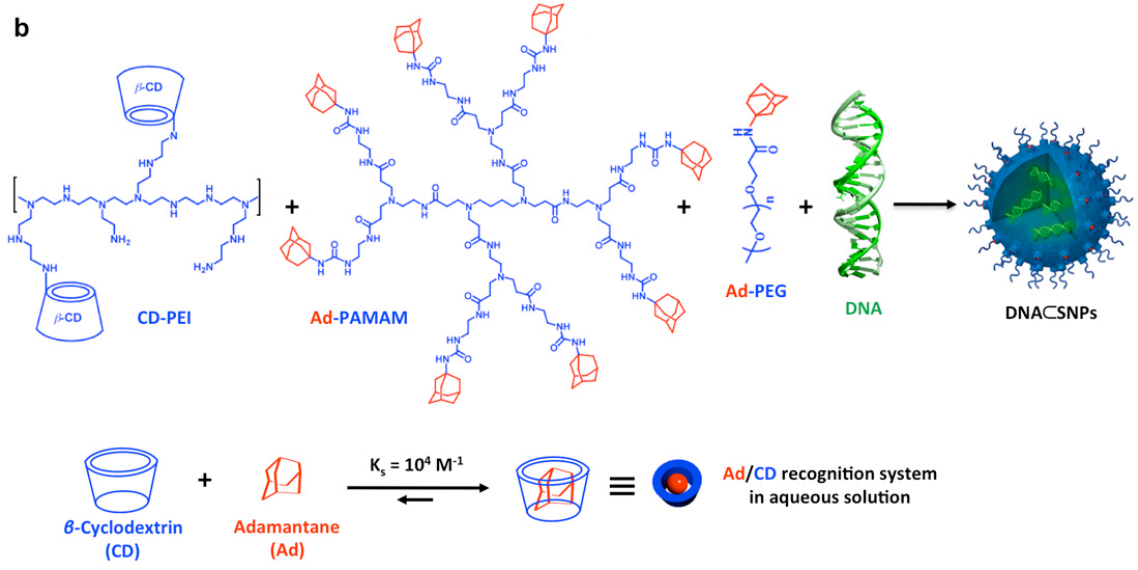

b
β-CD
CD-PEI
Ad-PAMAM
Ad-PEG
DNA
DNA⊂SNPs
β-Cyclodextrin (CD)
Adamantane (Ad)
K_s = 10^4 M^-1
Ad/CD recognition system in aqueous solution

Figure 16. (previous page) Catalytic Delivery NanoSubstrates (CDNS). a, Schematic illustration of the unique catalytic mechanism that governs the highly efficient biomolecular delivery of CDNS platform. Cells first settle onto an Ad-SiNWS, resulting in "transient defects" on the cell membranes due to enhanced local topographic interactions between cell membranes and Ad-SiNWS. Upon exposure of biomolecules□SNPs to Ad-SiNWS, multivalent molecular recognition between the Ad motifs on SiNWS and the CD motifs on the biomolecules□SNPs leads to local enrichment of biomolecules□SNPs from the surrounding solution/medium onto Ad-SiNWS. Consequently, the enriched biomolecules□SNPs on Ad-SiNWS dynamically detach and enter the Ad-SiNWS-immobilized cells through the "transient defects", achieving a highly efficient delivery of biomolecules. Such a unique operation mechanism allows for the repeated use of Ad-SiNWS, as well as the repeated delivery of multiple batches of biomolecules. b, Supramolecular assembly of biomolecules□SNPs from the three molecular building blocks (i.e., CD-PEI: CD-grafted branched polyethylenimine, Ad-PAMAM: Ad-grafted polyamidoamine dendrimer, Ad-PEG: Ad-grafted polyethylene glycol) and biomolecular payloads (e.g., DNA plasmids, siRNA, and transcription factors). (Hou et al, 2012)

Figure 17

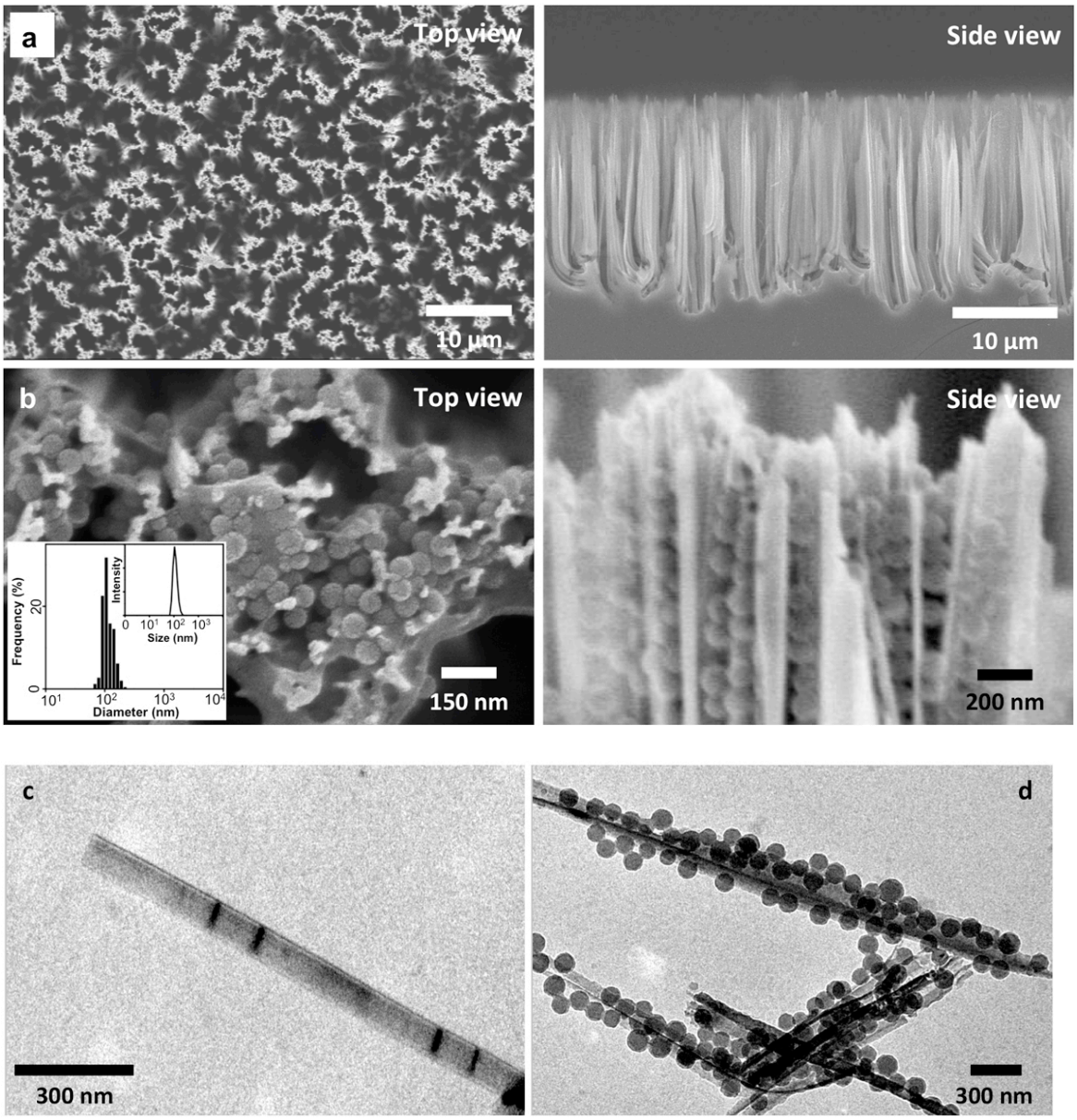


Figure 17. Electron microscopy characterization of the morphologies and structures of CDNS. a, SEM images of the Ad-SiNWS, which were prepared from wet-etching followed by covalent functionalization of Ad. The diameters and lengths of Ad-SiNWS are ca. 100–200 nm and 15-20 µm, respectively. b, Upon exposure of 100-nm pEGFP□SNPs in the solution/medium to Ad-SiNWS, the resulting pEGFP□SNPs-grafted Ad-SiNWS were examined by SEM. The narrow size distribution (106 ± 5 nm) of pEGFP□SNPs on Ad-SiNWS agrees with that observed by DLS measurements (inset). c and d, Free Ad-SiNWS and pEGFP□SNPs-grafted Ad-SiNWS were released from the substrates, and their morphology and sizes were further examined by TEM. (Hou et al, 2012)

Materials and Methods

In our attempts to differentiate hESCs and hiPSCs to SMPCs, we have used two main methods. The directed differentiation method consists of exposing the cells to growth factors known to be expressed during the course of development from stem cell to muscle cells. The direct reprogramming method overexpresses transcription factors known to be master regulators of the myogenic program. However, for each experiment type we begin in the same way, by predifferentiating the cells to the mesoderm lineage. We do this by plating the cells on Matrigel at a concentration of 384,000 cells per well of a six well tissure culture plate in mTeSR serum free stem cell media with 5ng/ml BMP4. Following this initial step we have varied our protocols in several ways in an attempt to find the most successful protocol to differentiate hPSCs to SMPCs.

Directed Differentiation Experiments:

Tom's first directed differentiation experiment was to culture pre-differentiated hESCs (line H9) and hiPSCs (DMD patient derived line 5017) in a Chemically defined media (CDM) consisting of IMDM (50%), (F12 50%), Bovine Serum Albumin (BSA) (5mg/ml), Lipids (1x), Glutamine (1x), Insulin-Transferrin-Selenium (ITS) (1x), Monothioglycerol (MTG) (450um). Controls were grown in this media only. Experimental conditions consisted of 1) FGF (20ng/ml) and Ly (10μM), 2) FGF (20ng/ml), Ly (10μM) and LiCl (5mM), 3) FGF (20ng/ml), Ly (10μM) and Wnt7a (25ng/ml). Samples were taken at days 18 and 30 for analysis by qPCR.

I conducted a second directed differentiation experiment consisted of culturing pre-differentiated hESCs (line H9) in human skeletal muscle media pre-conditioned on human skeletal muscle cells (hsmm cm). Cells grown in this media were the controls. Experimental conditions were 1) Wnt7a (25ng/ml), and 2) LiCl (5mM). Timepoints were

taken at days 0, 7, 16, & 24. QPCR was performed on samples taken at these timepoints,as well as immunofluorescent staining.

Our third and fourth experiments consisted of culturing predifferentiated hESCs (line H9) in CDM alone as the control, and to this media we added the following growth factors as experimental conditions: 1) FGF (20ng/ml) and Ly (10μM), 2) Bio (5μM) and Noggin (100ng/ml). For the fourth experiment samples were taken for qPCR at day 8.

For the third experiment, we attempted to culture the cells in the same conditions as the third experiment for 4 days, after which we changed their culture conditions as follows. The control cells in CDM were switched to Myogenic Induction media (MIM), consisting of IMDM (base), Fetal Bovine serum (FBS) (15%), Horse serum (10%), Chick embryo extract (1%), Ascorbic acid (50μg/ml), Monothioglycerol (4.5mM), and bFGF (5ng/ml). The cells with added growth factors were cultured in the same MIM media with the following growth factors added: FGF (5μg/ml), Wnt7a (25ng/ml), Noggin (100ng/ml), SHH (25ng/ml), and FGF8 (100ng/ml). QPCR timepoints were taken at day 0, 6, and 18. Immunofluorescent staining was done at day 18. These third and fourth directed differentiation experiments yeilded no usable data upon qPCR data quality analysis tools, and so will not be discussed in the results.

Direct Reprogramming Experiments

All direct reprogramming experiments thus far have used supramolecular nanoparticles (SNPs) containing the Pax3 gene cloned into the PCMV6-Entry vector (Origene #PS100001) and/or the Pax7 gene cloned into the PCMV6-AC-GFP vector (Origene #PS100010).

For the first direct reprogramming experiment H9 cells pre-differentiated to mesoderm lineage with BMP4 were plated onto the CDNS platform in 6 well plates at a density of $1.5*10^6$ cells/well in human skeletal muscle media. Pax3 and Pax7 SNPs were added to the wells for 6 hours per day, and samples were taken at day 15 for qPCR. Control wells were plated on the platform and cultured in the same media, but were not exposed to any SNPs.

The second direct reprogramming experiment consisted of culturing pre-differentiated hESCs (line H9) in Myogenic Induction media (MIM) on the CDNS platform, and exposed to SNPs containing both Pax3 and Pax7 for 6 hours a day. Samples were taken at day 10 and 19 for analysis by qPCR, and immunofluorescence staining for Pax7 and Pax3 was done at day 19.

The third direct reprogramming experiment we conducted consisted of culturing pre-differentiated hESCs (line H9) in MIM on the CDNS platform. Control cells received no SNPs, with the following experimental groups: 1) Pax3 and Pax7 together, or Pax7 alone. SNPS were applied every day for the first four days, and every other day for the remainder of the experiment, each SNP exposure lasting 6 hours. Samples were taken at day 7 and day 14 timepoints for analysis by qPCR. Additionally at each timepoint cells from each condition were replated in 6 well plates and chamber slides in both MIM and hsmm cm. At day 24 slides and wells were fixed and stained for Pax7 and MyoD.

The fourth direct reprogramming experiment consisted of culturing pre-differentiated H9s and patient derived fibroblasts (line 5017) on the CDNS platform in MIM media. Cells were exposed to SNPs containing Pax7 for 24 hours per day, every day for the first four days, and every other day for the remainder of the experiment. After

14 days, cells were analyzed by Fluorescence-activated cell sorting (FACS). Cells were sorted for the presence or absence of the cell surface marker M-Cadherin. Cells were then grown for 7 days in MIM media, and then switched to a differentiation media consisting of DMEM and 2% B-27. They will be analysed by either immunofluorescent staining or qPCR.

Cells collected at experimental timepoints were used to isolate RNA, from which cDNA was made, which was then used for quantitative analysis via qPCR.

Results-

Directed Differentiation experiments yield expression of embryonic muscle markers

Tom's first directed differentiation experiment culturing DMD patient derived hiPSCs (line 5017, predifferentiated with BMP4) in CDM+Fly in the presence or absence of Wnt7a or LiCl yielded cells which were analyzed by qPCR for the muscle progenitor markers Pax3 and Neural cell adhesion molecule (NCAM), as well as neural cell marker Pax6. The results showed a heterogenous population of muscle and neural type cells, with muscle type cells predominating (Figures 18a,b,c). NCAM expression was highest in the CDM+FLY+Wnt7a condition at day 14, while Pax3 expression was highest in the CDM+FLy+Wnt7a+LiCl condition at day 14.

Figure 18a

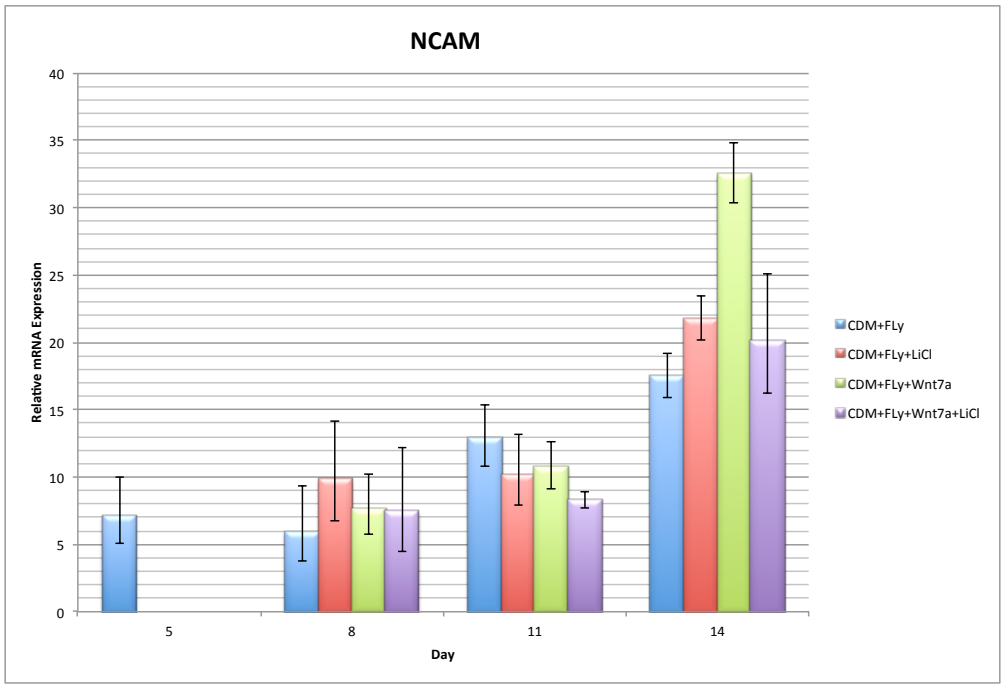


Figure 18b

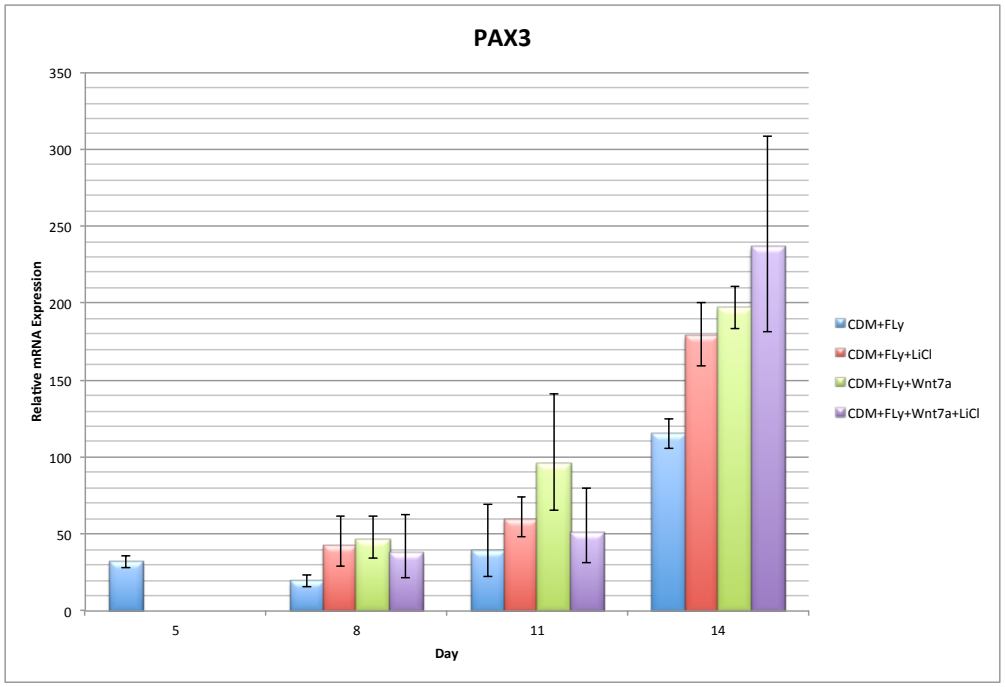


Figure 18c

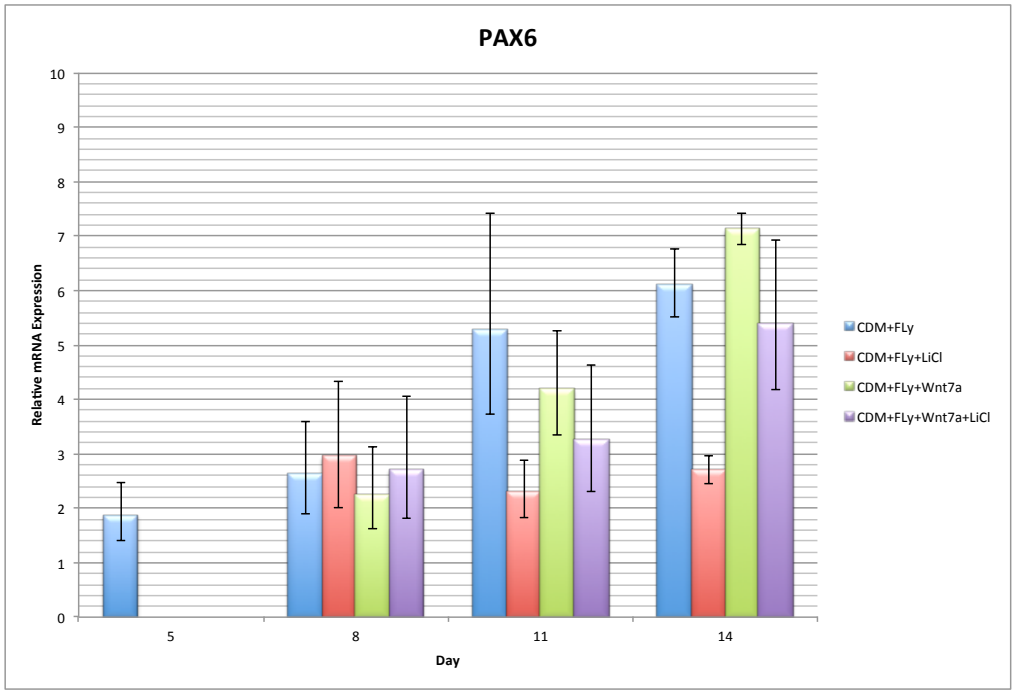
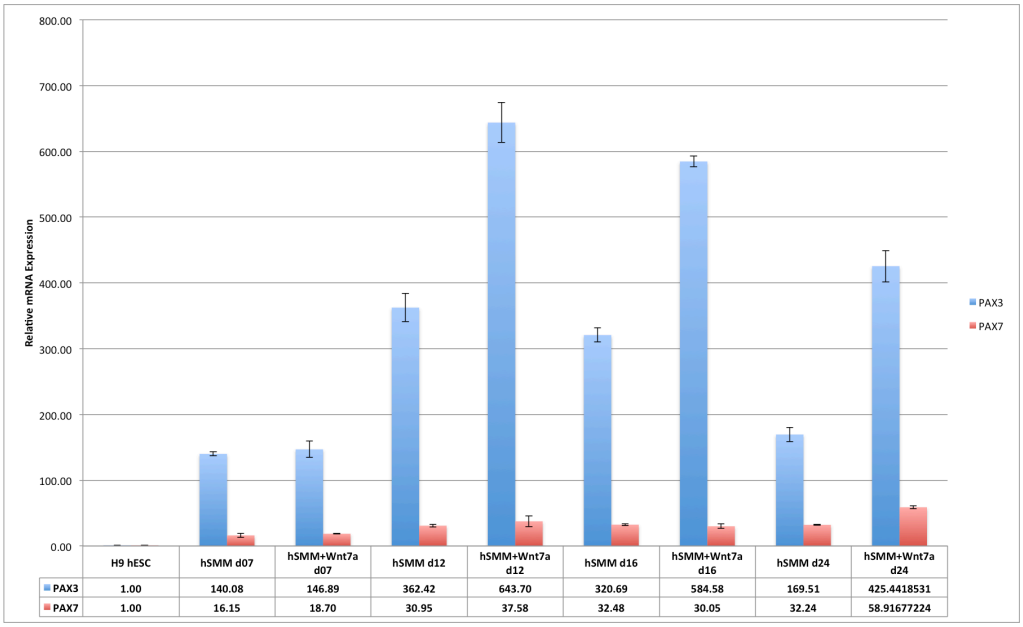


Figure 18. (previous pages) Relative Expression of NCAM, Pax3, and Pax6 in directly differentiated cells. DMD patient derived hiPSCs (line 5017) predifferentiated on matrigel in mTeSR + 5ng/ml BMP4, 2 days grown in CDM, CDM+Fly+LiCL, CDM+Fly+Wnt7a, & CDM+Fly+Wnt7a+LiCl. Relative expression of NCAM (a), Pax3 (b) and Pax6 (c) as determined by qPCR

My second directed differentiation experiment culturing hESCs (line H9, pre-differentiated in BMP4), in human skeletal muscle media (hsmm) conditioned on human skeletal muscle cells in the presence or absence of Wnt7a. The control was H9 cells grown in media formulated to maintain pluripotency. The cells were analyzed by qPCR for Pax3 and Pax7, with Pax3 being expressed much higher than Pax7 in both conditions (Figure 19). The hsmm+Wnt7a condition yielded approximately double the Pax3 expression compared to the hsmm condition. The highest overall Pax3 expression was at day 12 for both conditions, after which expression was reduced. Pax7 expression was uniform throughout the trial for both conditions, except for the day 24 timepoint in the hsmm +Wnt7a condition where it nearly doubled it's expression relative to the rest of the samples. The prevalence of Pax3 with low Pax7 expression suggests this protocol results in embryonic type muscle lineage cells. In order to derive SMPCs, another as yet unknown step would need to be added to this protocol to reduce Pax3 expression and induce Pax7 expression.



	H9 hESC	hSMM d07	hSMM+Wnt7a d07	hSMM d12	hSMM+Wnt7a d12	hSMM d16	hSMM+Wnt7a d16	hSMM d24	hSMM+Wnt7a d24
PAX3	1.00	140.08	146.89	362.42	643.70	320.69	584.58	169.51	425.4418531
PAX7	1.00	16.15	18.70	30.95	37.58	32.48	30.05	32.24	58.91677224

Figure 19. Relative expression of Pax3 & Pax7 in directly differentiated cells. HESCS (line h9)) predifferentiated 2 days on matrigel in mTeSR + 5ng/ml BMP$, grown in hsmm media conditioned on hsmm cells in the presence or absence of Wnt7a (25ng/ml). Relative expression of Pax3 and Pax7 as determined by qPCR.

Direct reprogramming yields some, not all, skeletal muscle progenitor makers; no fused myotubes upon differentiation conditions.

The directed differentiation experiment culturing mesoderm predifferentiated hESCs cells (line H9) on the CDNS platform in Myogenic Induction media and exposure to SNPs containing plasmids expression vectors with Pax3 and Pax7 together and Pax7 alone was analyzed by qPCR for expression of Pax3, Pax7 and MyoD (Figure 20a). While MyoD was never shown to have been expressed, Pax3 and Pax7 were each shown to have been overexpressed. Most interestingly, conditions containing supramolecular nanoparticles (SNPs) containing Pax7 alone yielding the highest amounts of Pax3 and Pax7 expression at each timepoint (7 and 14 days), with expression levels increasing from day 7 to day 14. The major drawback of this test was that we were unable to distinguish between expression by the plasmid and expression endogenous to the cells. Difficulty in culturing cells on the CDNS substrate surface and collecting cells from the CDNS substrate surface limited the amount of RNA extracted for each sample, and thus limited the number of primers testable for each sample. In electing to test for muscle markers instead of a negative control in the form of a neural marker in the face of this RNA shortage ultimately limited the conclusions which this analysis could prove. Seeking to analyze a different SMPC marker, cells were removed from the chip and replated in both MIM and hsmm cm media and stained for M-cadherin (M-CAD). The resulting extracellular M-CAD staining is indicative of SMPC cell fate (Figure 20b).

Figure 20a

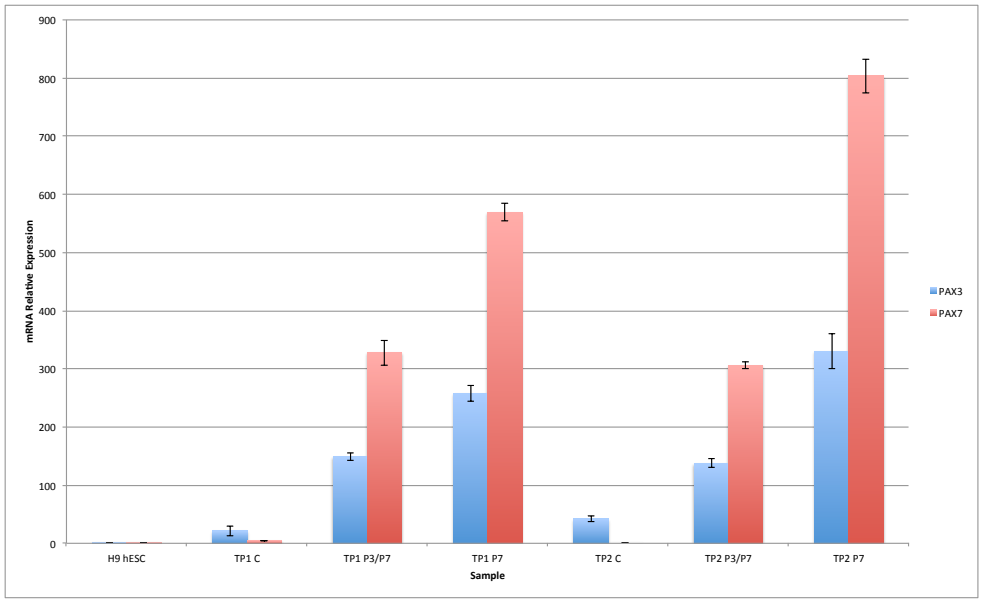


Figure 20b

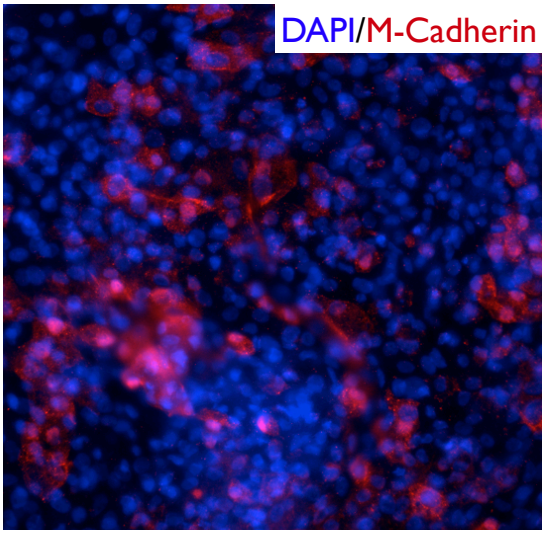


Figure 20. Expression of muscle progenitor markers in directly reprogramed cells. hESCs (line H9) predifferentiated on matrigel in mTeSR + 5ng/ml BMP4, plated on CDNS platform in Myogenic Induction Media. A) Relative quanity of Pax3 and Pax7 in three conditions: C: Control (no SNP), P3/7: Pax3 and Pax7 packaged in SNPs, and P7: Pax7 packaged in SNP. Timepoints are 7 and 14 days. B) Immunofluorescent staining for DAPI and M-Cadherin after replating. Control plate was lost due to leakage of slide well.

A further test was conducted comparing mesoderm predifferentiated hESCs (line H9) to DMD patient derived fibroblasts (line 5017), both plated on the CDNS platform and exposed to SNPs containing PAx7 expressing plasmid vectors. These cells were analyzed and sorted FACS for M-CAD. The 5017 line showed more M-CAD in the control than the experimental group, while 16.4% of the Pax7 treated H9s expressed M-CAD verses 9.2% of the control H9s (Figure 21). These cells were plated and grown in proliferation conditions for one week (MIM media) and then differentiation conditions for one week (DMEM+B-27[2%]) and stained for Myosin Heavy Chain (MHC), a marker of fused myotubes. No MHC staining was seen in any sample, though the cells were too sparse to have fused.

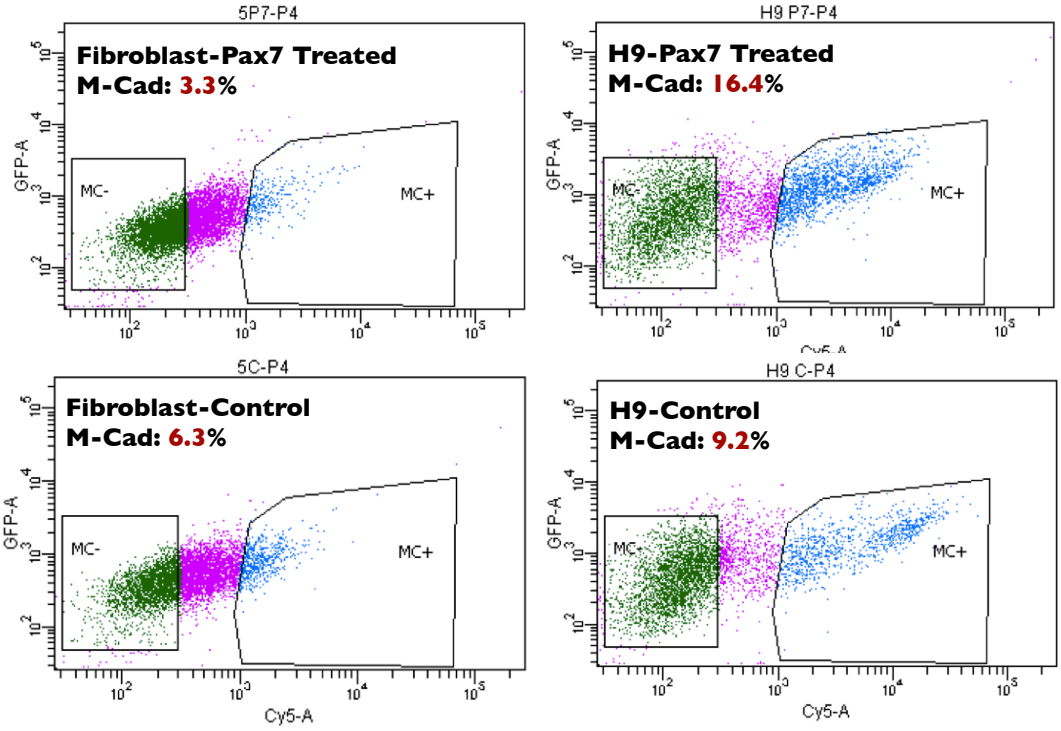


Figure 21. M-Cadherin expression in directly reprogramed cells. hESCs (line H9) predifferentiated on matrigel in mTeSR + 5ng/ml BMP4 and DMD patient derived fibroblasts (line 5017), plated on CDNS platform in Myogenic Induction Media, exposed to Pax7 packaged in SNP, control no SNP. FACS sorted for M-Cadherin.

Discussion

Directed differentiation

The CDM+Fly+/-Wnt7a+/-LiCl experiment showed the most promising results in the CDM+Fly+Wnt7a+LiCl condition at the last time point (day 14). High levels of Pax3 and NCAM are indicative of mesoderm lineage, early muscle cells but could also be neuronal. This experiment should be repeated and extended for longer time periods, as well as tested for Pax7 via qPCR, via immunofluorescence for M-Cadherin and other SMPC markers, then exposed to conditions inducing terminal differentiation and stained for Myosin Heavy Chain, a marker of fused muscle tubes. Presence of fused muscle tubes would indicate that at some point a myogenic progenitor was present in the experiment.

The hsmm+/-Wnt7a experiment showed the highest Pax3 expression at day 12, but relatively little Pax7 expression. This experiment could also be repeated and tested via immunofluorescence for M-Cadherin and other SMPC markers, then exposed to conditions inducing terminal differentiation and stained for Myosin Heavy Chain. The high expression of Pax3 and low expression of Pax7 is indicative of embryonic muscle progenitor cells. In order to induce Pax7 expression, some as-yet-unknown step must be added to this protocol.

The Barberi group has recently published a paper in which they derive PAX3+/PAX+ skeletal muscle progenitor cells from hESCs by first activating the canonical Wnt pathway by a novel method (Borchin et al, 2013). Rather than inducing this pathway with a Wnt ligand, they instead use a small molecule (CHIR 99021) capable of inhibiting the GSK3β componant of the pathway which normally phosphorylates β-catenin leading to it's destruction in the absense of a Wnt ligand induced signal (see Figure 10). The GSK3β inhibitor was used for four days. This lead to a cellular identity

typical of the dorsal dermomyotome, the source of skeletal muscle progenitor cells. These cells were then successfully expanded by the adition of FGF2 from days 4 through 18. Then the cells were expanded in a standard cell culture media (DMEM-F12) supplemented with insulin, transferrin, and selenium for days 19 through 35. At day 35 cells were FACS sorted for muscle-specific nicotinic acetylcholine receptor AChR, the chemokine receptor CXCR4, and hepatocyte growth factor receptor CMET/HGF. HESCs transformed in this way were able to form fused myotubes, a significant funtional test that none of the experimental cells in this thesis were able to complete. The simplicity of this protocol in terms of number of growth factors used (two) and markers sorted for (three), make it a potentially ideal protocol for deriving SMPCs from hIPSCS for use in clinical treatment of human DMD patients. However this paper did not include any animal model engraftment studies, which would be the next step before clinical trials in animals could begin.

I encountered a poster presentation at the 2013 ISSCR conference in Boston by the Genea Biocellcompany of Sydney, Australia. Their poster outlined a protocol they had developed using a proprietary set of growth factors and/or small molecules used in a stepwise and precisely timed fashion to differentiate hESCs to skeletal muscle. The number of unnamed factors used was reported to be between 6 and 50 in total. They have not published these findings in a peer reviewed scientific journal, but have published a press release on Dr. Leslie Caron's ability to differentiate hESCs to skeletal muscle without cell sorting or genetic manipulation and with high yields (Genea Biocell, 2013). A separate page on the Facioscapulohumeral Dystrophy Global Research Foundation website describes in general terms Dr. Caron's stepwise differentiation of hESCs to first

myoblasts, then myocytes, and finally fused myotubes with a set of unnamed growth factors (FSHD, 2014). Personal communication with Genea Biocell suggested they are currently in the final stages of validating the protocol. Neither of these press releases mentions the SMPC cell fate, as they seem to be focused on the terminally differentiated muscle, though their stepwise differentiation protocol may yield a SMPC cell they have not yet characterised in Dr. Caron's published papers

Another recently published paper uses the growth factors FGF2 and Epidermal growth factor (EGF) along with a variaton on the embyroid body differentiation technique to derive human muscle progenitor cells (Hosoyama et al, 2014). Embyroid bodies are hESC colonies which have been lifted off of the tissue culture dish with an application of trypsin, after which they spontaneously form spheres and are grown in non-adherant cell culture flasks. These spheres have often been replated onto an adherent cell culture plates, where they differentiate into various cell types. This technique has been used by many stem cell researchers. However, a problem arises when long culture times are required by a particular experiment. In prolonged culture, the center of embyroid bodies become necrotic, as the spheres grow in size with cell division, and the cells in the center exude waste products and become nutrient starved. To solve this problem, embyroid bodies can be cut to an ideal size using a tissue chopper. This device was developed for chopping whole tissues for histological experiments, but works well for chopping the embyroid bodies. In this way they can be maintained at an ideal size allowing for diffusion of waste and nutrients to and from the center of the colony. Using this technique in conjunction with high concentrations of the noted growth factors for six weeks yielded hESC and hIPSC derived muscle progenitor cells capable of producing

fused myotubes after two weeks in differentiation conditions. The EZ sphere technique facilitated the long culture times required for this experiment to be successful.

Direct reprogramming

The failure of overexpression of Pax3 and/or Pax7 via CDNS to induce fused myotubes in treated cells subsequently exposed to differentiation conditions may be attributable to several factors. Some of these factors relate to the interaction of the cells with the CDNS platform, while others relate to the gene overexpression.

The difficulty in getting cells to attach to and later unattach from the nanowires of the CDNS substrate gives us several causes for concern. The low experimental yields acquired in terms of cell numbers available for analysis and terminal differentiation experiments severely limits the conclusions that can be made from these experiments. Several trials yeilded low RNA concentrations, making QPCR difficult and error prone. As fusion of myocytes into multinuclear myotubes requires a high cell concentration, a low experimental cell number yeild would limit the possibility of successful post-treatment fusion even if the satellite cell fate has been successfully differentiated. Furthermore high trypsin concentrations, extended trypsinisation times, and mechanical force by repeated washes with the electric pipetter at pressures much higher than used in standard cell culture practice were necessary to remove the cells from the surface of the CDNS platform. These steps likely had a deleterious effect on the cells, and many cells were likely lost in the process.

It is possible that the experimental level of PAX7 overexpression achieved in this set of experiments is insufficient to induce the myogenic program. The 800 fold relative expression of PAX7 timepoint seems high, but it is possible that it is not enough to effect a cell fate change in the pluripotent cells. This would be supported by the low expression of GFP seen in our FACS analysis. While several expression vectors using stronger

promoters were tried in short term tests in hopes of increasing the efficiency and strength of the transfection, none exceeded the performance of the pCMV promoter in the original mammalian overexpression vector containing Pax7 aquired from Origene. It is still possible that another as-yet-untested promoter would yeild higher expression at some threshold we have not yet met, and that direct reprogramming to SMPC cell fate could be achieved in this way.

On the other hand, excessively high overexpression via the CDNS platform could be the reason for our treated cell's inability to form fused myotubes. This seems unlikely as the fluorescent GFP marker was expressed at low levels, so plasmid gene expression is likely low unless the GFP is being silenced somehow inside the cell. However, it is possible that the overexpression vector could have integrated into the pluripotent cell genome. The CDNS system is designed to be a transient overexpression system without integration, but as overexpression increases so does the risk of genetic integration. If such integration occurred in these experiments the cells would highly express Pax7, which would arrest the cells in the SMPC fate and prevent further differentiation. Pax7 is downregulated *in vivo* during terminal differentiation to fused myotubes. Gene integration of our overexpression vector would prevent this downregulation and in doing so prevent terminal differentiation. Any such cells would be non-functional and clinically irrelevant. In order to detect such an integration, treated cells could be sequenced and the genome scanned for the pCMV promoter and the GFP tag. Unfortunately, the terminal differentiation experiment was among the last trials conducted in this study, and sourced from a CDNS platform with very low cell number yeild. As high cell concentrations are required for myocyte fusion, all available cells were used in the terminal differentiation

experiment. They were plated in the smallest available slide chambers to get the maximum cell concentration for the low cell number yeild, exposed to differentiation conditions, fixed with paraformaldehyde and stained with relevant antibodies. If this experiment had yeilded more cells, the sequencing test described above could have been completed.

It is possible that the green fluorescent protein (GFP) tag used to verify expression of the plasmids containing PAX3 and PAX7 interfered with the expression of these transcription factors.

The qPCR analysis shown in figure 15a only shows expression of PAX3 and PAX7, the same transcription factors overexpressed in the experiment. As such, it does not distinguish between genes overexpressed by the plasmid and genes expressed by the cells, which is a significant weakness. Durring this analysis, primers for N-Cam and MyoD were also included, however they did not show any expression. We considered using the same PAX6 marker of neural cells as a negative control, but the low RNA concentration from the experiment limited the amount of primers I could test for, and we elected to test for more SMPC markers which did not show up. The lack of a negative control in this experiment is not ideal.

I encountered several problems using cells on the CDNS platform. The platform (or chip, as we called it) is opaque, so I could not visually assess the cells growing on the platform surface. Visual assessment by microscope of the number, density and relative health of cells growing in a standard transparent cell culture plate is essential for maintaining cell viability in standard cell culture practises. Using an opaque growing surface left me without a good sense of how the cells on that surface were growing. For

these experiments the rectangular chips rested in the circular wells of standard tissue culture plates, so I was able to visualize cells in the transparent spaces around the edges of the platform where cells that did not attach to the platform had congregated. However, this was not informative as to what was happening on the platform itself. The concentrations of RNA extracted from cells removed from the platform for use in qPCR analysis was often quite low, and indicative of low plating efficiency on the substrate. This slightly improved when I attempted lysing cells directly off of the substrate rather than removing them from the substrate with trypsin first, though the levels of RNA were often still low. Low RNA concentration can lead to unreliable qPCR results, which I experienced with some regularity throughout the course of these experiments.

Our highest levels of Pax3 and Pax7 expression were seen at the second time point (day14) when only overexpressing Pax7. However we were unable to detect MyoD expression via qPCR. I attempted to remove cells from the CDNS platform and replate them in differentiation conditions to test their ability to form fused myotubes and thus demonstrate myogenic potential. However, it was very difficult to get sufficient numbers of viable cells off of the platform. Myotubes will not fuse unless they are sufficiently dense, and even though I plated mine in the smallest possible chamber slide well, their concentration was still too low to induce fusion. Our concern is that cells are becoming embedded into the nanowire structure of the platform over the course of the multi-week experiments. As these experiments are carried forward, we intend to address this issue by removing the cells from the platform once per week, use FACS or microfluidics to sort for SMPC markers, then replate cells back onto the chip platform. We will soon have the opportunity to test a new version of the CDNS platform which was thus designed to

release cells when exposed to 4°C by changing the confirmation of the nanowire structure in response to low temperature.

Furthermore, when I was preparing the cells from the CDNS platform for FACs sorting, I lost many cells at the filtration step. When I could get cells to come off of the platform, they often did so in large aggregates, which could not pass through the filter meant to passage only single cells. As these experiments are carried forward, we intend to alter the protocol at this step, re-trypsinizing cells as many times as necessary to break them up into single cells. This could drastically change the results of FACS sorting experiments if we obtain all the cells from the platform instead of just a small fraction of them, and potentially result in enough cells to replate at sufficient density to differentiate to fused myotubes if the differentiation has been successful.

As the CDNS platform proved to be so problematic in these experiments, we must conduct a new set of experiments to evaluate it's ability to transform various cell types compared with other transient gene overexpression methods. In these experiments we should attempt to differentiate hESCs to neural cells, and differentiate fibroblasts to SMPCs. These experiments should be conducted on both the CDNS platform and an alternate transient gene overexpression system. The results of these experiments should help elucidate the usefulness of the CDNS for transforming specific cell types.

Our collaborator Dr. Tseng has also developed a microfluidics system to capture and release cells of specific types (Hou et al, 2013). This system was developed to capture circulating tumor cells, but could be adapted to capture SMPCs. The microfluidics system is housed in a "microchip", and contains a serpentine course through which blood or cell growth media can flow, with nanostructures to induce ideal

amounts of turbulence for thorough mixing of the fluid, and vertically positioned nanowires featuring cell-specific adhesion molecules. The cells are captured by coming into contact with multiple adhesion molecules in a manner similar to velcro, and so they have nicknamed this technology "nanovelcro". The adhesion molecules are anchored to nanowires which are temperature sensitive, such that they capture cells at 37°C and release them at 4°C. This allows researchers to efficiently retain their desired cell type and to keep those cells viable. This microfluidics system could be used as an alternative to FACS sorting in our experiments if it indeed shows increased viability after sorting.

It would be interesting to do a +/-Wnt7a and +/-LiCl condition when using the CDNS platform during direct reprogramming with overexpression of PAX7. Wnt7a has been implicated as influencing satellite cell expansion, and LiCl increases the effects. As the CDNS platform has thus far given a low cell number yield, expansion of the cell yeild could greatly enhance the chances of success. Furthermore, the lab of Dr. Helen Blau has published a paper outlining the use of bioengineered soft substrates to enable the successful expansion of murine muscle progenitors, which has proven impossible until now (Gilbert et al, 2010). To achieve this they engineered a cell culture substrate which was pliable and mimiced the pliability of the basal lamina of muscle cells. This paper utilized satellite cells extracted from mouse adult muscle tissue, and should be replicated with human adult satellite cells. Assuming it is successful in human cells, if we could utilize a similar pliable substrate to replate the cells after treatement on the CDNS platform, we may be able to expand any SMPCs in that cell population.

A paper recently published by the Puri group showed another reason why PAX3 and PAX7 overexpression alone did not yeild any functional SMPC cells in this study.

They showed that hESCs do not express the BAF60C subunit of chromatin remodeling factor SWI/SNF which is expressed in adult somatic cells, and that this factor is required for MyoD mediated differentiation to the myoblast cell fate (Albini et al, 2013). Furthermore, BAF60C was shown to enable the differentiation to bypass the mesoderm stage of differentiation. The Puri group did not attempt to isolate and expand the satellite cell or SMPC as this thesis does, instead concentrating on the ability to generate muscle tissue. The paper detailing the Puri group's findings was published after the work in this thesis was completed, but will be taken into account as the project continues. The Darabi group whose results we are trying to replicate used no such epigenetic modifiers in their differentiation protocol (Darabi et al, 2012). Perhaps their differentiation was successful because lentiviral integration of PAX7 results in strong enough PAX7 expression to overwhelm the hESC's lack of BAF60C, or upregulate BAF60C. If the level of PAX7 expression in our experiments is insufficient to induce functional SMPCs, modification of our transient PAX7 overexpression protocol by the addition of simultaneous overexpression of BAF60C or supplementation with BAF60C may have a synergistic effect sufficient to induce the MyoD mediated generation of myoblasts and the subsequent division to myocytes and fused muscle. The lack of BAF60C could also be why we were unable to see any MyoD expression in the QPCR analysis, as the absense of BAF60C likely blocked it's transcription.

The elimination of epigenetic modifiers preventing direct reprogramming could be the key to increasing the efficiency of this technique. A recent paper showed that the key to highly efficient reprogramming of mouse and human adult somatic cells to IPSCs via the viral expression of the Oct4, Sox2, Klf4 and Myc transcription factors is the

elimination of the Mbd3 component of the Mbd3/NuRD nucleasome repressor complex (Rais et al, 2013). Mbd3 was knocked out via iRNA, and reprogramming efficiency subsequently was remarkably increased to 100% compared to 20% in the control. While this study used virus-mediated integrating transformation which is more likely to achieve high overexpression than transient overexpression, knocking out epigenetic modifiers could greatly increase the efficiency of the transient overexpression techniques. The method they used to determine the key epigenetic molecule to conduct an siRNA screen to knock out a host of candidate epigenetic modifiers. If the addition of BAF60C to our SMPC differentiation protocol is insufficient to allow for efficient reprogramming, a similar siRNA screen could be used to seek other epigenetic barriers to reprogramming.

Due to the varying embryonic developmental pathways leading to trunk, head and limb muscle, it is unclear if any SMPCs developed by our protocols would be functional in regenerating muscle of all three types. Engraftment studies would need to be conducted in all three muscele tissues of a dystrophy mouse model. Furthermore, this project has not addressed the regeneration of cardiac muscle and smooth muscle, each of which expresses dystrophin and is subject to degeneration in DMD patients. As one of the major causes of mortality in DMD patients is cardiac failure, regeneration of cardiac muscle with functional dystrophin would be required to cure the disease.

Satellite cells have been proved to be the major contributor to regenerating damaged muscle, but they are not the only Skeletal muscle progenitor cell. Another SMPC of interest in potential treatments of DMD is the pericyte, as it has unique properties. Pericytes are cells which reside in or near the capillaries in multiple tissues, and those in the capillaries of skeletal muscle are able to migrate into the muscle in

response to damage. However, in addition to muscle repair, they are also involved in fat deposition. Pericytes expressing Nestin (Type 2) contribute to muscle regeneration, while those not expressing Nestin (Type 1) contribute to adipose deposits (Birbrair et al, 2013). Satellite cells have not shown great potential to migrate throughout the muscle in engraftment studies. The number of satellite cell injection engraftments in a DMD patient would be excessive. The ability of pericytes to move from capillaries into the muscle interests researchers in that it suggests a possible solution to the tissue wide engraftment problem. Pericytes have a different developmental lineage than satellite cells, and so different protocols would be necessary to differentiate them from hESCs. It would likely be necessary to learn to control the expression of Nestin in any hESC derived pericytes in order to minimize the amount of fatty deposition and maximize the amount of muscle regeneration in dystrophy patients or animal models.

Though I did not proceed far enough in this experiment to try it, as this study progresses we intend to test the capacity for muscle regeneration of our experimentally derived cells by injecting them into one of the improved mdx mouse model which has been immunocompromised. We would first have to prove that our experimentally derived cells are capable of expansion and differentiation into fused myotubes *in vitro*. If this step is succesful we would then conduct an *in vivo* test to determine the ability of the experimentally derived cell's ability to engraft in damaged muscle of immunosuppressed *mdx* mice. We would use a human-specific dystrophin antibody or fluorescently labeled marker to determine if the experimentally derived cells could regenerate the damaged muscle and repopulate the skeletal muscle progenitor cell niche.

It is possible that direct reprogramming to specific cell fates need not start with a pluripoent cell. Mice fibroblasts have been directly reprogrammed to functional neurons using the transcription factors Brn2, Myt1l, and Ascl1, bypassing the pluripotent state all together (Vierbuchen et al., 2010). This work has been replicated in human cells with the BRN2 and MYT1L transcription factors plus the miR-124 microRNA (Ambasudhan et al, 2011).

Conclusions

All the results from both the directed differentiation and direct reprogramming methods described in this thesis are preliminary in nature. These preliminary results showed sufficient potential for the lab to obtain an NIH R01 grant to continue investigating these methods and optimizing protocols for the differentiation of skeletal muscle progenitor cells. We will continue to examine developmental signals in myogenesis and to monitor advances in SMPC differentiation in order to improve our protocols. As SMPC differentiation and dystrophin gene therapy advances, so does potenital for a cure to Duchennes muscular dystrophy.

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