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Isolation and characterization of urine-derived stem cells as a novel modeling tool to study hereditary neuromuscular diseases

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DEDICATION

“ Where there is a will there is a way”

This is for you, Mom. Thanks for always being there for me

To My Father, my lovely sisters and my brothers

To My Friends, my teachers and my students

To everyone who supports me

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ABSTRACT

Background: Urine derived stem cells (USCs) have become a novel source for generating human induced pluripotent stem cells (iPSCs) that can be used as a disease model for understanding the pathogenesis, disease mechanism and exploring drug-screening platforms for rare human diseases. The isolation of stem cells from urine is considered to be noninvasive, simple, affordable, ubiquitous, and readily accepted by patients. Therefore, it could be used as a diagnostic tool for many human genetic disorders.

Aims: This study aimed to set up a protocol for the isolation of stem cells from urine specimens and to use these cells for functional genetic studies in Duchenne Muscular Dystrophy (DMD), a rare X-linked recessive inherited disease caused by a variety of dystrophin gene mutations.

Methods: In the present study USCs were isolated from urine samples collected from two healthy donors and one DMD patient with a deletion of exon 45. The isolated USCs were characterized by surface markers.To profile the dystrophin transcript in both native and myogenic transformed USCs we used DMD gene micro-fluidic card (FluiDMD), a TaqMan gene expression assay that profiling the entire dystrophin transcript including all exon-exon junctions. Furthermore, myotubes obtained from MyoD-transformed DMD USCs were treated with antisense oligoribonucleotide (AON) for dystrophin exon 44 skipping and the exon skipping percentage was quantified. The AON 44 is a known experimental drug used for the exon skipping therapy approach to reframe dystrophin transcript in DMD boys. Finally, the dystrophin protein expression was analyzed using immunostaining analysis and Western blotting.

Results: This study demonstrated that native urine-derived stem cells expressed the full-length dystrophin transcript, and the dystrophin mutation was retained in the cells of the patient with DMD, both at DNA and RNA level, while the dystrophin protein was detected solely in control cells after myogenic transformation according to the phenotype. The treatment with antisense oligoribonucleotide against dystrophin exon 44 induced skipping in both DMD native and DMD MyoD-transformed urine-derived stem cells, with a successful therapeutic transcript-reframing effect, as well as visible protein restoration in the latter.

Conclusion: This study suggested that native urine stem cells could represent a non invasive and cost effective human cell model to study the dystrophin transcript and can be used as a source to explore DNA and RNA profiles and to test new drugs for therapeutical approaches in DMD and other neuromuscular diseases.

ABSTRACT ITALIANO

Basi scientifiche: Le cellule staminali dervanti dalle urine (Urine derived stem cells o USCs) sono state recentemente identificate come fonte di cellule staminali pluripotenti o iPSC. Queste cellule sono estremamente interessanti poichè rappresentano un modello cellulare utilizzabile per studiare e meglio comprendere le patologie umane, in un ampio spettro di funzioni, dalla patogenesi, ai meccanismi eziologici, a nuove terapie. Le loro caratteristiche di staminalità le rendono inoltre adatte per valutare nuovi farmaci sperimentali. Le urine si possono ottenere in modo completamente non invasivo e di conseguenza i pazienti e in generale gli individui sono molto complianti per tale esame, che puo essere ripetuto nel tempo. Le cellule del sedimento urinario, incluse le cellule staminali, sono quindi facilmente ottenibili e rapidamente isolabili. Esse possono anche essere utilizzate come strumento per la diagnosi genetica e proteica, e la loro natura non invasiva le rende una ottima sorgente cellulare per monitorare i trattamenti sperimentali presumibilmente di un numero svariato di malattie genetiche con diversa origine.

Scopo: Questo studio ha avuto l'obiettivo di mettere a punto un protocollo e una strategia per isolare cellule staminali da urine di pazienti e controlli, e in seguito utilizzare le cellule per valutare in esse l'assetto genetico, trascrittomico, proteomico del gene distrofina, sia in pazienti Duchenne che in controlli. La distrofia muscolare di Duchenne (DMD) è una patologia ereditaria recessiva legata al cromosoma X e causata da una ampia varietà di mutazioni nel gene distrofina.

Metodi: In questo lavoro le cellule urinarie staminali sono state isolate da urine di controlli e di un paziente DMD. Il paziente DMD aveva una mutazione per delezione che coinvolgeva l'esone 45. Le USCs isolate sono state caratterizzate per i marcatori di superficie. Per studiare il trascritto ditrofina sia nelle USCs native che in quelle rese miogeniche tramite trasfromazione con fattore MyoD sono state utilizzate delle fluidic cards, una metodica che si basa su un saggio quantitativo TaqMan. Le fluidcards per il gene DMD (FluiDMD) nel profilano l'intero trascritto, comprese le giunzioni esone-esone e le diverse isoforme. Le USCs trasformate con MyoD sono state differenziate in miotubi e trattate con molecole 2'OMePS antisenso (esone 44) per indurre un exon skipping terapeutico. Lo skipping dell' esone 44 determina infatti nei pazienti DMD con delezione dell'esone 45 un recupero della trascrizione e della conseguente espressione della distrofina nel sarcolemma, che è stata visualizzata tramite immunoistochimica e western blotting.

Risultati: il nostro studio ha dimostrato che le USCs native esprimono fisiologicamente la distrofina a livello trascrizionale ma non la protein e che nel paziente DMD il profilo

genotipico è riprodotto nelle cellule sia a livello del DNA che dell' RNA. Al contrario, la proteina distrofina è presente solo nelle USCs trasformate con MyoD e quindi rese miogeniche del controllo. Il trattamento con antisenso ripristina la sintesi della proteina nel paziente DMD tramite lo skipping favorevole dell'esone 44. L' effetto dell'antisenso è visibile sia nelle USCs native (trascritto) che in quelle rese miogeniche (trascritto e proteina).

Conclusioni: In conclusione, noi abbiamo dimostrato che le USCs rappresentano un ottimo modello cellulare sia per la diagnosi che per approcci terapeutici in vitro per la Distrofia muscolare di Duchenne. La loro natura completamente non invasiva ne facilita l'utilizzo e le condizioni standardizzate di isolamento e crescita supportano la loro applicazione anche per screening di nuove molecole terapeutiche. Non ultimo, la loro origine mesenchimale e la loro pluripotenzialita' le candidano anche come sorgente per terapie cellulari.

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Introduction and Literature Review

1.1. Stem Cells

Stem cells are mother cells and are considered as the origin of all tissue cells; they are a class of undifferentiated cells with the ability of self-renewal, and division into multilineages with a specific function. The main types of stem cells are: embryonic stem cells (ESCs), adult stem cells and induced pluripotent stem cells (iPSCs). All types of stem cells are able to develop into various cell types; they can differentiate into cells of the blood, heart, bones, skin, muscles, brain, etc. Stem cells are recognized as valuable research tools and have been used in different clinical applications in a wide range of diseases (1).

1.2. Types of stem cells

1.2.1. Embryonic stem cells (ESCs)

Embryonic stem cells are also known as early stem cells, they derived from the early stage of human fetus development from tissues of four or five days old, in the phase of a blastocyst. The blastocyst is composed of two parts: the embryoblast which forms the inner cell mass and the trophoblast which is the outer cell mass. During the development, the trophoblast becomes part of the placenta, while the embryoblast becomes the source of embryonic stem cell which has the capacity to develop into any type of cells in the body. Since the discovery of human ESCs; many scientists are keen on using them for exploring new drugs, and discovering their potential in immunotherapy, and regenerative medicine. However, there are many considerations in using human ESCs due to ethical controversies and the difficulty in acquiring an appropriate quality of the oocytes (1).

1.2.2. Adult stem cells

Adult stem cells or somatic stem cells start to exist after the embryonic development and can be found in both children and adult. They can be obtained from different types of tissues such as the brain, bone marrow, blood, blood vessels, skeletal muscles, skin, and liver. The activation of adult stem cells in the human body takes place when there is a disease or tissue injury, these cells are divided into several cell types from the originating organ or even reform the entire original organ. They are not only differentiate into the cells of their tissue of origin, but there are some evidence provided their ability of transdifferentiation (1).

1.2.2.1. Mesenchymal stem cells (MSCs)

Definition

Mesenchymal stem cells (MSCs), or multipotent mesenchymal stromal cells, are type of adult stem cells characterized by being adherent to the plastic surface giving them the ability to perform colony forming unit fibroblasts-like cells and to proliferate extensively in vitro as well as in vivo. They called mesenchymal stem cells (MSC); because they are able to differentiate into variety of specialized mesoderm-type cells such as osteoblasts, chondrocytes, myocytes, adipocytes, they can also develop into neuronal cells and recently identified to develop into beta-pancreatic islet cells.

The first description of mesenchymal stem cells was given by Friedenstein in Russia who identified these cells from murine bone marrow, nearly half a century ago and then followed by Owen in the United Kingdom (1-3).

Sources

Mesenchymal stem cells can be isolated from different tissues that have an efficient population of cells and showing the characteristics of MSCs mainly from bone marrow, other sources are adipose tissue, umbilical cord blood, dental tissues, endometrium, limb bud, menstrual blood, peripheral blood, salivary gland, skin and foreskin, synovial fluid and Wharton's jelly.

Although there are many studies on native MSCs, they still remain poorly characterized. On the other hand, cultured MSCs can be characterized by several surface markers such as CD44, CD73, CD90, CD105, CD166, CD271, and Stro-1 together with the absence of hematopoietic surface markers CD14, CD34, CD45, and HLA-DR. However, there are no specific markers for their characterization (4-8).

Applications

Mesenchymal stem cells have been known as an outstanding subject in modern research era due to its distinctive biological features; which vary according to their tissue of origin, way of isolation and expansion in vitro and also due to its clinical applications. It has a therapeutic potential to repair various types of tissues through stem cell transplant, so it is an excellent candidate to be used in regenerative medicine. MSCs have an immune moduation effects and by producing soluble factors, they can induced both immunosuppressive and anti-inflammatory responses (9, 10).

These cells are able to migrate toward the injured tissue and the sites of tumor (11, 12). Many trials using MSCs are still ongoing in different conditions such as orthopedic injuries, graft versus host disease (GVHD) following bone marrow transplantation (BMT), cardiovascular diseases, autoimmune diseases, and liver diseases. Another prominent

application of MSCs is in the genetic modification by overexpressing antitumor genes, which can be used as anticancer therapy in clinical settings (13).

1.2.2.2. Induced pluripotent stem cells (iPSCs)

The discovery of induced pluripotent stem cells or iPS cells has become one of the most important breakthroughs over the last decade. iPSC technology has been a successful modeling tool for many diseases. The invention of this technology was achieved by Takahashi and Yamanaka in 2006. In their first experiment they succeeded to reprogramme murine fibroblast cells to a pluripotent stage, and then in the following year, they successfully applied this protocol to human fibroblast cells. Since the development of this technology, many laboratories have used iPSC as a disease model to understand the pathogenesis, disease mechanism and to discover new drug-screening platforms (14).

Induced pluripotent stem cells are adult stem cells from skin, liver, stomach or other mature cells reprogrammed by the introduction of four transcription factors: Oct3/4 (octamer-binding transcription factor 3/4), Sox2 (sex determining region Y), Klf4 (kruppel-like factor 4) and c-Myc (Avian Myelocytomatosis virus oncogene cellular homologue) that transform it into a cell with the same properties of the embryonic stem cells. These cells display ESC-like morphology. iPSCs express pluripotency markers, so they can differentiate into all cell types of the body except for cells in extra-embryonic tissues such as the placenta. Both cells share the same level of expression and epigenetic states and are able to develop three germ layers in vitro and in vivo (14-16).

There are variety of sources available for producing iPSC (Table 1.1). The procedures for collecting most of them are considered to be invasive, however iPSCs derived from urine have many advantages over all other sources as it is safe, simple, affordable, ubiquitous, and readily accepted by patients (17, 18).

Donor sources	Cell types	Obtained process
Fibroblasts	Facial dermal fibroblasts, periodontal ligament fibroblasts, and gingival fibroblasts	Invasive (biopsy)
Keratinocytes	Keratin-dense epithelial cells	Not strictly noninvasive
Melanoma cells	Skin melanocytes	Invasive (skin punch biopsies)
Adipose stem cells	White preadipocytes and adipose-derived mesenchymal stem cells	Invasive (liposuction)
Cord blood	Cord blood-derived stem cells and endothelial cells	Noninvasive
Peripheral blood	Mononuclear, T- , and myeloid cells	Minimal invasion (venipuncture)
Neural cells	Neural stem cells	Invasive
Astrocytes	Human astrocytes	Invasive
Hepatocytes	Primary human hepatocytes	Invasive
Amniocytes	Human amniotic fluid-derived cells	Invasive (amniocentesis)

Table 1.1 Different sources for generating human iPSC (18).

1.3. Urine-derived stem cells

Urine-derived stem cells (USCs) are a subpopulation of cells having the same biological features of mesenchymal stem cells. USCs can be isolated from human urine as well as animal urine (e.g. monkeys, pigs and rabbits) using simple, noninvasive and cost-effective methods.

USCs are characterized by expressing MSC/pericyte markers and some key cell surface markers such as CD44, CD73, CD90 (Thy-1), CD105 (endoglin), CD133, CD146, NG2, and PDGF-rβ. These cells lack the expression of general hematopoietic cell marker CD45, hematopoietic stem cell markers (except for MHC-1), endothelial cell markers (CD31 and CD34), or human leukocyte antigen (locus) DR (HLA-DR). The absence of these markers is important to prove that they are not derived from endothelial or hematopoietic progenitor cells.

Human USCs have many advantages with respect to other types of MSCs: It is easy to be collected regardless the age, gender, ethnic group, body type, or the health status of the donor (excluding those with urinary tract infection and anuria). Pure stem cells can be isolated without the enzymatic digestion.

These cells show telomerase activity which indicates their ability to generate more cells which are not teratomas or tumors (19, 20).

1.3.1. The origin of USCs

Urine sediment contains several morphologically different types of cells that may originate from the blood, epithelial cells lining the urinary tract, or they may be microorganisms such as bacteria or yeast.

Epithelial cells lining the urinary tract that usually present in the urine include podocytes, renal tubular epithelial cells, urothelial cells from urinary tract lumen, and stratified squamous epithelium cells from the distal urethra. The majority of these cells are somatic cells so they are not able to grow in vitro. Few renal cells like podocytes and renal tubular epithelial cells are able to grow in vitro but for limited passages. The expression of the telomerase activity by the USCs is responsible for their proliferation for up to 16 passages.

The origin of USCs is considered to be from renal tissue, this idea is supported by many evidence including USC clones obtained from gender-mismatched kidney transplant patients demonstrated X/Y chromosome characteristics, this proves that USCs were from the upper urinary system. It has been identified that USCs expressed genes for kidney-related markers such as PAX8, NR3C2, and L1CAM by different techniques such as Real-time PCR, Western blots, and immunocytochemical staining. It has been found that there are no differences between USCs derived from fresh human urine obtained from the upper

urinary tract and voided USCs in terms of morphology, cell phenotypes, growth pattern, and differentiation capacity indicating that the voided USCs originate from the upper urinary tract (20-22).

1.3.2. Applications of USCs

Urine stem cells (USCs) possess the capability to differentiate into multipotential cell lineages such as osteogenic, chondrogenic, adipogenic, myogenic, neurogenic and endothelial cell types. Following in vivo implantation, induced USCs can form functional endothelium, urothelium tissue, podocytes, smooth and skeletal muscles, bone, cartilage and fat tissue.

Recently, USCs have been identified as a highly competent strategy for different applications especially in cell-based therapies and tissue engineering. They are mainly involved in replacing the injured, diseased and aged cells. They have a profound impact on the restoration of the function and histological structure of the tissue, or organ of origin such as urogenital reconstruction (20, 22-24).

iPSCs generated from USCs receive a great deal of attention, they have many advantages in respect to other iPSCs sources and they have opened new avenues for studying diseases especially rare human diseases (Fig 1.1). They offer a remarkable cellular transplantation therapeutic strategy. Both USCs and iPSCs derived from USCs play a vital role in exploring and a better understanding of the molecular and cellular mechanisms underlying disease progression. Moreover, they are substantially contributed to study the potential of different pharmacological and genetic therapeutic approaches (18).

The first attempts to isolate USCs were done in 1972 (25), subsequently many researchers repeated these attempts (26, 27). On the other hand, the first generation of iPSCs from urine-derived stem cells was achieved by Zhou *et al* in 2012 (28).

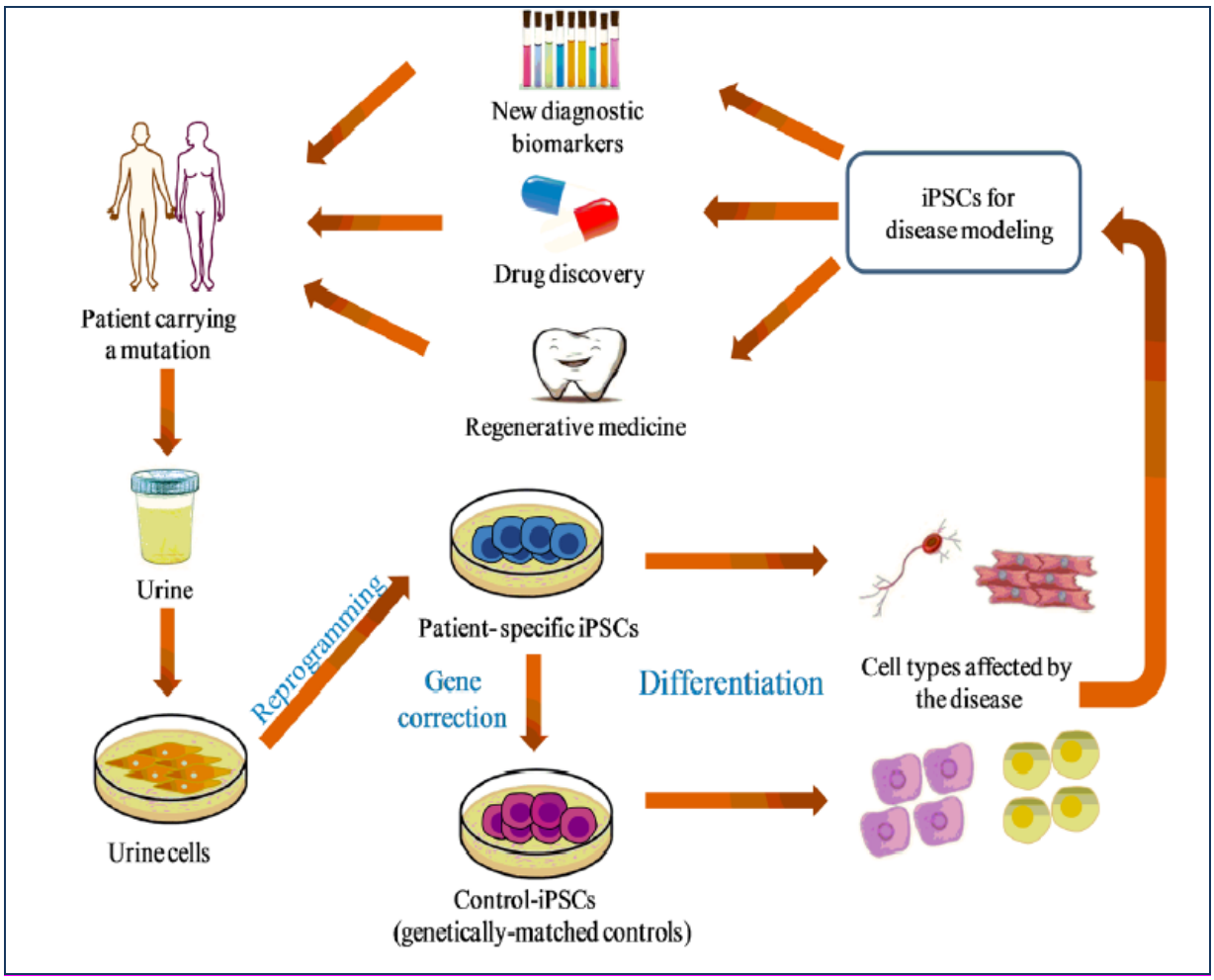


Figure 1.1 A diagram showing iPSCs derived from USCs representing a disease model with various applications in studying rare human diseases. Cells are isolated from patient urine samples and reprogrammed into iPSCs. Isogenic controls are created with gene-editing tools and then differentiated into diseased cells that can recreate crucial aspects of the disease *in vitro*. Patient-specific disease models can be used to identify new diagnostic biomarkers and to screen effective and novel drugs as well as to replace cells or tissues in regenerative medicine (18).

The isolation of stem cells from urine specimens and their clinical applications have been reported in many recent studies. However, only a few studies examine their applicability for investigating muscular dystrophy.

Guan and his colleagues reported that iPSC isolated from urine specimen of Duchenne muscular dystrophy (DMD) patient were easily generated and well differentiated into beating cardiomyocytes and recapitulate the DMD phenotype (29).

It has been reported that USCs provide advantages for cell therapy and tissue engineering applications in bladder regeneration as they originate from the urinary tract system (30).

Many studies presented the importance of USCs as they have shown compatibility with bone tissue engineering (31), they could be applied for gene therapeutic approaches to improve medical conditions of patients with epidermolysis bullosa (EB) (32), and they

provide unique features and advantages for tissue repair in the genitourinary system (33, 34).

Jouni and collaborators verified that iPS cells derived from human urine stem cells could be used as an in vitro human cell disease model for studying a patient with long QT syndrome, a cardiac arrhythmic disease and recapitulate cardiac arrhythmia phenotypes (35).

It has been reported that urine-derived induced pluripotent stem cells are a feasible modeling tool to investigate the pathogenic mechanisms of paroxysmal kinesigenic dyskinesia PKD (36).

Kim *et al* demonstrated that direct reprogramming of urine-derived cells could be an effective and reproducible procedure for generating human myogenic cells (37).

It has been also reported that urine-derived human induced pluripotent stem cells are a useful model to study PCSK9-mediated autosomal dominant hypercholesterolemia (38).

It has been demonstrated that the generation of systemic lupus erythematosus-specific induced pluripotent stem cells from urine sample was feasible and efficient and could provide convenient model to study disease pathogenesis, drugs screening, and gene therapy (39).

It has been shown that the multipotential differentiation of human urine-derived stem cells represent the USCs as one of the most promising cell sources for cell therapies in the treatment of different urologic disorders such as urinary incontinence, renal insufficiency, erectile dysfunction, vesicoureteral reflux and for regeneration of bladder and urethral tissue (20).

1.4. Hereditary neuromuscular diseases

Hereditary neuromuscular disease is a general term that described diseases of a series of functional entities called motor units. Each motor unit composed of a motor neuron that located either in the anterior horn of the spinal cord or in a number of motor nuclei of the brain stem. The axons of these motor units travel through a peripheral nerve toward a particular muscle in which several muscle cells are contacted by means of neuromuscular junctions.

Neuromuscular diseases are named according to the specific site of the lesion within the motor unit. When the lesion occurs in the motor neurons it is called spinal muscular atrophies (SMA). Diseases of the axons are known as peripheral neuropathies. If it affects the muscles, then the disease is called a primary myopathy. Muscular dystrophies

are the most common form of myopathies. Hereditary neuromuscular disorders have a relatively high incidence, especially among children. Its classification is a rather complex issue due to the variation in many factors such as the pattern of inheritance, the genetic mutation, incidence, symptoms, age of onset, the rate of progression, and prognosis. The main classification of neuromuscular disorders includes the following conditions: Muscular dystrophies, Myotonic disorders, Congenital myopathy, Metabolic myopathies, Inherited auto-immune myositides, Spinal muscular atrophies and Hereditary motor and sensory peripheral neuropathy (40).

A list of neuromuscular diseases in which the causative gene is identified can be found on the website of the Neuromuscular Disorder Journal, organ of the World Muscle Society (WMS), in the dedicated link www.musclegenetable.fr/.

1.5. Muscular Dystrophy

Muscular dystrophy (MD) is a group of genetic noninflammatory disorders, characterized by progressive degeneration and weakness of the voluntary skeletal muscles which is responsible for the body movement. In some forms of MD, the cardiac muscles and other organs are also affected. MD occurs due to mutations in the genes encoding specific muscle proteins, or proteins networking on the muscle environment and functions. These disorders differ in terms of age which also determined the severity of the disease. Some can appear in infancy or childhood while others may not appear until middle age or later. Some types of these inherited disorders affect only males; some have mild symptoms that progress very slowly while others develop severe muscle weakness leading to death in the late teens to the early 20s. The term of dystrophy is due to the profound reshaping of the muscle structure that undergoes to a massive muscle loss, followed by adipose tissue and then connective tissue replacement. This is the typical morphological picture observed in the muscle biopsy.

Conte and Gioja in 1836 reported the first description of MD as they described cases of two brothers at age 10 years with progressive weakness of muscles. These two cases later developed general muscle weakness and hypertrophy in various types of muscles, which is now recognized as the main characteristics of milder Becker MD. At that time their discovery did not achieve recognition since many thought that it was a case of tuberculosis (41).

Later, in 1852 Meryon reported that a family with four boys were affected by significant muscle changes without central nervous system abnormality. He gave the first description of MD and its transmission (41).

Guillaume Duchenne was a French neurologist, in 1868, he gave a comprehensive account of the most common and severe form of the disease and he called it "paralysie musculaire pseudo-hypertrophique". Due to his contributions to understanding muscle diseases Duchenne MD, now bears his name (41).

Muscular dystrophy has been classified according to three main factors: the clinical phenotype, the pathology, and the mode of inheritance. Based on the mode of inheritance they classified into; sex-linked MDs which include Duchenne, Becker, Emery-Dreifuss, autosomal recessive MD including Limb-girdle form, and autosomal dominant MDs that include: Facioscapulohumeral, distal, ocular, oculopharyngeal (41).

1.6. Duchenne muscular dystrophy (DMD)

Duchenne muscular dystrophy (DMD) is a fatal type of muscular dystrophy. This rare genetic disorder is the most common muscle disease of childhood, affecting about 1 out of every 3,600 newborn male.Very rarely it may affect girls (approximately 1 in 50 million girls) and it occurs across all races and cultures. It may occur due to new genetic changes in the child without a known family history of DMD, however, the risk is increased with a family history of Duchenne muscular dystrophy.

DMD is an X-linked recessive inherited neuromuscular disorder, occurs due to a variety of dystrophin gene mutations, the gene encoding the dystrophin protein which is responsible for the stability of the cell membrane in skeletal muscle cells. This mutation causes a defect in the dystrophin protein and lead to the loss of functional dystrophin which results in progressive muscle degeneration and dysfunction (42-45).

1.6.1. Dystrophin: Gene and protein

Dystrophin is the largest known human gene measuring about 2.4 Mb. This gene is localized on the short arm of the X chromosome on locus Xp21 and it is composed of 79 exons. It encodes a 14-kb mRNA which is mainly expressed in skeletal and cardiac muscle with the presence of small amount in the nerve cells in the brain. It is very complicated as it contains three independently regulated promoters that represent the major sites of dystrophin expression: the brain (B), muscle (M), and Purkinje (P) promoters, giving rise to the full length dystrophin isoforms (Dp427 b-m-p, respectively). It also has at least four internal promoters that splice into exons 30, 45, 56, and 63 and give rise to shorter

dystrophin transcripts which are known as Dp260 retinal (R), Dp140 brain-3 (B3), Dp116 Schwann cell (S), and Dp71general (G) promoters respectively.

The differential splicing of dystrophin RNA produced various transcripts, which encode a large number of protein isoforms. Dystrophin protein is 427-kDa, this large rod-shaped cytoskeletal protein is a member of the β-spectrin/α-actinin protein family. Dystrophin is organized into four separate domains based on sequence homologies and protein-binding capabilities. These domains are the actin-binding domain at the NH2 terminus and it contains between 232 and 240 amino-acid residues depending on the isoform. The central rod domain is composed of 25 repeating units that are similar to the triple helical repeats of spectrin and contains about 3000 residues. The cysteine-rich domain consists of 280 residues and the last carboxy COOH-terminal domain consists of 420 residues.

Dystrophin is a sarcolemma-associated protein found at the inner surface of muscle fibers which connects subsarcolemmal cytoskeleton to the sarcolemma by binding cytoskeletal F-actin through its N-terminal domain and the dystrophin-glycoprotein complex (DGC) through its C-terminal domain. It connects each muscle cell's structural framework (cytoskeleton) with a protein complex and other molecules outside the cell (extracellular matrix) exerting a bridging function between sarcomer and sarcolemma protein complexes (Fig 1.2).

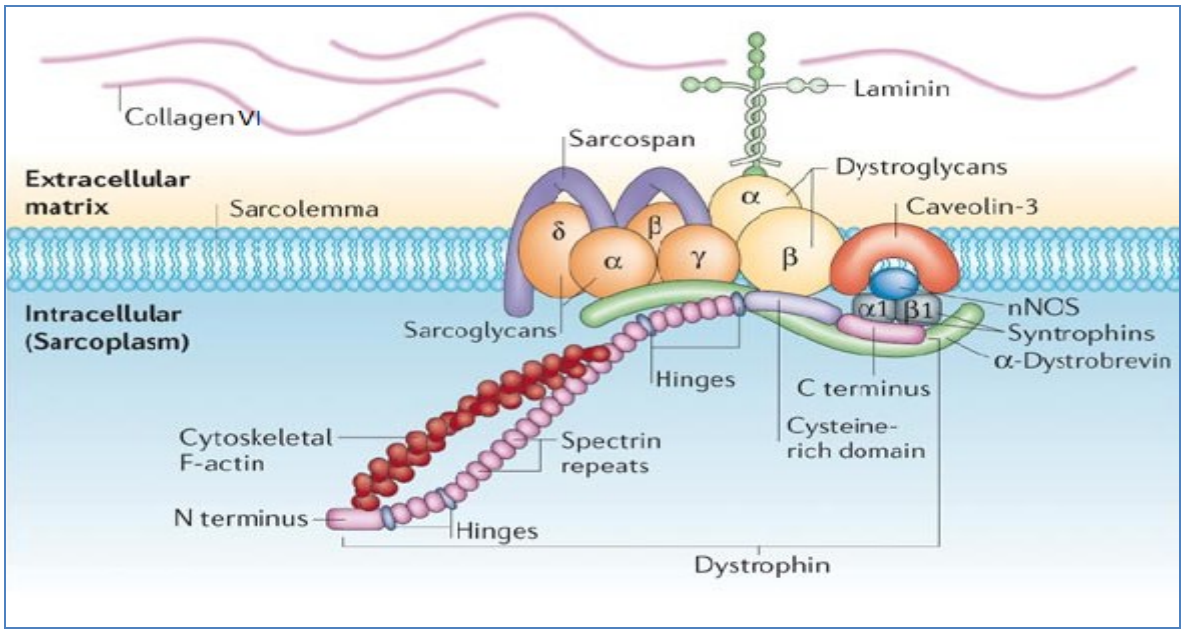


Figure 1.2 Schematic diagram showing the relation between the cytoskeletal protein dystrophin, the dystrophin-associated protein complex in the membrane and the extracellular matrix in normal muscle (46).

The main function of dystrophin together with the protein complex is to strengthen the muscle fibers and stabilizes them during muscles contract and relax. In addition to its mechanical function dystrophin-glycoprotein complex suggested to have a role in cell signaling acting as a transmembrane signaling complex.

Beside the full-length dystrophin, the four internal promoters give rise to shorter dystrophin proteins that lack the actin-binding terminus, while retaining the cysteine rich and carboxy-terminus domains that contain the binding sites for dystroglycan, dystrobrevin, and syntrophin.

They generate protein products of 260 kDa (Dp260), 140 kDa (Dp140), 116 kDa (Dp116), and 71 kDa (Dp71). These isoforms are commonly alternatively spliced and they are thought to be involved in the stabilization and function of nonmuscle dystrophin-like protein complexes (42, 47-53).

1.6.2. Mutations in the dystrophin gene

Mutations occur in dystrophin gene can cause DMD disease or Becker muscular dystrophy (BMD). About 65% of dystrophin mutations are intragenic deletions, while the frequency of duplications is about 10%. The other 25% is due to small mutations (nonsense or frame-shifting mutations), or other smaller rearrangements, and rare atypical mutations may occur as less than 1% (54).

Deletions and duplications in dystrophin gene can occur almost anywhere in the gene, although there are two deletion hotspots identified. The first one is located in the region of exons 2–19 with genomic breakpoints commonly found in introns 2 and 7, while the other one is at the 3′ end often involving exons 44-48 and neighboring regions. The huge intron 44 is a very frequent site of breakpoints (54, 55).

It has been known that there is no relation between the size and the position of the deletion within the DMD gene and the clinical phenotype observed. This means when the deletion leads to the expression of an internally truncated transcript that maintains the normal open reading frame, the result will be abnormal, smaller but functional version of dystrophin product. This pattern is consistent with a BMD phenotype. On the other hand, if the deletions and duplications disrupt the reading frame (frame-shift) translational, then many premature terminations of translation will lead to the production of a shorten protein. This results into extremely low levels/absence of dystrophin expression due to mRNA or protein instability and is associated with DMD phenotype.

In some cases like deletions in exons 32-44, 48-51 and 48-53, the dystrophin might be normal or near normal. This is due to the fact that the central and the distal rod-domains

are likely to be functionally dispensable and deletions in these domains have been associated with some signs like cramps, hyperCkemia, and myalgia but not with weakness at least in the childhood. In general, when a large part of rod domain is deleted without affecting the C and N-termini, this result in BMD phenotype (56, 57).

There are exceptions to the reading frame rule such as in patients with BMD who carry out of frame deletions; and this can be explained either by exon skipping event due to an alternative splicing, or due to the presence of an alternative translation initiation beginning in DMD exon 6 (58-60).

Finally, mutations in the DMD gene can cause X-linked cardiomyopathy that leads to the elimination of the cardiac gene expression of dystrophin. This case is associated with ventricular wall dysfunction, dilated cardiomyopathy, and cardiac failure in the absence of skeletal myopathy. Mutations affecting the transcription of the muscle-specific dystrophin promoter (Dp427m) selectively abolish the expression of the protein in the heart (and not in the skeletal muscle where the brain isoform (Dp427b) can be upregulated if the muscle isoform is not present) (54, 58, 59, 61, 62).

1.6.3. Signs and symptoms of DMD

One-third of DMD boys do not have a family history of the disease, because the involved gene might be a site of de novo mutations. A high index of suspicion is in the family history when a sibling has been previously affected. The disease affects only boys, however, girls are usually carriers and very rarely mildly affected. At birth, there are no signs of abnormality noted in the patient and the first clinical manifestations of the muscle weakness do not begin until the child begins to walk. Children with DMD have a delay in ambulation; usually, do not begin to walk until about age 18 months or later (63).

The main sign of muscular dystrophy is progressive muscle weakness. The signs and symptoms of DMD typically start to present between the ages of 2 and 3 years, and may include: frequent falls, trouble running and jumping, Gower's sign; difficulty getting up from a lying or sitting position, waddling gait, walking on the toes, large calf muscles, muscle pain and stiffness and mental deficits. While still ambulatory, the child may have minimal deformities. However, a trained pediatrician can observe the failure to thrive in DMD boys, and then can diagnose the disease quite earlier, even if the child is 12 months old.

Loss of ambulation is the second important phase in DMD patients in which there is usually a rapidly progressive course of muscle or tendon contractures and scoliosis. This phase usually appears at the ages of 7 and 13 years, in some cases they become wheelchair-bound by age 6 years. In this phase, the muscle weakness may lead to

respiratory failure. The third phase of DMD is mainly characterized by a respiratory infection that progresses extremely rapidly despite its initial benign course. The main cause of death is usually due to respiratory failure as a result of underlying progressive nocturnal hypoventilation and hypoxia or from an acute cardiac insufficiency (64).

Becker muscular dystrophy is a milder form of DMD and it has the same signs and symptoms, but are milder and slowly progressed as patients have some functioning dystrophin. Symptoms usually start to appear in the teens, however it may not occur until the mid-20s or even later.

1.6.4. Diagnosis of DMD

1.6.4.1. Clinical diagnosis

An early diagnosis of muscular dystrophy is considered as the main step for the follow up of child health status. Diagnosis of Duchenne muscular dystrophy is done by several ways. It is started with the clinical diagnosis by taking a patient and family history, and performing a physical examination. The suspicion of DMD is increased if the family has a history of Duchenne muscular dystrophy or a woman who carries a mutation in the dystrophin gene. Delay in treatment makes the DMD boys become wheelchair dependent before age 13 years. The clinical exam checks the child's muscles and development. Muscular involvement is usually assessed by dynamic tests such as Gower's manoeuvre, time to get up from the floor and to climb 4 standard steps in seconds. Since 2010 both the North Star Ambulatory Assessment (NSAA) and the 6-minute walk test (6MWT) have been widely used, providing information on different aspects of the motor function of ambulant boys affected by DMD (65, 66).

1.6.4.2. Serum CK test

Serum creatine kinase (CK) determination may represent an important preliminary test in all types of muscular dystrophy. Muscle cells in the body need CK to function so that the progressive degeneration of the muscles released this enzyme into the blood. Serum creatine kinase is greatly elevated during the course of the disease in DMD patient (50-200 times higher than normal) and it reaches a peak between 3 and 5 years old. However, it decreased with increasing age in children with DMD. This test could reflect the rate of muscle decay due to the characteristic changes of serum creatine kinase. It may also discriminate if the patient will develop a severe or milder phenotype (67, 68).

1.6.4.3. Genetic Testing and Genetic Counseling

Confirmation of the diagnosis of DMD requires a genetic testing in order to identify the type of mutation that causes the disease. The results can also provide clinical

information required for genetic counseling, prenatal diagnosis, and consideration for future mutation-specific therapies. On the other hand, genetic counseling can offer many benefits for families living with Duchenne or Becker muscular dystrophy. It can provide information about the cause, the typical symptoms and course of the disorder, and can provide them with the available diagnostic and genetic testing options.

If the family has a history of DMD with a known mutation, then the genetic counselor will select a test method that will detect this type of mutation. If it is not known, there are many different types of genetic tests to be used.

The minimum level of genetic diagnostic testing is the quantitative analysis of DMD genes identifying the most common types of mutations which are exonic deletions or duplications, followed by qualitative approach represented by full gene sequencing (42, 69, 70).

Although multiplex PCR is considered to be the basic method that is widely available and the least expensive one, this approch can not detect duplications and does not cover the whole gene and it is not suitable for screening females carriers (71, 72).

Therefore, the quantitative method to test all exons of the DMD gene is multiplex ligation-dependent probe amplification (MLPA). This method detects deletions and duplications and can be used for detecting the mutations in female carriers. Any mutations identified by using this technique need to be confirmed by an alternative method, due to the possibility of a single nucleotide polymorphism (SNP) under a probe or primer binding site (71).

A more recent quantitative analysis of DMD gene is the oligonucleotide-based array comparative genomic hybridization (array-CGH). This method identifies the copy number variation (CNV) in the entire genomic region of the DMD gene and allows the identification of intronic and 3′ and 5′ flanking regions so that complex rearrangements and intronic alterations, as well as the exact mutation break-points, are all detected. False positive results that may occur due to SNPs are reduced in CGH arrays using a high density of probes (42, 73, 74).

The quantitative analysis that results in the identification and full characterization of a dystrophin mutation does not require any further investigation. On the other hand, if the deletion or the duplication is not detected, then a qualitative analysis is required. Full sequence analysis of the entire coding region of DMD gene on genomic DNA could detect small mutations (small deletion or insertion, single base change, and splicing mutation). The variety of technologies available for genome sequencing includes Sanger Gene Sequencing which is widely used, and Next generation sequencing that based on

sequencing millions of copies of the DNA fragments simultaneously, increasing DNA sequencing output and reducing the time and cost necessary to fulfill the genetic diagnosis, however, it requires a high level of laboratory automation. Sequencing of genomic DNA may not detect 1% of atypical mutations that result from complex rearrangements or deep intronic changes. Therefore, these rare types of mutations require the RNA analysis of muscle tissue using RT-PCR and sequencing (42, 73, 74).

Fluidic card is a new, accurate, rapid and easy-to-use platform based on TaqMan Real-Time systems. This diagnostic approach is designated to detect DMD exon junctions from RNA extracted from a variety of patients' tissues. This novel technology can define the full-exon composition, identify changes in mRNA decay, and profile the dystrophin isoforms present. By using this technology all deletions/duplications and splicing affecting mutations can easily be detected (42, 75).

Muscle biopsy is also considered as a necessary diagnostic step when the mutation of DMD gene is not detected in the blood samples by genetic diagnosis. Muscle biopsy can be obtained by single-core needle biopsy providing a small sample, however, large samples can be obtained using the conchotome technique without an open surgical procedure. Dystrophin protein is examined on the muscle biopsy by immunocytochemistry and immunoblotting, providing information about the presence or absence of this protein, its amount, and molecular size. It can distinguish the phenotype whether it is DMD or BMD. Positive biopsy diagnosis of DMD should always be followed by genetic testing (69).

Full characterization of the DMD gene mutation is important to correlate the predicted effect of the mutation on the reading frame of the gene, which is the major determinant of the phenotypic variability seen in dystrophinopathy. It helps to understand the pathways and mechanisms that regulate expression as well as to determine eligibility for the mutation-specific treatments currently in trials (69).

1.6.5. Therapeutic Interventions for DMD

There is no curative treatment available for DMD. However, the currently available treatment aimed at prevention and management of the disease complications, by slowing the progression of the illness and improving muscles strength and function.

Many studies in animal models have been carried out to develop a possible therapy to reduce both primary and secondary pathologic effects of DMD using mdx mouse. This animal model of DMD has spontaneous point mutation in exon 23 that result in the absence of the dystrophin protein in the muscle. Unlike the human DMD, this model present very mild symptoms until old age, probably due to the presence of utrophin, however it shows DMD skeletal muscle phenotype with a gradual loss of in force/power

with age, high creatine kinase (CK) levels, muscle necrosis and problems in the respiratory system as a result of the degeneration of diaphragm (42, 69, 76).

1.6.5.1. Corticosteroids

Glucocorticoids are the main drugs (off label) treatment currently available for DMD. Since it was suggested in 1974, many clinical trials have been carried out to define the optimal dose, the age of initiation and frequency (77). Over two decades, Glucocorticoids have been used in DMD treatment showing an improvement in muscle strength, although they are associated with several side effects. Prednisone/Prednisolone and Deflazacort, an oxazoline derivative of prednisolone, are the main Corticosteroids that extended the period of ambulation and enhanced their functionality in daily activities.

These drugs can be administrated daily or intermittent, however, the daily use of these drugs has been proved to be more effective than alternative regimens. There are three regimens of Glucocorticoids commonly used; 0.75 mg/kg/day is the indicated dose of prednisone, a higher dose of 1.5 mg/kg/day and a lower dose of 0.3 mg/kg/day are less effective. The other two regimens are 0.9 mg/kg/day deflazacort, and an intermittent administration of 0.75 mg/kg/day prednisone for 10 days on and 10 days off. Both prednisone and deflazacort are effective in treating DMD in the short-term trials (six months to two years), slowing the decline in motor function and improving the muscle strength, without considerable clinical side effects. Moreover, many studies in the long-term treatment trials that extended over 3 years with prednisone or deflazacort reported that patients showed a significant improvement in ambulation and cardiac function with a reduction in the risk of progressive scoliosis and respiratory dysfunction leading to an increase in life expectancy. Steroids therapy has a side effect that should be considered and managed as well. The main side effects of chronic glucocorticoid therapy are a reduction in the patient's height and weight gain. Weight gain is less common when treated with Deflazacort, but it causes more cataracts than prednisone.

Other side effects that should be monitored and are less frequent if the indicated doses have been used include vertebral fractures, cushingoid facies, arterial hypertension, delayed puberty, acne, hirsutism, behavior disorder, immunosuppression and gastrointestinal problems. Initiation of glucocorticoid treatment is an individual decision, based on functional state and also considering age and pre-existing risk factors for adverse side-effects. Initiation of glucocorticoid is recommended in the plateau phase at age 4–6 years, and it is not recommended for a child under 2 years due to the progression in motor skills. Glucocorticoid treatment in non-ambulatory patients could preserve the function of

the upper limb, reduce the risk of scoliosis and slow impairment of respiratory and cardiac function (42, 76).

1.6.5.2. Stem cell-based therapies

In the last few years stem cells therapy has been considered as one of the most promising approaches for the treatment of various human diseases including muscular dystrophy. Stem cells are self-renewal and are able to differentiate into myogenic cells that replace dystrophin and lead to muscle regeneration.

There are two methods used for the treatment of DMD patients using stem cell therapy. The first method is done by transplantation of stem cells isolated from DMD patients and genetically altered in vitro to restore dystrophin expression. The second method is by transferring allogenic stem cells derived from an individual with functional dystrophin to DMD patients.

Many studies reported that transplantation of myoblast-derived from healthy donors is not a successful treatment for DMD patients as no functional or clinical improvement has been achieved. The limitation of this method is due to the limited survival rate of the transplanted cells, the impossibility of migration via circulation and its rejection by the hosts' immune system.

Stem cell populations with myogenic potential, Mesoangioblasts (MABs) are a promising treatment for DMD. They have the advantage of distributing across the blood vessel, and efficiently ameliorate the dystrophic phenotype. Many clinical trials are still ongoing on the efficiency and effectiveness of intra-arterial mesoangioblasts transplantation (42, 78, 79).

1.6.5.3 Gene Therapy

Another promising therapeutic approach for DMD is the gene therapy. Gene therapy is based on the replacement of the defective gene with an effective alternative gene that supports cell function or repair gene mutations. The aim of gene therapy in DMD patients is to deliver a functional copy of the dystrophin gene to all muscle cells in order to restore the expression of dystrophin.

There are many viral vectors used for DMD gene delivery. Adeno-associated virus (AAV) is considered as the most effective therapeutic gene transfer to a wide variety of cells and tissues including muscles. Retroviruses and lentiviruses are other viral vectors available for gene transfer. The selection of the viral vector depends on the target cell, immunogenicity, and required a duration of transgene expression. There are some challenges facing DMD gene therapy; this includes the limited capacity of the viral vector

AAV (~4.7 kb) to fit the large size of dystrophin cDNA (13 kb of sequence). To address this challenge transplicing technology has been used to produce mini or micro- dystrophin gene that can fit the packaging capacity of the viral vector. Another challenge is the potential for an immune response triggers by transgene expression, or from a previous exposure to the adeno-associated virus which may lead to the rejection. Some studies on mdx mice reported that mini- or micro-dystrophin transferred by rAAV6 and rAAV9 induced dystrophin expression but with triggering host immune response. The development of rAAV2/8, a chimeric vector restore the production of the dystrophin protein without immunological response. Further studies are still carrying out to define the immunogenicity of AAV serotypes and transgenes and the potential need for immunosuppression (42, 79-81).

1.6.5.4. Exon Skipping

Exon Skipping is a new splicing therapeutic approach that has been discovered over 20 years ago. The aim of this therapy is to manipulate the pre-mRNA splicing in order to reconstruct the disrupted reading frame by using a splicing component that induced the skipping of the mutated exon or intron. A number of genetic disorders concentrated on using exon skipping as a possible therapeutic approach such as thalassemia and DMD. Exon skipping for the treatment of DMD based on reforming the pre-mRNA splicing motif to transform the frame-shifts mutation into an in-frame mutation, thus changing DMD phenotype into BMD-like phenotype. This goal can be accomplished by using antisense oligonucleotides (AONs) with 20–30 nucleotides in length, designated to bind an intronic and exonic mRNA sites where the mutation occurred and skipped their translation by the splicing machinery and producing truncated but functional dystrophin protein.

The two main antisense oligonucleotides that are currently tested in clinical trials and have different chemical structures are Phosphorothioate oligonucleotides (PS) or 2′O-methyl-phosphorothioate oligonucleotide (2′OMePS) also known as Drisapersen and is developed by Prosensa Holding N.V., and morpholino phosphorodiamidate oligomers (PMO), named eteplirsen developed by Sarepta Therapeutics, Inc (42, 79, 82, 83).

The majority of DMD mutations (90%) localized in the hot spot region (comprising exon 45 to exon 53) can be treated by exon skipping, including the most common mutation in exons 49–50, 48–50, 47–50, 45–50 and a deletion of exon 52 that lead to frameshifts and absence of dystrophin (82, 83).

However, it is not suitable for the less common mutation (10%) such as mutations located between exon 64 and exon 70, which are crucial for protein function, when the deletions

revoke all actin-binding sites in the N-terminal region or affect the first or the last exon, and large chromosomal rearrangements such as translocations.

Exon skipping could be directed towards a single exon or double exon according to the type of mutation which sometimes requires skipping of two different exons to restore the reading frame. It has been reported that the efficiency of the double exon skipping is slightly lower than single exon skipping (84).

Many clinical trials are evaluating antisense oligonucleotides (AONs) that target exon 51. This AONs induced the skipping of exon 51, and restore the reading frame with the production of shorter but functional dystrophin (42).

The outcome of using exon skipping therapy is promising and need further research, to design specific oligonucleotide drug for other types of deletions.

1.6.5.5 Stop-Codon Read-Through: Mutation Suppression

Stop-Codon Read-Through or Mutation Suppression is another therapeutic means of treatment for nonsense DMD mutation that estimated to be around 15% of the total DMD mutations. The nonsense mutation result in the formation of a premature termination codon due to the fact that an amino acid encoding sense codon is elucidated as a stop codon, therefore the outcome is a truncated non-functional dystrophin. There are many molecules that significantly support the "read-through" of these premature stop codons and recognize them as a sense codons. Thus, lead to the remodeling of mRNA transcript and reconstruct dystrophin function. The available molecules currently used in clinical trials for Stop-Codon Read-Through therapy are suppressor tRNAs, Aminoglycosides, and Ataluren (PTC124).

Some studies carried out in mdx mice treated with the suppressor tRNA gene showed an increased in dystrophin expression, however, this treatment is not so far a good option as it has some limitation like the rejection by the host immune system and its toxic effect.

Aminoglycosides such as gentamicin are another compounds used for stop codons read through, and the effect of gentamicin has been studied in mdx muscles showing that partial dystrophin function was restored. The main limitation of this treatment is the requirement of a high dose of gentamicin which considered to have side effects such as toxicity to kidney and ototoxicity.

Ataluren or Tranlsarna, previously known as PTC124 is an oral administrated drug and now considered as the best option for stop codons read through. It shows a great benefit in mdx mice and it has a successful improvement in restoring the expression of dystrophin when applied to DMD patients. Ataluren or Tranlsarna get the approval of

being a treatment for ambulatory DMD patients with nonsense mutations at age 5 years and above (42, 79, 80).

1.6.5.6. Utrophin Modulation

Utrophin Modulation is another therapeutic strategy for the treatment of all DMD patients regardless the type of dystrophin mutations. Utrophin and dystrophin share the same functional and structural characteristics. Utrophin has slightly less molecular weight (395 kDa) and localized at the sarcolemma in utero. Utrophin production is started during the development of the muscle fiber until it becomes mature then it is stopped and progressively subrogate by dystrophin. It is also produced in order to repair the damaged muscle fiber, so that in the case of DMD in both human and mdx mice it is spontaneously elevated as a result of dystrophin absence to keep the proper functioning of the muscles.

An orally administered drug called SMT C1100 or Ezutromid is a small utrophin modulator molecule developed by Tinsley and collaborators and it has been proved to be an effective alternate to dystrophin when tested in human and in mdx mice as well. It promotes the expression of mRNA and increases utrophin level which modulates the phenotype of DMD and improves the muscle function (42, 85, 86).

Further studies are still ongoing to evaluate the safety and tolerability of high doses of SMT C1100 in DMD patients.

1.7. Collagen VI-related myopathy

Collagen VI-related myopathy, is a form of muscular dystrophy contains several types of rare inherited genetic disorders with varying degree of severity. The mildest form is Bethlem myopathy, while the most severe one is Ullrich congenital muscular dystrophy (UCMD). The intermediate forms of these disorders are moderate in severity including autosomal dominant limb-girdle muscular dystrophy and autosomal recessive myosclerosis myopathy.

Collagens represent an important constituent of the extracellular matrix (ECM) of the skeletal muscles. Collagen type VI (ColVI) is a member of the collagen family and it is mainly found in the extracellular matrix (ECM) of the skeletal muscles and the major cell type responsible for its deposition is the interstitial fibroblasts. ColVI is also abundantly expressed by skin fibroblasts. Although myogenic cells contain factors that can control ColVI secretion, they do not express it. ColVI plays an important role in maintaining muscles' strength and stability, as it is able to bind to ECM components, bridging cells to the surrounding connective tissue and organizing the tissue architecture of skeletal muscles, tendons, bone, and cartilage (87).

It has characteristic molecular properties with three different ColVI peptide chains called α1(VI), α2(VI) and α3(VI), that encoded COL6A1, COL6A2, and COL6A3 genes respectively in human.

Mutations in the genes encoding ColVI chains are responsible for causing different forms of Collagen VI-related myopathy. These mutations lead to the reduction or abnormal production of type VI collagen. The lower amount of ColVI is associated with severe signs and symptoms in early life. These disorders are generally characterized by muscle weakness and joint deformities.

The manner of inheritance of Collagen VI-related myopathy is variable. It can be an autosomal dominant pattern such as in Bethlemmyopathy or autosomal recessive manner like in Ullrich congenital muscular dystrophy.

Diagnosis of Collagen VI-related myopathy is based on the clinical features, high level of serum creatine kinase concentration, muscle or skin biopsy for collagen VI immunolabeling and the molecular diagnostic tests for the mutation in the COL6A1, COL6A2, and COL6A3. Primary skin fibroblast cultures derived from skin biopsy are widely used to study ColVI mutations in Bethlem myopathy and UCMD (88, 89).

1.8. Urine derived stem cells (USCs) and Duchenne muscular dystrophy (DMD)

Hereditary neuromuscular diseases are composed of a wide variety of genetic defects, which require a proper disease model for understanding their pathological mechanism and exploring new therpeutical strategy to cure them.

Different animal models are available for muscle diseases providing a valid in vivo modeling tool, however the major disadvantages of using animal models are being expensive, time consuming and presenting limited number of alleles involved in muscle diseases.

Recently human cell model and especially induced pluripotent stem cells (iPSCs) have been widely used as an appropriate modeling tool for many disorders. iPSCs have been successfully reprogrammed to produce muscle-like lineages, but the process for generating these type of cells is considered to be time consuming with a different consequence among culture systems.

Human myoblast cell lines provide a useful model for normal skeletal muscles when they differentiated into multinucleate myotubes, however obtaining disease-specific myoblast from patients requires human muscle biopsy which is considered to be invasive and sometimes harmful for patient with limited muscle mass.

Direct programming of fibroblasts is still considered as the most useful in vitro skeletal muscle model, however the procedure for obtaining dermal fibroblasts from skin biopsy is invasive with limited access from children.

Using a noninvasive patient-specific in vitro model for muscle diseases that can be accessed easily especially from pediatric populations is highly required.

Urine specimen has become a valuable source for isolating urine stem cells which have been considered as a novel, noninvasive and powerful modeling tools for human-specific diseases and for generating pluripotent patient-specific cell lines.

In this study, we investigate the applicability of urine-derived stem cells as a disease model for DMD by studying the dystrophin gene expression. We also evaluate the potential of USCs as a noninvasive diagnostic tool to substitute the muscle biopsy, which is often required from DMD patient. Finally, we test the efficacy of antisense treatment on USCs derived from DMD patient. The findings of this study will adopt USCs as a useful disease model for DMD and other neuromuscular disorders.

1.9. Aims of study

Urine derived stem cells have been recognized by many researchers as a prominent subject in modern research era due to its biological importance and clinical applications. However, few studies have examined the potential benefits of USCs in the field of muscle diseases.

The collection and isolation of USCs is considered to be noninvasive, simple, affordable, ubiquitous, and readily accepted by patients.

The aim of this study is to set up a protocol for the isolation of stem cells from urine specimens collected from both healthy donors and DMD patient.

Then the isolated urine-derived stem cells will be used as a noninvasive disease model for functional genetic studies in Duchenne Muscular Dystrophy (DMD). In this study, we propose using USCs as an alternative diagnostic method to muscle biopsy for the diagnosis of DMD patients when the genetic mutations are undetectable.

In addition to the dystrophin gene, we will analyze the expression of collagen VI gene in order to explore the validity of USCs to detect various transcripts not only associated with the sarcolemmal protein of the muscle cells but also make up a part of the extracellular matrix that surrounds muscle cells and connective tissue. This will confirm the usefulness of USCs in the transcriptomic profiling of others muscle genes.

The main important goal of this study is to validate the feasibility of USCs for testing and screening therapeutic molecules and drug candidates, without iPSC reprogramming. This will suggest that direct reprogramming of urine stem cells with the transcription factor MyoD can create a useful model for drug screening and functional genetic studies in Duchenne muscular dystrophy (DMD).

This will be performed by treating myotubes obtained from MyoD-transformed DMD USCs with antisense treatment and the efficacy of the treatment will be determined by the dystrophin protein restoration.

The findings of this study could represent USCs as a noninvasive human cell model for studying DMD and suggest it as a novel modeling tool for other neuromuscular diseases.

1.10. Objectives

General objective

Isolation and characterization of urine-derived stem cells as a novel modeling tool for functional genetic studies in Duchenne Muscular Dystrophy (DMD).

Specific Objectives

1. To set up a protocol for the isolation of stem cells from urine specimens collected from two healthy donors and one DMD patient with a deletion of exon 45.
2. To characterize the isolated USCs by surface markers.
3. To identify the DMD gene mutation by genomic and transcriptomic analysis.
4. To evaluate the dystrophin transcript after myogenic transformation of USCs.
5. To assess the antisense treatment of native USCs and myogenic USCs using antisense oligoribonucleotide (AON) for dystrophin exon 44 skipping and validate the efficacy of USCs as in vitro model for drug screening.
6. To verify the presence of dystrophin protein before and after treatment using Immunofluorescence analysis and Western blotting.
7. To examine collagen VI gene expression in USCs.

Materials and Methods

2.1. Study design and isolation of human USCs

Urine samples were collected from three individuals: two healthy control subjects and one patient diagnosed with DMD (deletion of exon 45 identified in blood sample by MLPA technique), for research purposes (Table 2.1). All individuals signed an informed consent before urine collection. Progenitor stem cells were isolated and cultured from urine specimens as described by Zhou and colleagues (28), albeit with some of our modifications. Briefly, first-morning mid stream urine specimens (200–350 ml) were collected and centrifuged at 400 g for 10 min at room temperature and washed with phosphate-buffered saline (Gibco PBS) supplemented with an antibiotic/antimycotic solution (Sigma-Aldrich). After discarding the supernatant, the urine sediment was mixed with 1 ml of primary medium, and each sample was plated into a single well of a 12-well plate, coated beforehand with 0.1% gelatin (Millipore). Every 24 hrs, 1 ml of primary culture medium was added to each cell preparation. After 96 hrs of plating, the primary culture medium was removed leaving only 1 ml in each well and adding 1 ml of proliferation medium to each well. The proliferation medium was changed daily by discarding 1 ml and adding 1 ml.

Sample	Disease	Mutation	Gender
DMD	DMD	Del. ex45	M
Donor 1	Healthy Control	/	M
Donor 2	Healthy Control	/	M

Table 2.1 Subjects enrolled for the isolation of urinary stem cells (DMD:Duchenne Muscular Dystrophy).

Media reagents: The primary medium contained Dulbecco's modified Eagle's medium (DMEM)/ high glucose (EuroClone) and Gibco Ham's F12 nutrient mix (1:1), supplemented with 10% (v/v) fetal bovine serum (FBS), antibiotic/antimycotic solution (Sigma-Aldrich), and REGM (renal epithelial cell growth medium) Single Quot kit (Lonza). On the other hand, the main components of the proliferation medium included REGM Bullet Kit and RE cell basal medium, (Lonza) and mesenchymal proliferation

medium (DMEM/ high glucose, 10% [v/v] FBS, 1% [v/v] Gibco GlutaMAX, 1% [v/v] non essential amino acids [NEAA (Gibco)], 1% antibiotic/antimycotic solution, basic fibroblast growth factor [bFGF, 5 ng/ml, ProSpec], platelet-derived growth factor [PDGF-AB, 5 ng/ml, ProSpec], epidermal growth factor (EGF, 5 ng/ml), Lonza]) mixed at a 1:1 ratio.

2.2. Fluorescence-activated cell-sorting analysis

After isolation, USCs were expanded in the proliferation medium. Aliquots of USCs at passage 5–7 were analyzed by flow cytometry to evaluate epithelial cells (CD13, CD324), fibroblasts (fibronectin), stem cells (c-Kit, CD90, CD105), and bone marrow markers (CD34). Antibodies, excluding c-Kit, were conjugated with fluorochromes CD324 (E-cadherin) eFluor 660 (eBioscience) 0.5µg/100µl; anti-human fibronectin, CD34, AlexaFluor 488 (eBioscience) 10µg/ml; anti-human CD90 and CD105 with phycoerythrin (PE) (eBioscience) 0.25µg/100µl; and anti-human CD13 allophycocyanin (APC) (eBioscience) 0.125µg/100µl. USCs were incubated with antibodies against surface antigens. The primary antibody c-Kit anti-rabbit (Santa Cruz Biotechnology) was diluted 1:100, and the secondary antibody, Alexa Fluor 488 anti-rabbit, was diluted 1:1000. The presence of epithelial and stem cell markers was investigated. In all cases, USCs were incubated for 20 min at room temperature. Flow cytometry was performed with an FC 500 instrument (Beckman Coulter), and cellular debris and aggregates were gated out on the basis of forward and side scatter. Isotype-matched negative control subjects helped to define the threshold for each specific signal and to establish the appropriate gate for positive cells. Data were analyzed with Kaluza software (Beckman Coulter). The viability of the cell preparations was evaluated by propidium iodide (PI) staining, and apoptosis was analyzed in each cell preparation by annexin V expression.

2.3. OCT3/4 and NANOG quantification

Total RNA was extracted from cultured USCs with an RNeasy Micro kit (Qiagen) according to the manufacturer's instructions. RNA yield and purity were determined by spectrophotometry absorption at 260 and 280 nm. To obtain cDNA, an equal amount of mRNA (1µg) was reverse transcribed, using iScript reverse transcription supermix (Bio-Rad). mRNA expression was measured by quantitative real-time PCR, using a thermal cycler (iCycler; Bio- Rad). The reaction was performed in a 15µl volume, using SYBR green supermix kit (Bio-Rad). All measures were performed in triplicate. The reaction conditions were as follows: 95°C for 3 min (polymerase activation), followed by 40 cycles at 95°C for 5 sec and 60°C for 10 sec, and 81 cycles at 55°C for 15 sec. Quantified values were normalized against the input determined with the housekeeping gene β_2-

microglobulin (B2M), using the formula log $2^{-\Delta Ct}$. The following oligonucleotides were used (Table 2.2).

B2M	Forward: 5′-AGGACTGGTCTTTCTATCTCTTGT-3′ Reverse: 5′-ACCTCCATGATGCTGCTTACA-3′
OCT3/4	Forward: 5′-AGGAGAAGCTGGAGCAAAA-3′ Reverse: 5′-GGCTGAATACCTTCCCAAA-3′
NANOG	Forward: 5′-GGTCCCGGTCAAGAAACAGA-3′ Reverse: 5′-GAGGTTCAGGATGTTGGAGA-3′

Table 2.2 Oligonucleotides used for quantitative real-time PCR for B2M, OCT3/4 and NANOG

2.4. Telomerase activity

The catalytic activity of telomerase in USCs was assessed by quantitative PCR. USCs were homogenized in CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) buffer and centrifuged at 4°C. Two different protein concentrations, 0.5 and 1μg, were employed for the assay. USC extracts were incubated at 30°C for 30 min in a solution containing reverse transcriptase reaction mix and *Taq* polymerase (TRAPEZE RT telomerase detection kit; Chemicon, Temecula, CA). The HepG2 cell line was used as positive control, and serial dilutions of control template TSR8 were employed for quantification. CHAPS buffer in the absence of protein lysates was used as negative control. PCR cycling conditions were as follows: 1 cycle of 95°C for 2 min; 40 cycles of 94°C for 15 sec, and 59°C for 60 sec. Data were collected at the 59°C stage of each cycle.

Statistical analysis

Continuous variables are expressed as means ± standard deviation, whereas categorical variables are expressed as numbers and percentages. An unpaired T-test or Mann–Whitney U test was used as appropriate for comparison between two groups, and categorical variables were compared by chi-square test or Fisher exact test, as appropriate. All probability values reported are two-sided, and $P<0.05$ was considered statistically significant. Statistical analysis was performed with STATA 11.1 software (Stata, College Station, TX).

2.5. DMD urine-derived stem cell genotype characterization

MLPA analysis: multiplex ligation-dependent probe amplification (MLPA) is a reliable quantitative tool for the detection of the deletion and duplication in entire dystrophin gene containing 79 exons. Genomic DNA was extracted from urine-derived

stem cells with a Nucleon BACC3 kit (GE Healthcare Life Sciences). Mutation analysis was performed by MLPA as previously described (54). MLPA analysis was carried out with SALSA probe mix 034 and 035, according to the manufacturer's recommendations (MRC,Holland), thereby achieving the copy number screening of all 79 dystrophin exons. PCR products were analyzed with an Applied Biosystems ABI 3130 automated sequencer, using GeneScan software (Thermo Fisher Scientific).

2.6. Myogenic transformation of USCs

Myogenesis of urine stem cells from both healthy donors and the DMD patient was induced by infection with an adenovirus serotype 5 (Ad5) derived, the EA1-deleted adenoviral vector carrying the MyoD gene. USCs were incubated with Ad5 in the infection media (high-glucose DMEM, supplemented with 2% horse serum (GIBCO) and antibiotic/ antimicotic solution) for 4 hrs at 37°C, as previously described (90). MyoD transformed USCs were cultured in high-glucose DMEM supplemented with 2% FBS and antibiotic/antimycotic solution (differentiation medium) during differentiation into myotubes.

2.7. Expression analysis of dystrophin transcript

RNA extraction: total RNA was extracted from cells, using an RNeasy kit (Qiagen), and reverse transcribed with Applied Biosystems high capacity cDNA reverse transcription kit (Thermo Fisher Scientific), according to the respective manufacturers' instructions. Before cDNA synthesis, RNA was treated with DNase I (Roche) and checked for residual DNA contamination by real-time PCR analysis.

2.8. DMD gene micro-fluidic card (FluiDMD) analysis

FluiDMD is a gene-specific TaqMan®Low Density Array (TLDA) able to completely characterize the DMD gene transcript. It is an accurate and rapid gene/exome specific method based on Applied Biosystems 7900HT Micro Fluidic Card technology. FluiDMD is designed to simultaneously analyze 85 unique TaqMan Real-Time systems that recognize 76 of 78 DMD exon junctions, all DMD isoforms, and two endogenous controls (β-actin and 18S).

The dystrophin transcript analysis was performed as described by Bovolenta and colleagues (75). In brief, 250 ng of RNA from each sample was reverse-transcribed, using an Applied Biosystems high-capacity cDNA reverse transcription kit, in a volume of 20µl. To this quantity, 80µl of sterile water and 100µl of Applied Biosystems 2X universal master mix were added. A 100µl volume of mix per port was loaded into the fluidic card,

and then was centrifuged two times at 1200 rpm for 1 min on a Sorvall centrifuge after that it was sealed and finally were run on an Applied Biosystems real time 7900HT appliance (Thermo Fisher Scientific). The cycle threshold (C_t) values, obtained for all exon junctions and dystrophin isoform systems, were normalized using human β-actin as the housekeeping gene ($\Delta C_t = C_t$ exon junction system – C_t β-actin).

2.9. Expression analysis of dystrophin protein

2.9.1. Western blotting

Protein extracts were isolated from cell cultures, using 150µl of lysis buffer (20mM Tris-HCl [pH 6.8], 0.1% sodium dodecylsulfate [SDS], 150mM NaCl, 1% Nonidet P-40 [NP- 40], 0.5% deoxycholic acid, 1mM NaF, 0.1mM sodium orthovanadate, 2mM phenylmethylsulfonylsulfate [PMSF], protease inhibitors), at 3 days post differentiation. Protein concentration was determined by the Bradford method. Aliquots corresponding to 20µg (myoblasts derived from a control muscle, WT) and 60µg (MyoD-transformed USCs) of protein were loaded on a 6% polyacrylamide gel and separated by electrophoresis. Samples were transferred to a nitrocellulose membrane that was blocked with nonfat dried milk for 60 min at room temperature and incubated overnight at 4°C with Dys2 antibodies (diluted 1:50, Leica Biosystems). Amersham horseradish peroxidase-conjugated anti-mouse secondary antibody (diluted 1:1000, GE Healthcare Life Sciences) was used as the secondary antibody. Western blots were developed with an Amersham ECL Plus Western blotting detection system (GE Healthcare Life Sciences).

2.9.2. Immunostaining analysis

Cell cultures were grown on coverslips, and fixed in -20°C methanol. Samples were incubated for 30 min in PBS mixed with 4% bovine serum albumin (Sigma-Aldrich). All samples were labeled with both polyclonal antidystrophin antibody (H300, Santa Cruz Biotechnology) diluted 1:100 and monoclonal anti-myosin antibody diluted 1:40 (Dako), washed with PBS, and revealed with tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit and fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (diluted 1:100, Dako), respectively. The slides were mounted with a molecular probes antifade mounting medium (Thermo Fisher Scientific), and analyzed with a Nikon Eclipse 80i fluorescence microscope (Nikon Instruments).

2.10. Antisense treatment

Both native DMD USCs and myotubes obtained from MyoD-transformed DMD USCs were transfected with 2′-*O*-methyl phosphorothioate (2′OMePS) antisense

oligoribonucleotide for dystrophin exon 44 skipping (IDT Technologies) in the presence of polyethylenimine (PEI) (ExGen 500; Biomol) as transfection reagent.

2.11. Exon-skipping quantification

Exon skipping was quantified with Agilent 2100 bioanalyzer (Agilent Technologies). The region surrounding the DMD mutation, spanning exons 43–46, was amplified, and PCRs were performed via 30 cycles at 94°C (30 sec), 60°C (45 sec), and 72°C (80 sec), with Invitrogen Platinum *Taq* DNA polymerase (Thermo Fisher Scientific). PCR products were analyzed with Agilent high-sensitivity DNA chip that performs capillary electrophoresis and uses a fluorescent dye to bind DNA and determine both DNA concentration and size. The skipping percentages were calculated as the ratio between skipped transcript and total transcript (skipped transcript/ total transcript (skipped transcript + unskipped transcript) x100).

Skipping percentage was also quantified with FluiDMD cards, and calculated by the $\Delta\Delta C_t$ method: a comparison of the ΔC_t of ex43–44 and the ΔC_t of total transcript ($\Delta\Delta C_t = \Delta C_t$ ex43–44 – ΔC_t of total transcript (mean of ΔC_t from ex1–2 junction to ex28–29)). $\Delta\Delta C_t$ values were used to calculate $2^{-\Delta\Delta Ct}$ and the skipping percentage as $2^{-\Delta\Delta Ct}$ x 100.

2.12. Custom Micro-Fluidic Exome Array: Flui ColVI analysis

FLUI-ColVI is a TaqMan gene expression assay providing ABI real-time exon-exon junction systems. ColVI genes have 115 exons and FluiColVI is designed to cover all exons and exon-exon junctions of the three different ColVI transcripts (ColVIα1 (35 exons), ColVIα2 (30 exons), and ColVIα3 (50 exons)), with the presence of two endogenous controls (β-actin and GAPDH). The COLVI transcripts analysis was performed as previously described for FluiDMD.

In brief, 125ng of RNA from each sample was reverse-transcribed, using an Applied Biosystems high-capacity cDNA reverse transcription kit, and the Applied Biosystems 2X universal master mix were added to each sample. A 100µl volume of mix per port was loaded into the fluidic cards, which were run on an Applied Biosystems real-time 7900HT appliance (Thermo Fisher Scientific). The cycle threshold (C_t) values obtained for all exon junctions were normalized, using human β-actin as the housekeeping gene ($\Delta C_t = C_t$ exon junction system – C_t β-actin).

Results

3.1. Morphology and CD markers evaluation in USCs

USCs were isolated from both healthy donors and from DMD patient with a deletion of exon 45 within two weeks using the modified protocol of Zhou *et al*. The isolated USCs displayed a typical MSC phenotype, in accordance with previous studies (28, 29). The morphology of the isolated USCs in the culture appears in two types: type I appears as regular rounded cells and type II as spindle-shaped cells (Fig 3.1). There is no differences between USCs isolated from the control subjects and USCs isolated from the DMD patient in terms of morphological appearance, time of expansion, or in vitro survival rate: 90% of the cells were viable at passage 7 (P7), while less than 5% of the cells expressed the apoptosis-associated marker annexin V (Fig 3.2a).

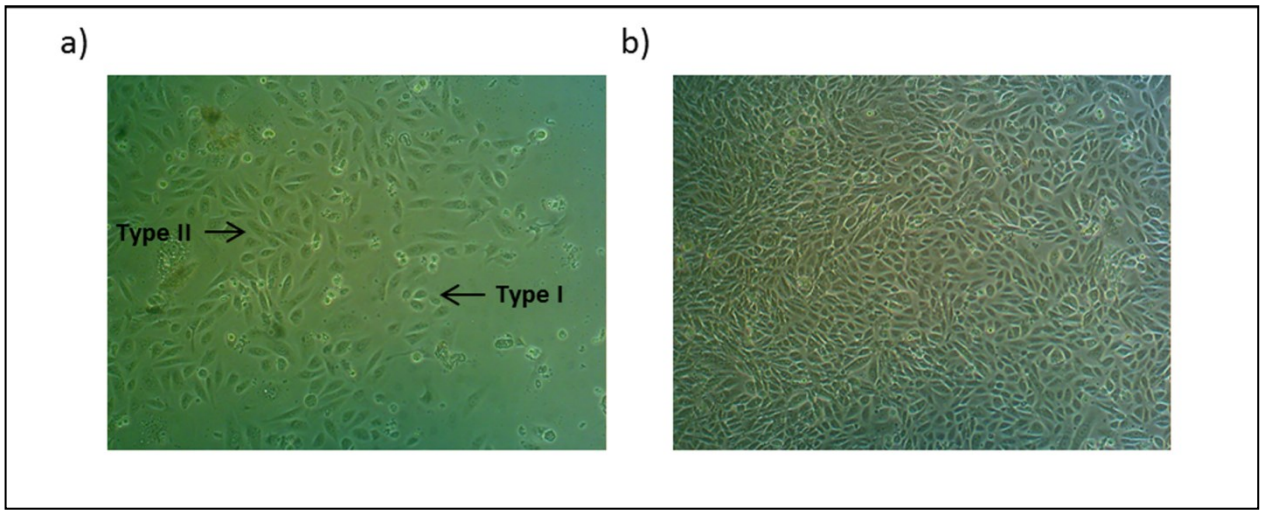


Figure 3.1 Morphology of urine stem cells (USCs) obtained from fresh Duchenne muscular dystrophy (DMD) urine sample at various time points after collection . Shown are type I and type II cells (arrows) on day 12 (a) and day 17 (b).

Variable percentages of USCs (85–95%) were positive for mesenchymal stem cell markers (CD90 and CD105) and for the fibroblast-associated protein fibronectin. On the other hand, USCs expressed low levels of stem cell factor receptor (CD117) and bone marrow derived epitopes such as CD34. Importantly, CD13 a renal epithelial marker which is also known as aminopeptidase N was expressed by a high percentage of cells. Unspecific positive labeling was excluded by isotype and negative control staining (Fig 3.2 b–d). At P5 and P7 a small fraction of USCs varying from 1 to 5%, expressed lymphocyte and mast cell surface markers namely CD4, CD8, and CD45 (Fig 3.2 b–d). Then, the evaluation of the proliferation capacity of USCs in vitro was assessed by stemness-related genes

expression (NANOG and Oct3/4) and telomerase activity and all were found to be present in all preparations analyzed. Notably, no differences were found between healthy and DMD donors (Fig 3.2 e). Thus, we established the conditions for the isolation and expansion of SCs from human urine samples in healthy control subjects and the DMD patient.

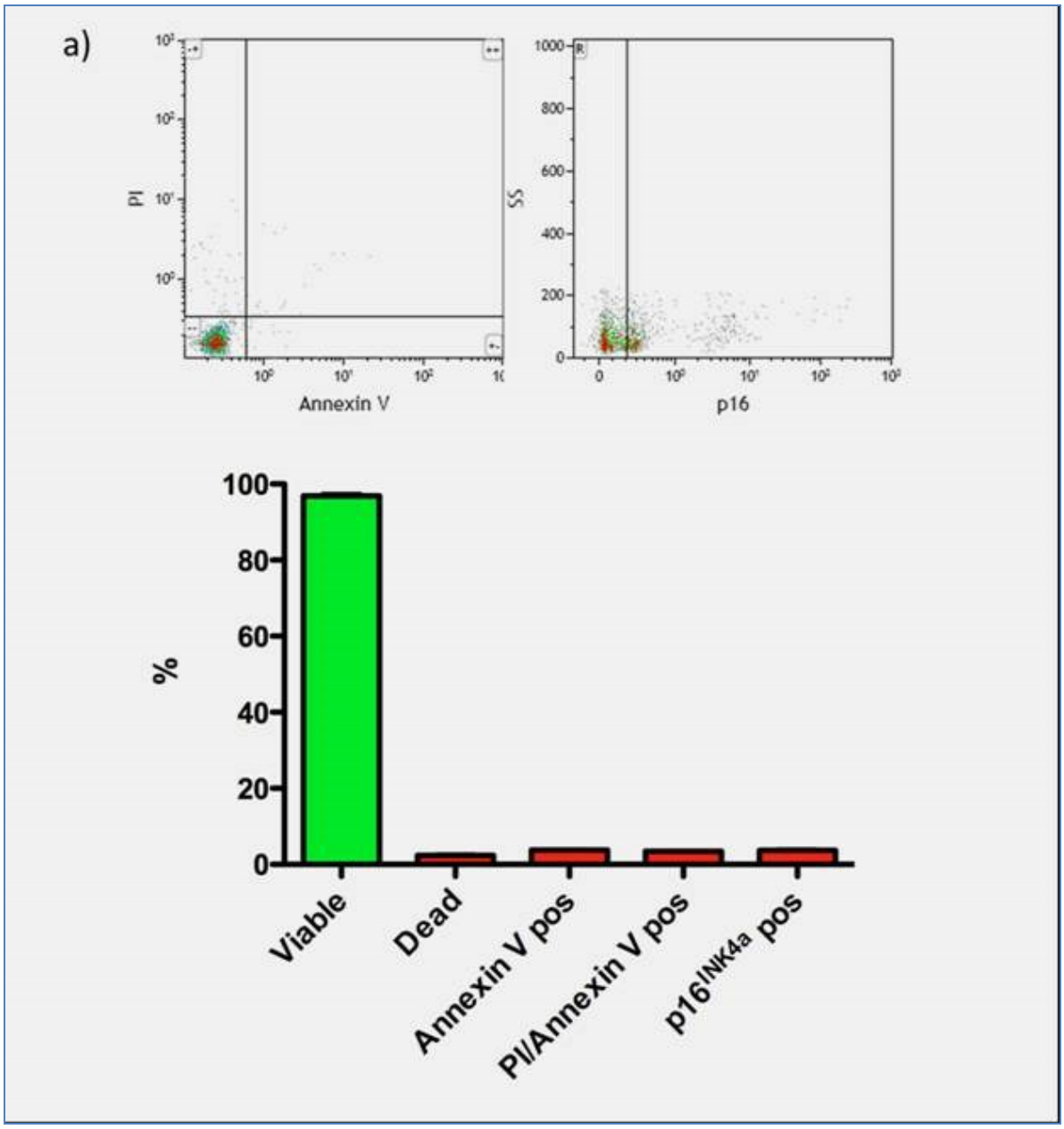


Figure 3.2 (a) Top: Bivariate distribution of propidium iodide (PI) and annexin V in prepared USCs (top left). Bivariate distribution of the senescence-associated protein p16 ^{INK4a}, and side-light scatter in USC preparation (top right). Bottom: Bar graph indicating the percentage of viable cells (green column) and dead cells, and the percentage of USCs positive for annexin V, PI/annexin V, and p16 ^{INK4a}, respectively (red columns). Each column shows the average and standard deviation.

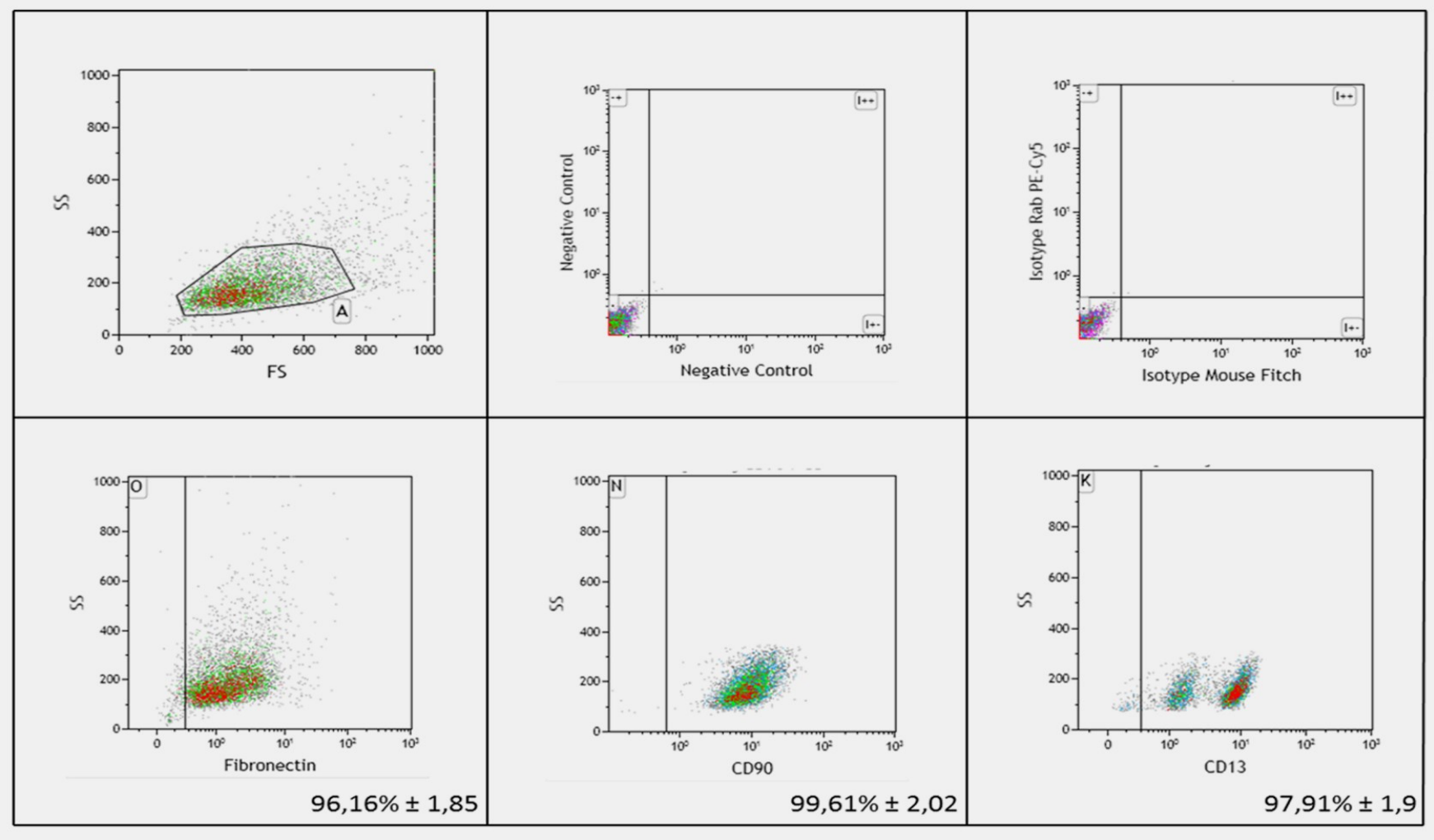


Figure 3.2 (b) Bivariate distribution of USCs in forward-and side-light scatter (top left). Expression of surface markers in isotype and negative control (top middle and right) is indicated. Bottom: Bivariate distribution of fibronectin, CD90, and CD13 in USC preparations. In each case the average and standard deviation are indicated.

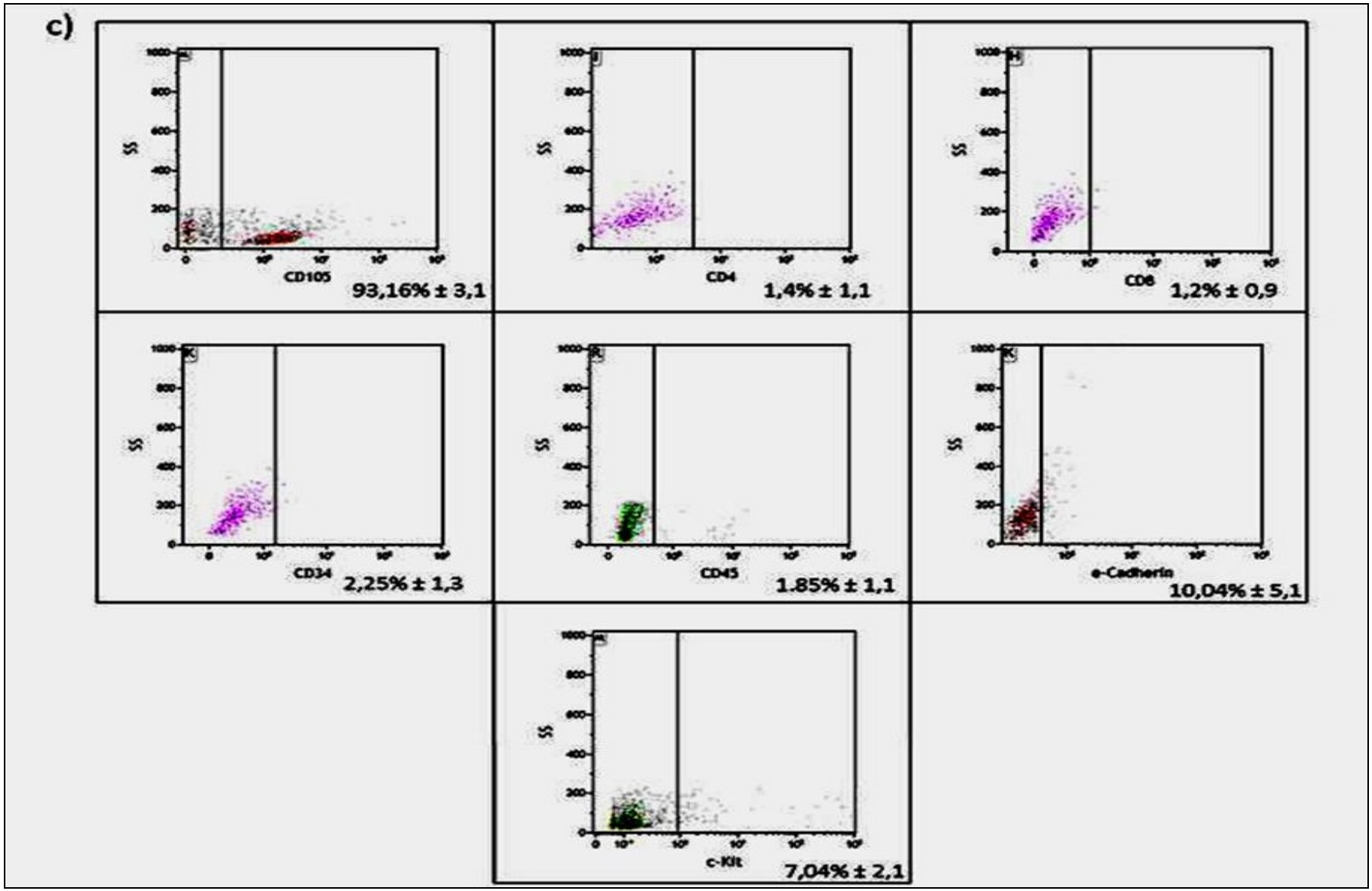


Figure 3.2 (c) Bivariate distribution of CD105, CD4, CD8, CD34, CD45, E-cadherin, and c-Kit in USC preparations. In each panel the average and standard deviation are provided.

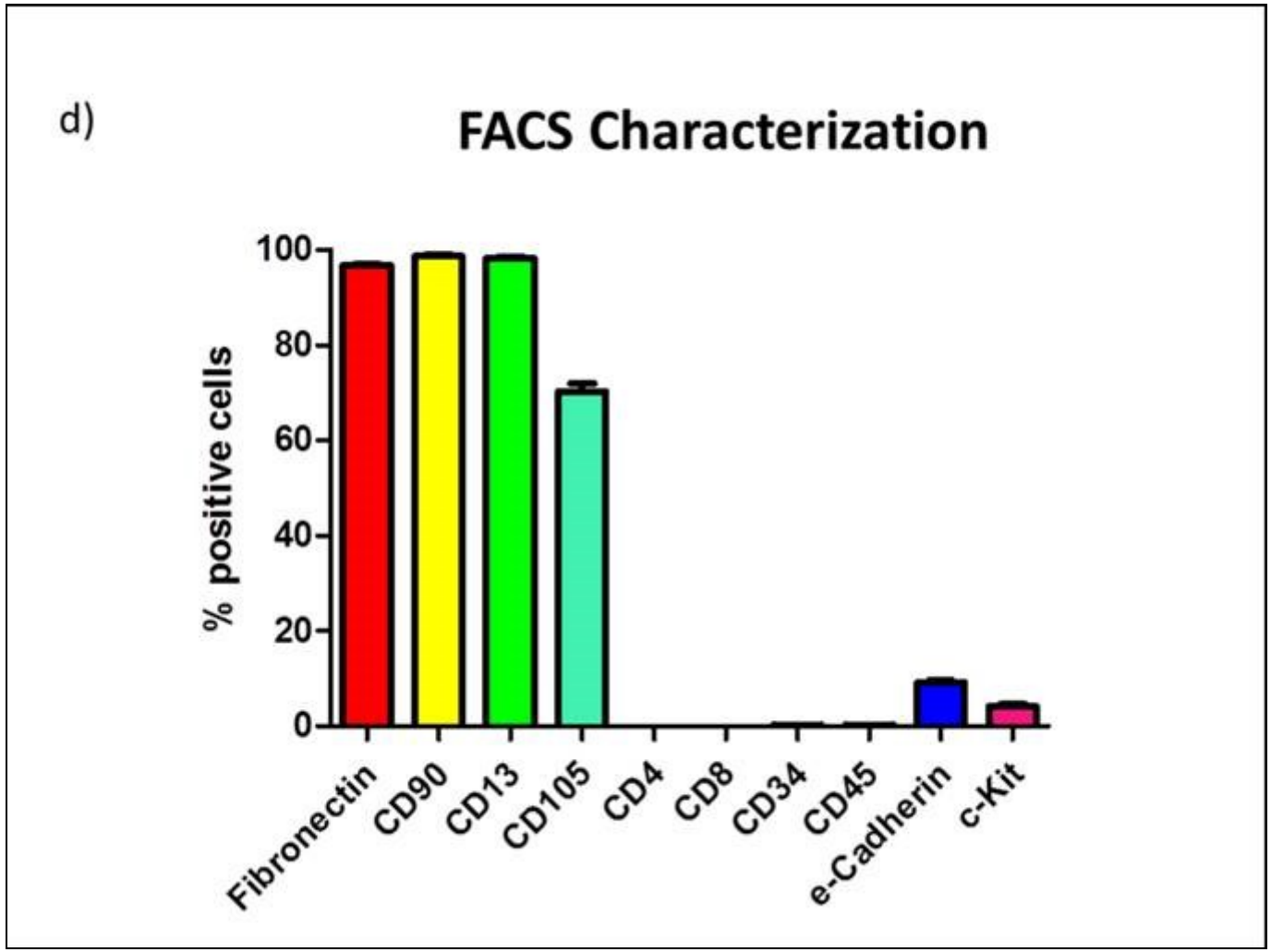


Figure 3.2 (d) Bar graph indicating the percentage of cells positive for each of the markers considered in the fluorescence-activated cell sorting (FACS) analysis.

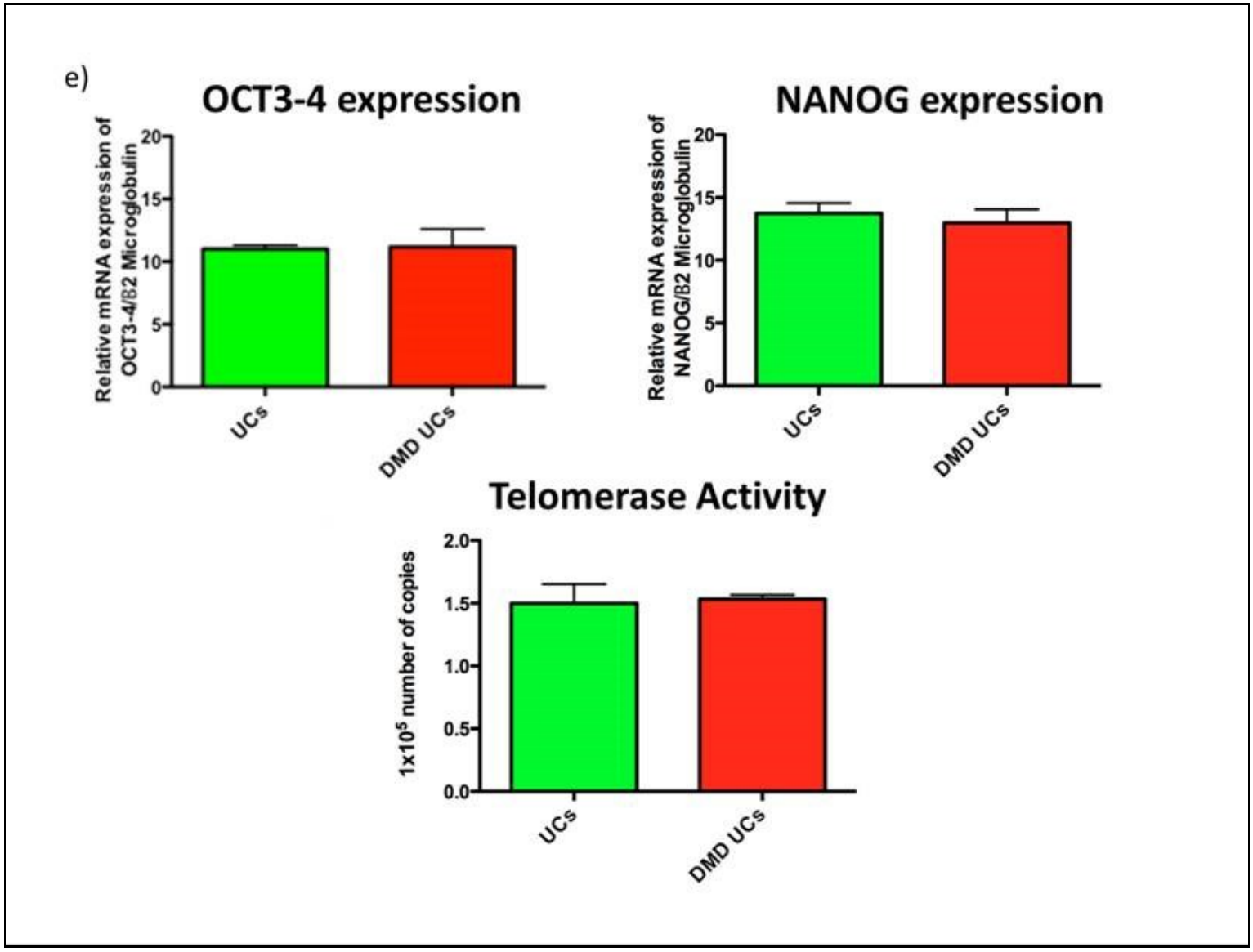


Figure 3.2 (e) Stemness-related genes expression and telomerase activity in USCs are shown at top and bottom, respectively. Average values and standard deviations are provided for USCs isolated from control subjects (green columns) and the patient with DMD (red columns).

3.2. DNA analysis in DMD urine-derived stem cells

MLPA analysis of DMD USCs confirmed the presence of an isolated exon 45 deletion, as previously identified in peripheral blood cells of the patient during routine diagnostic procedures (Fig 3.3).

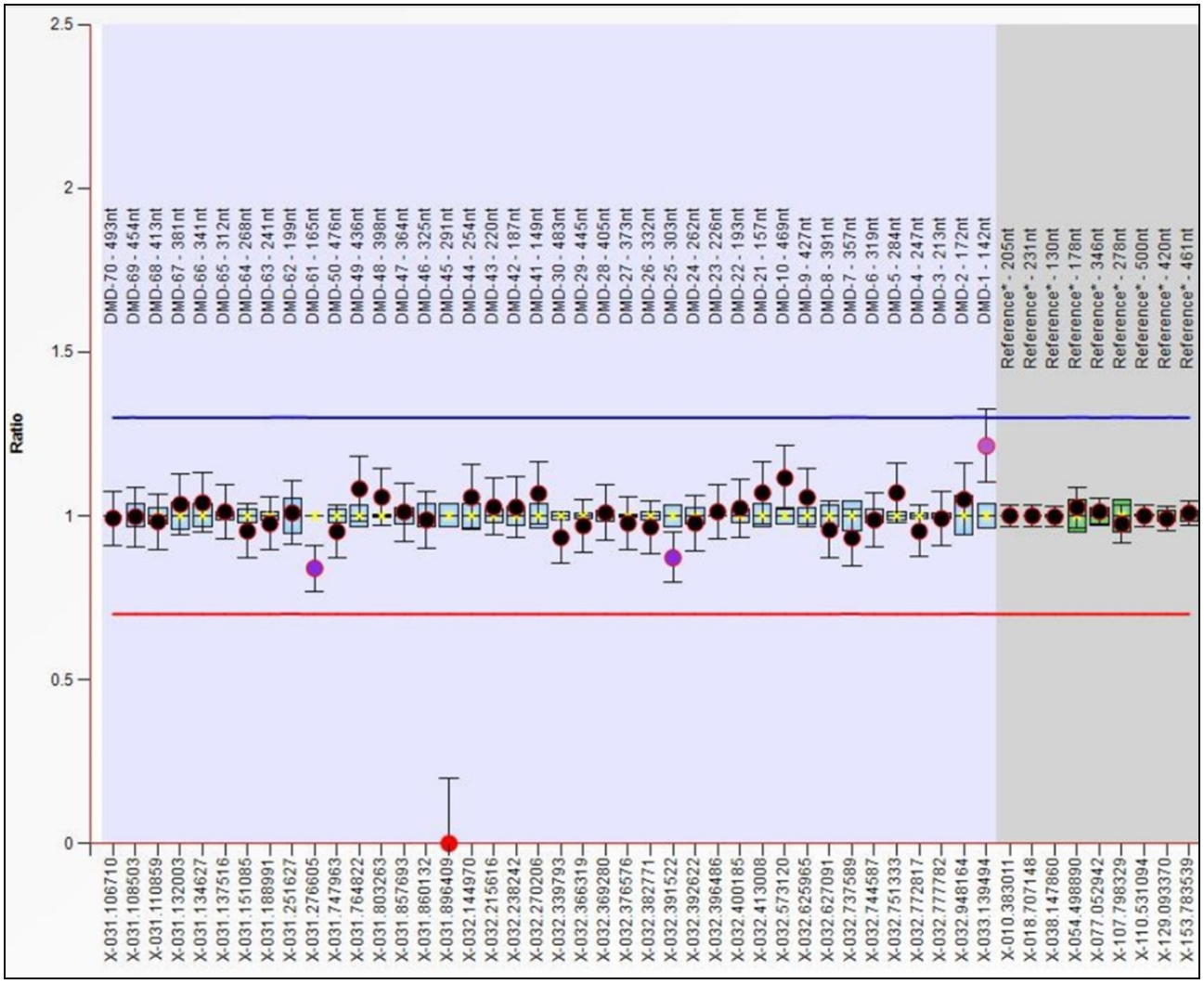


Figure 3.3 Multiplex ligation-dependent probe amplification (MLPA) profile of the whole DMD gene shows the isolated exon 45 deletion (red dot).

3.3. Expression profile of dystrophin transcript in native USCs and differentiated myogenic USCs

In order to establish USCs as a useful in vitro model for muscle disease studies, firstly we need to prove the presence of dystrophin transcript as a muscle cell-specific transcript. Hence, we studied both native USCs and MyoD transformed USCs from control subjects and the DMD patient.

USCs from both healthy donors and DMD patient were transformed into myogenic cells by infection with an Ad5-derived vector carrying the MyoD gene, and the resulting USCs-

MyoD were allowed to differentiate into myotubes. Then, the dystrophin transcript was evaluated in both native USCs and differentiated USCs-MyoD using the FluiDMD card. USCs isolated from the healthy control sample (Donor 1) revealed the presence of the full-length dystrophin transcript (Fig 3.4 a, blue bars) and all the junction–junction systems were detected, albeit at low levels, this including the full-length Dp427m (M), Dp427p (P), and the short Dp260, Dp116, Dp71 dystrophin isoforms. The Dp427b (B) isoform was detected only after myogenic transformation.

After myogenic transformation, there is an increase in the expression of all dystrophin transcripts for both single junction–junction systems and the specific dystrophin isoforms (Fig 3.4 a, red bars). The healthy control sample (Donor 2) presented an identical profile (data not shown).

In the DMD patient, the FluiDMD card precisely identified the deletion of exon 45 (Fig 3.4 b), demonstrating that USCs maintain the mutation in the dystrophin transcript. Like the healthy samples, the differentiation into myogenic cells enhanced the expression levels of all analyzed dystrophin transcripts.

(a)

(b)

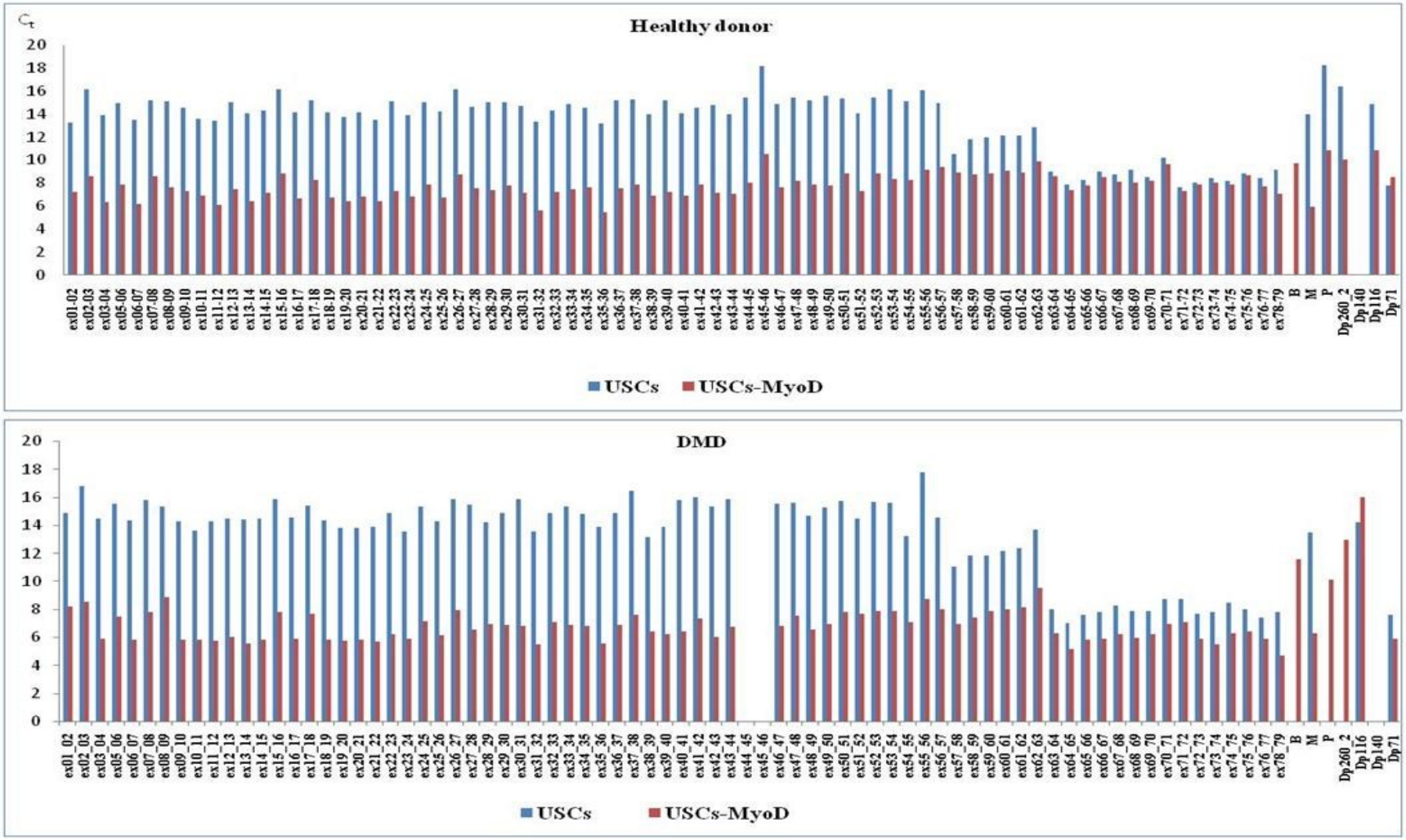


Figure 3.4 DMD gene microfluidic card (FluiDMD) profiles of USCs and USCs-MyoD (i.e., USCs transformed into myogenic cells by infection with an Ad5-derived vector carrying the MyoD gene) from healthy donor 1 (a) and a patient with DMD (b). In the healthy donor, FluiDMD identified the amplification of all exon junctions, from 1–2 to 78 -79 (a, blue bars). The absence of junctions between exons 44–45 and 45–46 demonstrates the mutation of exon 45 in the patient with DMD (b, blue bars). The red bars represent the transcript levels after myogenic transformation.

3.4. Exon skipping in USCs and USCs-MyoD treated with antisense oligonucleotide

The main target of our work was to determine the feasibility of using USCs for testing and screening therapeutic compounds, without the requirement of iPSC reprogramming. To this end, both USCs and USCs-MyoD from DMD patient carrying the deletion of exon 45, were treated with the 2′OMePS antisense oligoribonucleotide (AON) for exon 44 skipping. Then, the skipping was quantified by two different methods: (1) the $\Delta\Delta C_t$ method using FluiDMD card analysis, and (2) the Agilent 2100 bioanalyzer using their high-sensitivity DNA chip. The FluiDMD platform quantification of dystrophin exons showed the absence of off-target effects induced by the antisense treatment (Fig. 3.5 a and b). The skipping percentages were calculated using $\Delta\Delta C_t$, by comparing the expression level of the ex43–44 junction with respect to the total dystrophin transcript. This was performed by calculating the mean ΔC_t from junctions ex1–2 to ex28–29, this region was selected as a representative region of the total transcript driven by the full-length promoters, because the distal promoters lie within intron 29 (Dp260), intron44 (Dp140), intron 55 (Dp116), and intron 62 (Dp71) and we needed to rule out the effect of any additional expression on the total dystrophin transcript. This was particularly important in the case of Dp71, which was abundant in the USC culture.

The $\Delta\Delta C_t$ method revealed 7% physiological skipping of exon 44 in DMD USCs-MyoD, while AON treatment induced 30% new skipping.

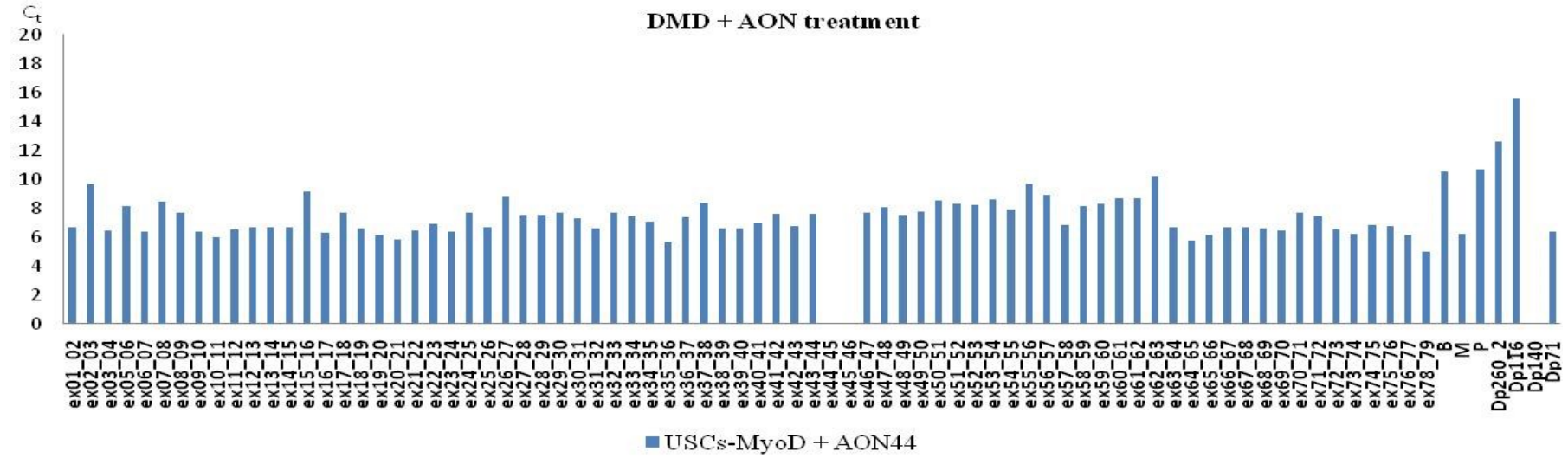
Figure 3.6 summarizes the results obtained by the Agilent high-sensitivity DNA chip, including the run on the bioanalyzer microgel (Fig. 3.6 A), showing the skipped and unskipped fragments, and the chromatogram representation, which indicates the size and the concentration of the amplified fragments, for both DMD USCs-MyoD (Fig. 3.6 B) and DMD USCs-MyoD treated with AON (Fig. 3.6 C).

The concentrations estimated by the chip were used to calculate the skipping percentages, as described in the Materials and Methods. The percentages of exon 44 skipping obtained by Agilent high sensitivity DNA chip revealed similar results to those obtained by the FluiDMD card. In particular, 4% physiological skipping and 33% AON-induced skipping. The use of both validated methods and their relative calculations lends robustness to our data.

The skipping quantification in native DMD USCs, using the high-sensitivity DNA chip, showed 9% physiological skipping and 35% AON-induced skipping, which is similar to the results observed in the DMD USCs-MyoD. FluiDMD data did not allow us to

accurately quantify the skipping in native DMD USCs, maybe due to the variable expression of exon junction-junction systems in the selected region used for the calculation of the ΔΔCt.

(a)



(b)

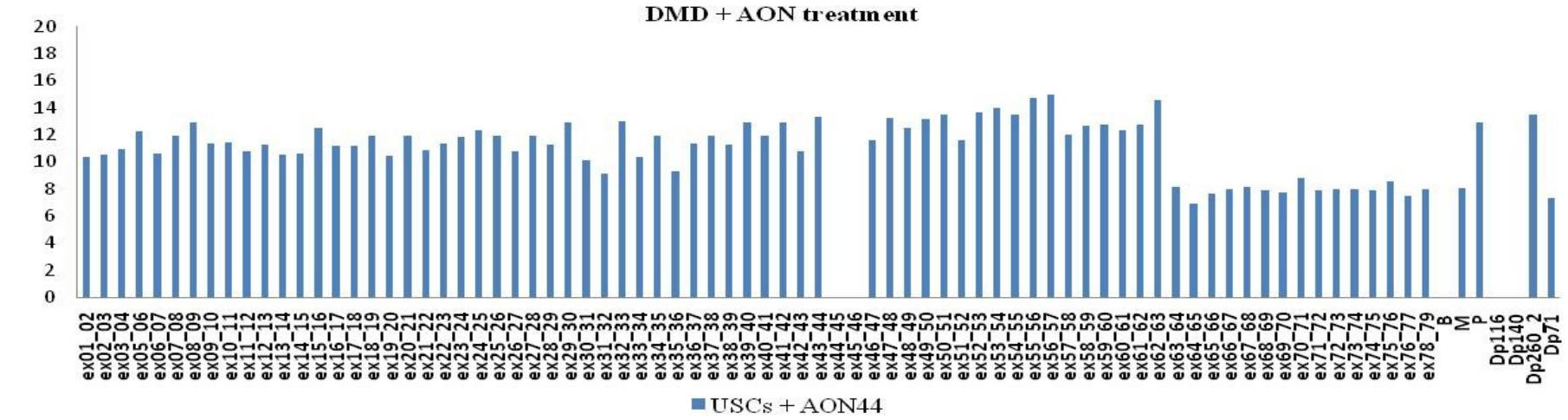


Figure 3.5 FluiDMD analysis of dystrophin transcript profiles after antisense treatment in USCs MyoD (a) and USCs (b) from a patient with DMD. The difference in expression of exon junction 43–44 was used to calculate exon 44 skipping in order to evaluate the efficacy of the treatment.

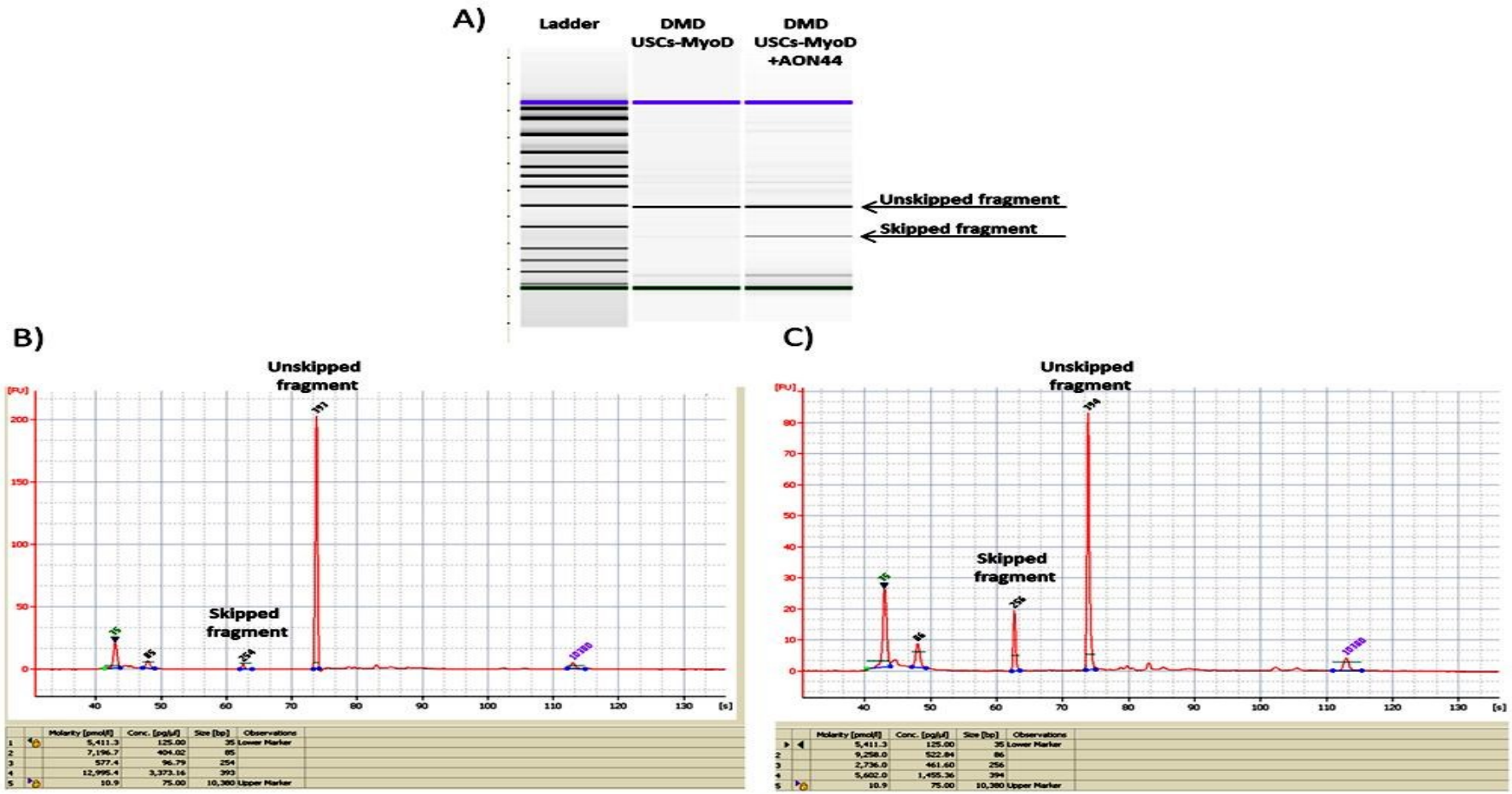


Figure 3.6 Agilent 2100 bioanalyzer microgel (A) shows the run of both skipped and unskipped transcripts in DMD USCs-MyoD before (lane 2) and after (lane3) antisense oligonucleotide (AON) treatment. The chromatograms show the size of the amplified fragments of both DMD USCs-MyoD (B) and DMD USCs-MyoD treated with AON (C). Concentrations are reported in the two tables below the chromatograms.

3.5. Muscular protein analysis

Dystrophin protein expression was analyzed in both native USCs and USCs-MyoD, derived from both healthy donors and DMD patient. Immunofluorescence analysis, performed with anti-dystrophin antibody, revealed the absence of dystrophin protein in all native USCs analyzed (Fig. 3.7 A). On the other hand, the protein was rescued only in the MyoD-transformed control USCs (Fig. 3.7 B). The molecular weight of the protein was verified by Western blot, and the full-length 427-kDa dystrophin protein was detected only in USCs-MyoD derived from the healthy controls (Fig. 3.8). The Western blot of USCs-MyoD from the patient with DMD was negative, as expected (data not shown).

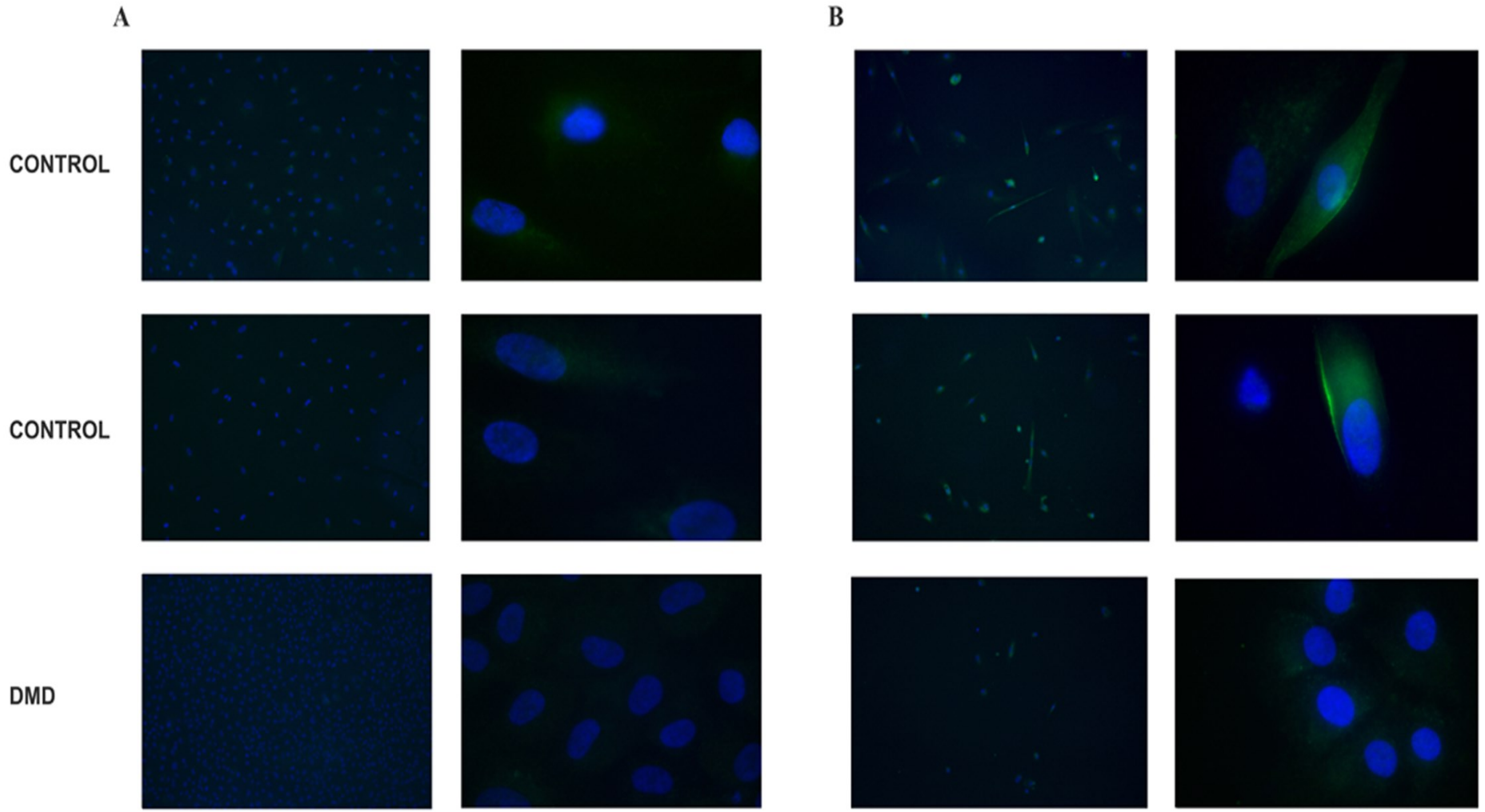


Figure 3.7. Immunofluorescence analysis of dystrophin (DYS) protein in USCs (A) and USCs-MyoD (B) from both healthy control subjects and the patient with DMD. Green staining shows the presence of dystrophin protein localized to the cell membrane in the healthy USCs after myogenic transformation.

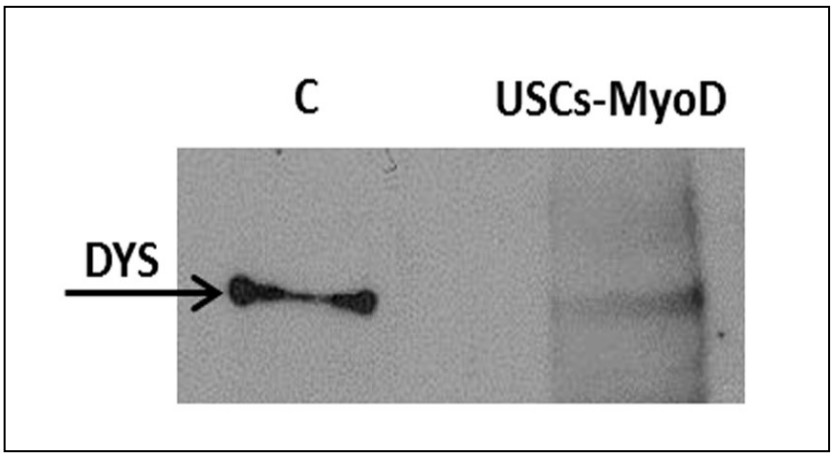


Figure 3.8. Western blot analysis of USCs-MyoD derived from a healthy donor, shows the presence of the dystrophin protein with the full-size molecular weight as the normal control (C).

Antisense treatment of DMD USC-MyoDs induced translation of the dystrophin protein, which was seen to be correctly localized at the sub-sarcolemma by immunofluorescence (Fig 3.9), while the western blot did not detect any dystrophin due to the low skipping percentage and consequent very low amount of the rescued protein. Figure 3.10 shows the double labeling of USCs-MyoD with anti-myosin and anti-dystrophin antibodies that appear co-localized at the sub-sarcolemma membrane.

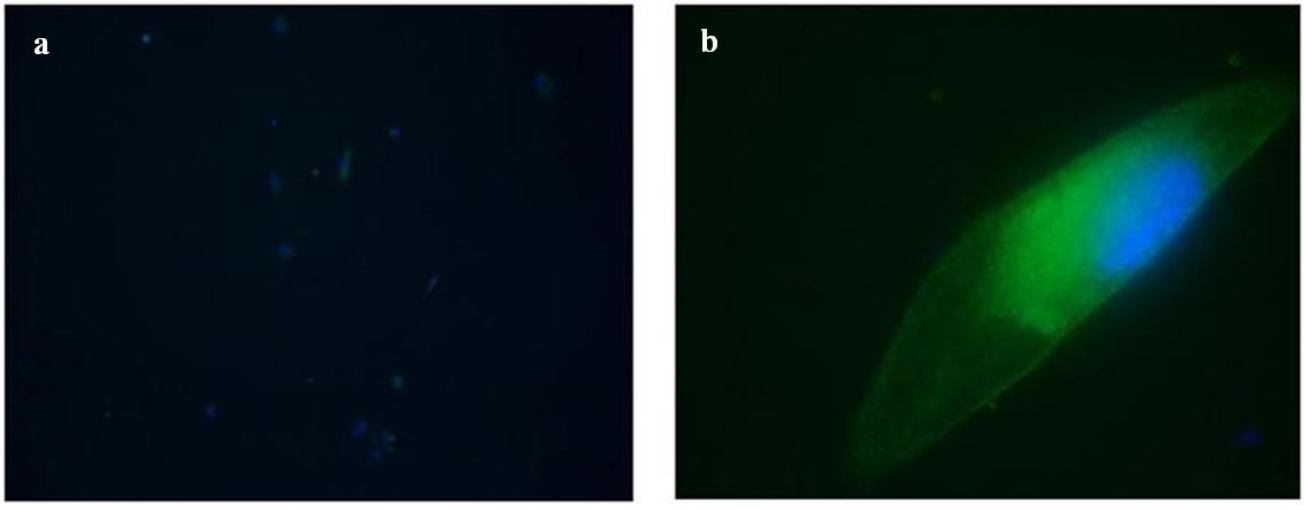


Figure 3.9 Immunofluorescence analysis of dystrophin protein in USCs-MyoD from the patient with DMD after antisense treatment. Original magnification (a) 10 x; (b) 100 x.

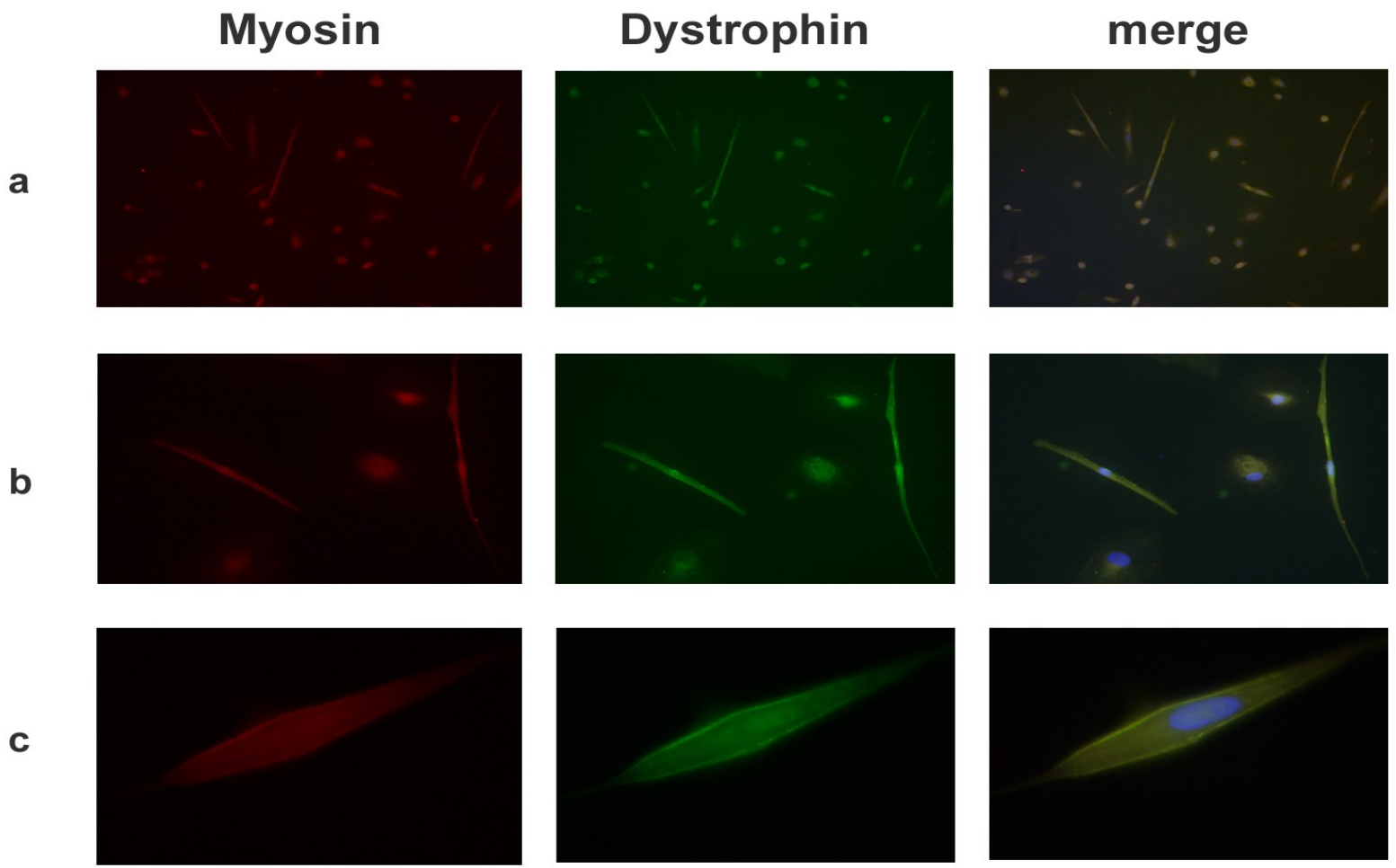


Figure 3.10 Immunostaining of USCs-MyoD from healthy donors, using double labeling with anti-myosin (red) and anti-dystrophin (green) antibodies. Original magnification: (a) 10 x; (b) 20 x; (c) 100 x.

3.6. Expression profile of COL VI transcripts in native USCs

In addition to dystrophin analysis, collagen VI (ColVI) transcripts were analyzed in both native and myogenic USCs derived from a healthy subject in order to test the presence of other transcripts related to muscular dystrophy. This was performed by using a specific microfluidic card (FLUI-ColVI), that we designed and we are using for the detection of collagen gene mutations.

Native USCs isolated from healthy control samples revealed the expression of all ColVI transcripts (α1, α2, α3), with the detection of all junction–junction systems (Fig 3.11 a and c). In ColVI α2 transcript we found that two junction-junction systems (ex11-ex12 and ex15-ex16) were not detected, but the adjacent systems that surround these regions confirm the presence of all exons. In Figure 3.11 we presented only the results obtained from the healthy donor 1, since the analysis of native USCs from the healthy donor 2 showed an identical profile (data not shown).

The analysis of MyoD-USCs of both healthy donors did not detect any transcripts, as expected (data not shown) due to the fact that myogenic cells do not express the ColVI.

Finally, the presence of both dystrophin and ColVI transcripts confirm that USCs could be used to detect various types of muscular transcripts with different cellular localization such as in sarcolemmal membrane, and the extracellular matrix.

From these findings, we can suggest that USCs could be a useful tool for the transcriptomic profiling of others muscle genes.

(a)

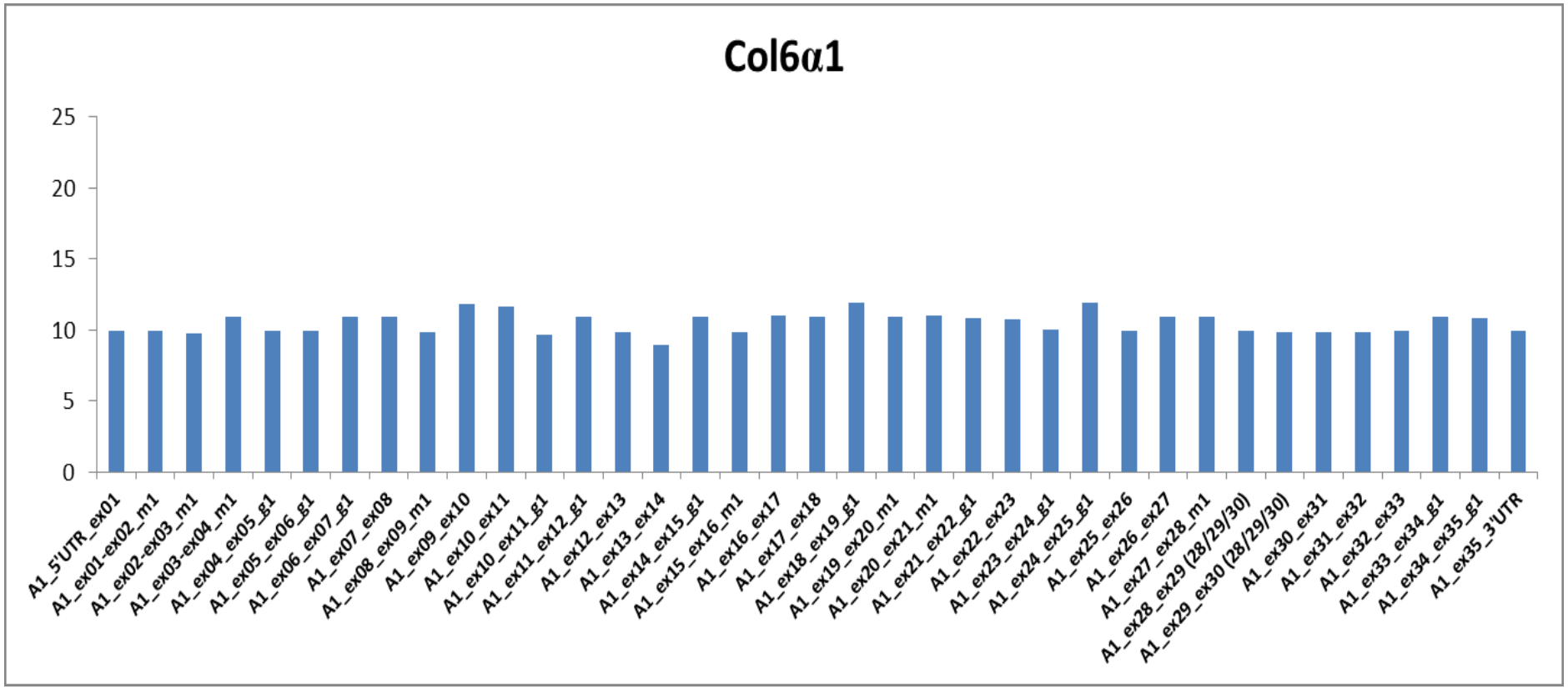


Figure 3.11 (a) ColVI gene microfluidic card (FluiColVI) profile of native USCs derived from a healthy control sample (Donor1). FluiColVI identified the amplification of all exon-exon junctions, in Col6α1transcript (35 exons).

(b)

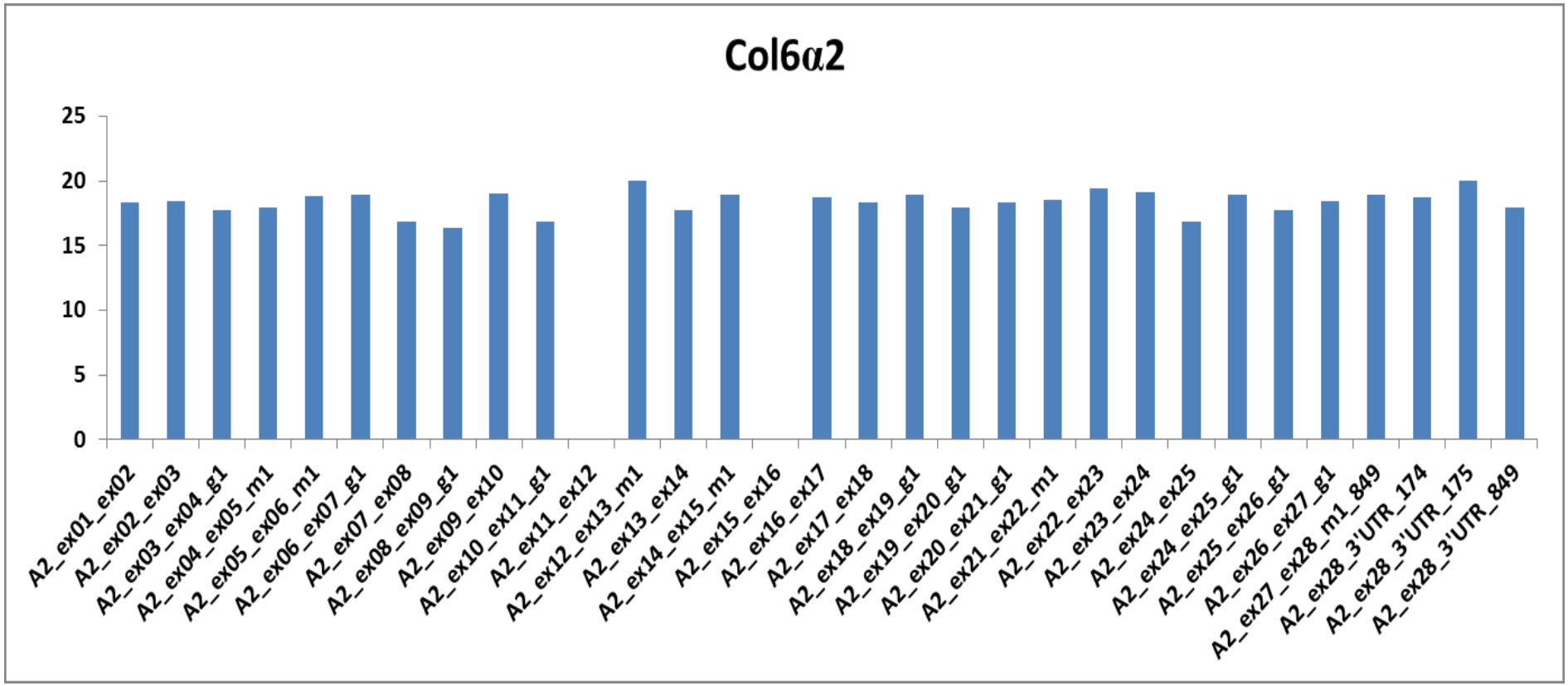


Figure 3.11 (b) ColVI gene microfluidic card (FluiColVI) profile of native USCs derived from a healthy control sample (Donor1). FluiColVI identified the amplification of all exons in Col6α2 (30 exons). Two junction-junction systems (ex11-ex12 and ex 15-ex16) were not detected but the adjacent systems that surround these regions confirm the presence of all exons.

(c)

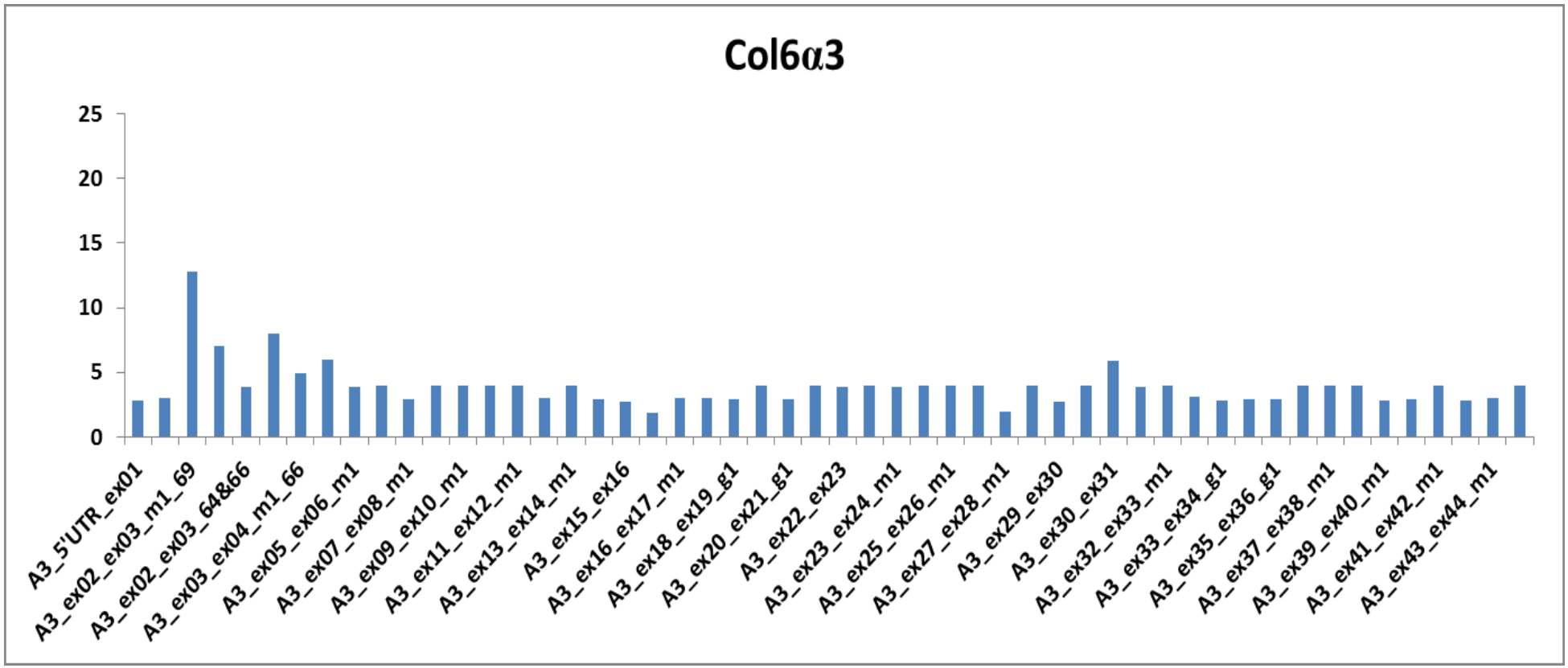


Figure 3.11 (c) ColVI gene microfluidic card (FluiColVI) profile of native USCs derived from a healthy control sample (Donor1). FluiColVI identified the amplification of all exon-exon junctions, in Col6α3 transcript (50 exons).

Discussion

Many attempts have been done to isolate stem cells from urine specimens and to discover their valuable application in studying various human diseases. However, only few studies described their importance in studying muscular dystrophy.

This study investigate the usefulness of stem cells derived from urine samples as a disease model for DMD, by evaluating their applicability in the diagnosis as well as in drugs screening.

In term of diagnosis we evaluate the ability of USCs to diagnose DMD gene mutation, so that it could be used as an alternative method for muscle biopsy. A muscle biopsy may be necessary for the diagnosis of some DMD cases, and the major disadvantages of muscle biopsies are the risks associated with it including the invasive procedure, using of anesthesia, the risk of muscle damage and infection, and formation of a scar after the surgical procedure.

Moreover, we discover their ability for the screening of drugs efficiency by testing one therapeutic approach which is antisense treatment; this could identify the role of USCs in the screening of new drugs candidates.

Firstly, we optimized a protocol for the isolation of USCs, this was done by applying some new modifications to Zhou protocol and it was successfully led to the isolation of USCs from both healthy controls and DMD patient within 2 weeks (28).

We found that first morning urine samples are the best samples for the successful isolation of USCs, however, it is also possible to use other urine samples collected during the day. Another main modification is the volume of urine samples; we recognized that increasing the urine volume can increase the possibility of obtaining USCs. We found that this protocol is effective for the isolation of USCs irrespective to the age and gender of the donor. In this study we select two healthy male controls and one DMD male patient, to examine the dystrophin gene expression in both.

In order to define the isolated cells as USCs, characterization of the cell surface markers was performed and we found that these cells were characterized by high expression of MSC surface markers, such as CD90 and CD105, together with markers typical of renal epithelial cells (i.e., CD13) and fibroblasts (fibronectin). Moreover, this class of cells retained a remarkable growth reserve in vitro, as shown by the low percentage of cells positive for the senescence associated protein p16 ^{INK4a} and the apoptotic marker annexin V. These cells also expressed a significant level of telomerase activity, indicating that they held characteristics compatible with stem/progenitor cells.

These characterizations allowed us to qualify the urine-derived cells as mesenchymal stem cells, as previously described (28).

USCs show many advantages in comparison with adipose-derived stem cells which are known as the most commonly used MSCs. They have higher differentiation capacity, longer life span in vitro and collected by noninvasive procedures (91).

When we compared this protocol with the available protocols used to obtain myogenic cells for muscle disease studies (e.g. muscle biopsy and skin biopsy), we found that there is no difference between these protocols in terms of timeline for generating myogenic cells. However, the main difference is the noninvasive procedure used for collecting urine stem cells as it does not require hospitalization and can be repeated easily as much as needed.

In this study, USCs were used to study the dystrophin gene expression. We found that USCs derived from DMD patient displayed the dystrophin mutation at both the DNA and RNA levels, and the dystrophin transcript was evidently detectable in both native and MyoD-transformed cells. The dystrophin transcript appeared to be correct in exon composition, with the expected exon-exon junctions, and therefore perfectly matched the RNA seen in skeletal muscle-derived cells.

DMD gene micro-fluidic card (FluiDMD) analysis is considered as an effective and sensitive tool for the detection of mutations and defining the full exon composition in the dystrophin transcript. In this study the deletion mutation was accurately detected by FluiDMD and we found that it was valid for molecular profiling of dystrophinopathies in USCs (75).

From this important finding we can suggest that USCs might be used for a variety of RNA analyses of patients with DMD. These cells could also be a beneficial tool in molecular genetic diagnosis, both for genotyping and for profiling the dystrophin transcript to detect exceptions to the frame rule, splicing choices, and generally speaking, all the cohorts of atypical mutations in dystrophinopathies (54).

These findings are in agreement with Guan and his colleagues study. In their study they successfully reprogrammed USCs derived from DMD patient into iPSC and differentiated it into cardiomyocytes, recapitulating cardiac phenotypes in vitro (29).

This is the first study demonstrated that the direct reprogramming of USCs with the transcription factor MyoD could represent a useful cell model for studying DMD (92).

These findings are also in concordance with what have been reported by Kim and his colleagues. They demonstrated that the direct reprogramming of urine-derived cells into human myogenic cells by inducible expression of MyoD is highly efficient and

reproducible strategy to generate an in vitro model of skeletal muscle from normal individuals as well as those with muscle diseases (37).

In addition to dystrophin gene, we used USCs to study the expression of collagenVI gene in order to discover the possibility of identifying various transcripts. We found that native USCs isolated from healthy control samples revealed also the expression of all ColVI transcripts, with the detection of all exon-exon junctions systems.

The expression of both dystrophin transcripts and ColVI transcripts in native USCs, means that it can be used directly without the need of MyoD transformation for studying various genes expression that encode sarcolemmal associated protein of the muscle cells such as the dystrophin, or making up part of the extracellular matrix that surrounds muscle cells and connective tissue like ColVI, so that it might be adopted for the transcriptomic profiling of others muscle genes in other muscular diseases (limb girdle phenotypes, congenital myopathies, etc.) if the specific genotype and cell phenotype would be recapitulated. Native USCs may foreseeably be used for diagnostic (DNA and RNA studies) and transcriptional profiling in patients with DMD, enabling the detection of variety of mutations (splicing mutations, splicing choices, deep intronic mutations, complex phenotypes) that can escape routine genetic testing.

In fact, we were successfully modulated the dystrophin gene splicing in DMD USCs-MyoD, using an antisense oligonucleotide. Treatment with 2′OMePS-backboned AON induced the skipping of exon 44 in native USCs DMD, and the antisense exon-skipping strategy was seen to restore the dystrophin protein in DMD USCs-MyoD.

We also found that the $\Delta\Delta C_t$ method using FluiDMD card analysis; and the Agilent 2100 bioanalyzer using their high-sensitivity DNA chip both are valid for the quantification of the skipping percentages. However, the Agilent 2100 bioanalyzer using their high-sensitivity DNA chip is more accurate than the relative quantification of the $\Delta\Delta C_t$ method using FluiDMD card analysis.

These results suggest that USCs may provide not only a valid model for genetic diagnosis and studying disease mechanisms, but also for testing drugs efficacy as a noninvasive source of myogenic cells, with consequent high patient compliance.

This finding is also in concordance with Kim et al study, which showed that urine-derived cells can be edited using CRISPR/Cas9 technology, so that it can be applied in genetic correction strategies with significant success especially for muscular dystrophy (37).

Furthermore, the immunofluorescence analysis showed that only USCs-MyoD derived from the control subjects were positive for correct localization of the full-length dystrophin protein at the subsarcolemma, as shown by myosin colocalization and

confirmed by Western blotting. On the other hand, the DMD USCs-MyoD carrying the out-of-frame dystrophin deletion (del. exon 45) were dystrophin negative, as expected.

This finding prove that we were able to recapitulate the dystrophin protein profiling in USCs, which indicate that they are appropriate modeling tool for drug discovery strategy and could play an important role in the identification as well as characterization of drugs candidates by analyzing their effect on protein expression (93).

Conclusion

Urine specimen is a valuable source for obtaining stem cells using simple, noninvasisve, affordable protocol, which is accepted by all subjects with great success of USCs isolation from different donors regardless the age and gender.

In this study we explore the significance of USCs in the diagnosis of DMD mutation and we confirm their usefulness to identify dystrophin gene mutation in DMD patient with a deletion of exon 45, so that it could be an alternative method for the muscle biopsy and a valuable diagnostic tool for DMD patients.

In addition to dystrophin gene we found that native USCs revealed the expression of all collagen VI transcripts (ColVI α1, ColVI α2, and ColVI α3).Thus, indicated that USCs can be used to detect various gene transcripts and can be a useful tool for studying other muscular diseases.

Micro-fluidic card (FluiDMD) analysis is an accurate tool to study the full exon composition, and to detect the pathogenic effect of all dystrophin mutations belonging to any category. In our study it defined the deletion mutation in USCs, so that it should be considered for the molecular profiling of dystrophinopathies.

USCs have been proved to be a valid model for the screening of drug efficacy, as shown by the result of an antisense oligonucleotide which was successfully modulated the dystrophin gene splicing in DMD USCs-MyoD and recapitulated the dystrophin protein. It could also be a beneficial tool in the discovery of new drugs candidates.

Moreover, we found that native USCs can be used as a disease model for drugs screening without transforming into USCs-MyoD, because both cells showed the same resuts of the skipping percentages.

We also demonstrated that $\Delta\Delta C_t$ method using FluiDMD card analysis could be useful in quantifying the skipping percentages giving similar results to the high-sensitivity DNA chip.

Although, we described only one case of DMD, the data that we obtained are promising. Further investigations to fully explore the use of this methodology as a clinical tool and an in vitro model to study other forms of muscular dystrophy require the deep genomic, transcriptomic and proteomic analysis of USCs and myogenic USCs in order to propose them as a modeling tool for other muscular diseases (limb-girdle dystrophy, facioscapulohumeral dystrophy, congenital myopathies, others) if the specific genotype and cell phenotype can be recapitulated as we demonstrated for DMD.

Therefore, we recommend to analyze the whole transcripts using RNA-sequencing (RNA-seq) and the proteomic profile of both native USCs and differentiated myogenic USCs in order to propose them as a model for different diseases.

Further researches are required for the evaluation of USCs as new DMD in vitro model for drugs screening and toxicity/efficacy studies, by testing different therapeutic approaches such as treatment with Translarna, Eteplirsen, and CRISPR/CAS9.

In addition, the possibility of inducing iPSCs from these urine-derived cells may open new perspectives in a variety of research applications; in the post-omics era, in which an enormous number of variations and putative new causative genes remain devoid of functional validation, USCs may represent a versatile tool for gene function exploration and genomic variation validation. Moreover, USCs might also be employed in tissue engineering, particularly with a view to autologous transplantation.

In conclusion, we demonstrated here that USCs from a patient with DMD are able to recapitulate the phenotype of the disease in vitro, thereby legitimizing further investigations to fully explore the use of this methodology as a clinical tool for personalized medicine. This work has been published by Falzarano *et al* (92).

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Abbreviations

ESCs	Embryonic stem cells
iPSCs	induced pluripotent stem cells
MSCs	Mesenchymal stem cells
Oct3/4	Octamer-binding transcription factor 3/4
Sox2	Sex determining region Y
Klf4	Kruppel-like factor 4
c-Myc	Avian Myelocytomatosis virus oncogene cellular homologue
USCs	Urine-derived stem cells
DMD	Duchenne muscular dystrophy
EB	Epidermolysis bullosa
PKD	Paroxysmal kinesigenic dyskinesia
SMA	Spinal muscular atrophies
MD	Muscular dystrophy
DGC	Dystrophin-glycoprotein complex
BMD	Becker muscular dystrophy
NSAA	North Star Ambulatory Assessment
6MWT	6-minute walk test
CK	Creatine kinase
MLPA	Multiplex ligation-dependent probe amplification
SNP	Single nucleotide polymorphism
CGH	Comparative genomic hybridisation
CNV	Copy number variation
AAV	Adeno-associated virus
AONs	Antisense oligonucleotides
2′OMePS	2′O-methyl-phosphorothioate oligonucleotide
PMO	Morpholino phosphorodiamidate oligomers
UCMD	Ullrich congenital muscular dystrophy
ColVI	Collagen VI
ECM	Extracellular matrix
FBS	Fetal bovine serum
REGM	Renal epithelial cell growth medium
bFGF	basic fibroblast growth factor
PDGF	Platelet-derived growth factor

EGF	Epidermal growth factor
PE	Phycoerythrin
APC	Allophycocyanin
PI	Propidium iodide
B2M	β_2-microglobulin
Ad5	Adenovirus serotype 5
TLDA	TaqMan®LowDensity Array
C_t	Cycle threshold
SDS	Sodium dodecylsulfate
PMSF	Phenylmethylsulfonylsulfate
TRITC	Tetramethylrhodamine isothiocyanate
FITC	Fluorescein isothiocyanate
PEI	Polyethylenimine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
RNA-seq	RNA-sequencing