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THE ROLE OF CaSR AND PTH IN HUMAN SKELETAL MUSCLE TISSUE

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ABSTRACT

The skeletal muscle regeneration process is impaired in muscular disorders such as sarcopenia, muscular dystrophies, and also this is a secondary disease present in other disorders and pathologies such as hypoparathyroidism. Pharmacological treatments are not available for these myopathies. Identifying suitable therapeutic targets and testing candidate drugs for ability to improve muscle regeneration require an *in vitro* cellular model to study the biology of this process in the patients. The satellite cells are the postnatal skeletal muscle stem cells which have been observed to be responsible for skeletal muscle regeneration. In our studies, we have established and characterized a primary cell culture of Human Satellite Cells (hSCs) isolated from human skeletal muscle biopsies. The established hSCs lines are capable of growth and can undergo myogenic differentiation, represents a reliable *in vitro* cellular model to study myogenic differentiation process and to identify new molecular targets that could be useful for the treatment of skeletal muscle disorders, characterized by an alteration of myogenesis.

In relation to this, we have analyzed the Calcium Sensing Receptor (CaSR) expression and its possible role during skeletal muscle *in vitro* myogenic differentiation. The results obtained from various techniques for CaSR gene and protein expression analysis and secondary messenger analysis in response calcium (Ca^{2+}) and CaSR drugs have demonstrated that this receptor is absent not only in human skeletal muscle tissues but also in the established hSCs lines and during *in vitro* myogenic differentiation. Additionally, we have not detected any effect of Ca^{2+} and CaSR drugs on *in vitro* myogenic differentiation. Taken together, our data support the fact that despite CaSR being a crucial molecule in physiology and pathologies, probably this receptor is not having any physiological role in skeletal muscle.

Since an alteration of skeletal muscle tissue is a complication in hypoparathyroidism and the direct effect of Parathyroid Hormone (PTH) in skeletal muscle myopathies is not clear, we have analyzed the expression of PTH main receptor, Parathyroid Hormone type-1 Receptor (PTH1R) in human skeletal muscle tissue, in hSCs lines and during myogenic differentiation. Data obtained have demonstrated the presence of PTH1R in human skeletal muscle tissue, in hSCs lines and during *in vitro* myogenic differentiation. Moreover, our data have also shown a significant increase of PTH1R expression during *in vitro* myogenic differentiation. Taken together, the data suggest a possible involvement of PTH in skeletal muscles for the first time. This finding could be very important to evaluate in future a potential therapeutic effect of PTH in myopathies.

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1 INTRODUCTION

1.1 Human Skeletal Muscle

Skeletal muscle is one of the three major muscle types, the others are the cardiac muscle and the smooth muscle, and all together form the muscular system. The muscular system is controlled through the nervous system, although some muscles such as the cardiac muscle can be completely autonomous. The muscular system, together with the skeletal system forms the musculoskeletal system. The musculoskeletal system is the most voluminous system of the human body and represents about the 80% of the total weight and consists of bones of the skeleton, cartilages, muscles, tendons, joints and other connective tissue that binds tissues and organs together and support the human body in movement and also protect the vital organs. The largest part of the musculoskeletal system is represented by the skeletal muscle, which accounts (on average) 40-50% of an adult male's and 30-40% of an adult female's body weight (1) (Fig. 1).

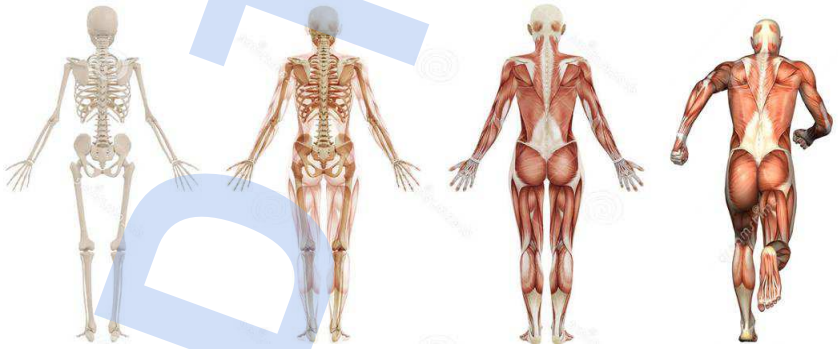


Fig. 1. *Human skeletal Muscle system.*

The skeletal muscles are under the voluntary control of the somatic nervous system, thus also referred as voluntary muscle, while smooth and the cardiac muscles are not under voluntary control and therefore designated as involuntary muscles (1). Skeletal muscles are striated in appearance and are composed of long cylindrical fibers with many peripherally located nuclei. Skeletal muscles are anchored to bones by tendons, and they are not only crucial for locomotion, maintaining

posture and resisting gravity, but also are essential to prevent an excessive movement of bones and joints. Thus, skeletal muscles play a crucial role in protecting the skeleton from excess movement and preventing skeletal structure damage or deformation. Skeletal muscles also protect internal organs (particularly abdominal and pelvic organs) by acting as an external barrier or shield to external trauma and by supporting the weight of the organs. Skeletal muscles are also responsible for subtle movements, such as facial expressions, eye movements, swallowing, urination, defecation, as well as respiration (1). On the other hand, the involuntary smooth muscles are non-striated in appearance and are composed of short spindle-shaped fibers with a single nucleus. The smooth muscles are found in the walls of several organs and structures, for example esophagus, stomach, intestine, blood vessels, uterus, etc. where they are responsible for involuntary movements such as moving food, involuntary control of respiration, secretions and blood flow in arteries (1). The involuntary cardiac muscles are striated in appearance like skeletal muscles, but unlike skeletal muscle, the cardiac muscle formed by special short branched muscle fibers and a majority of cardiac fibers are with only one nucleus. Cardiac muscles form the contractile walls of the heart and responsible for the powerful pumping of blood in Heart (1). The fig. 2 represents the muscular system in the human body.

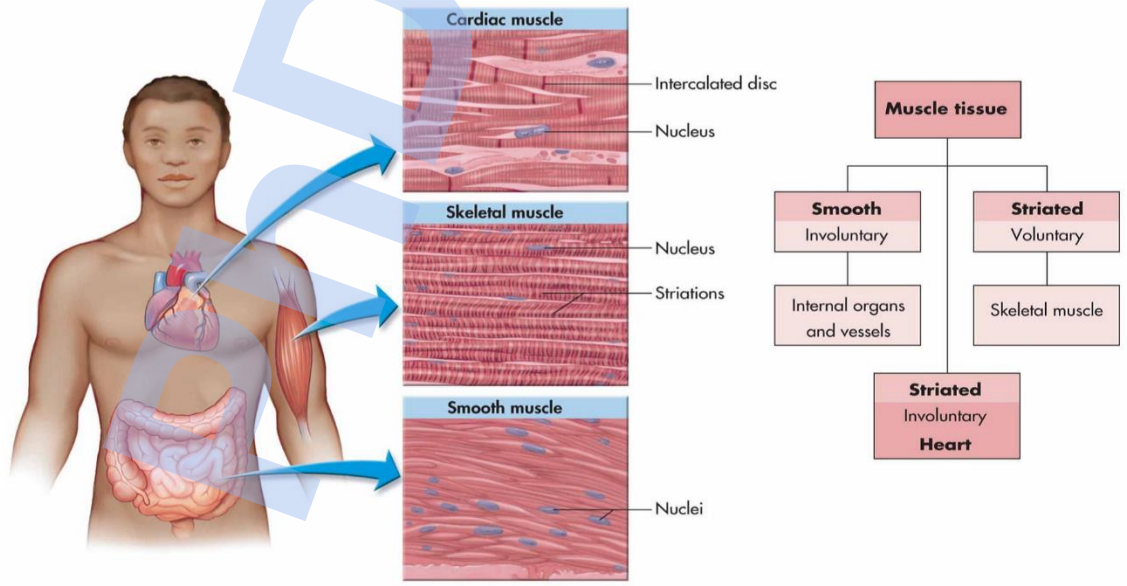


Fig. 2. *Human muscular system. The figure was taken from reference* (2).

Each skeletal muscle is an organ that consists of various integrated tissues; these tissues include the long cylindrical multinucleated skeletal muscle cells (called myofibers), blood vessels, nerve fibers, and connective tissues (Fig. 3). Myofibers can be quite large, with diameters up to 100 µm and lengths up to 30 cm in the Sartorius muscle of the upper leg (1).

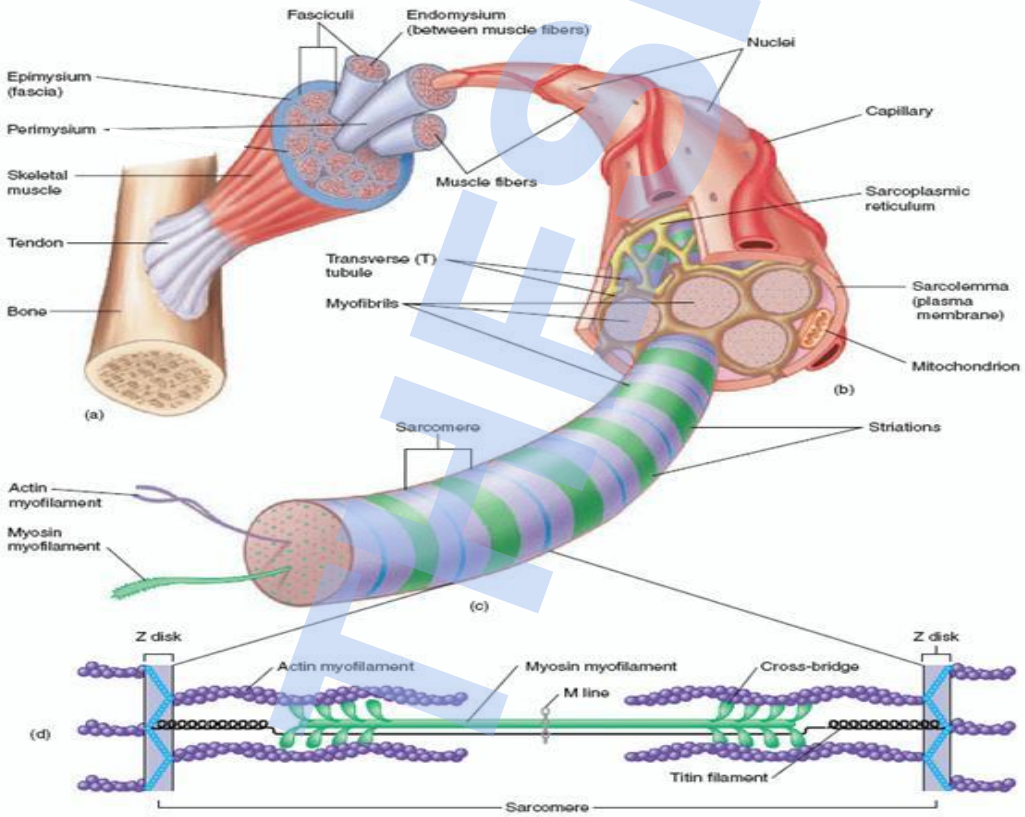


Fig. 3. *The internal structure of a skeletal muscle. The figure was taken from reference* (3). ***Epimysium:*** *The connective tissue that surrounds the entire muscle.* ***Perimysium:*** *The connective tissue that surrounds a fascicle (bundle of skeletal muscle fibers).* ***Endomysium:*** *The connective tissue that surrounds an individual muscle fiber.* ***Sarcolemma:*** *The cell membrane of an individual muscle fiber (or muscle cell).* ***Sarcoplasmic Reticulum (SR):*** *The endoplasmic reticulum of the muscle cell.* ***Transverse Tubules (T-tubules):*** *The extensions of the sarcolemma, which penetrates inside muscle fibers and tightly associate with the SR.* ***Myofibril:*** *A cylindrical bundle of contractile filaments within skeletal muscle cells.* ***Myofilaments:*** *The individual contractile proteins that make up the myofibril. There are two types of myofilaments; actin* ***(thin filament)*** *and myosin* ***(thick filament).***

During early development, embryonic myoblasts, each with its nucleus, fuse with up to hundreds of other myoblasts to form the long cylindrical multinucleated muscle cells. Multiple nuclei mean multiple

copies of genes, permitting the production of the massive amounts of proteins and enzymes needed for muscle contraction (4). Each myofiber communicates with blood vessels for nourishment, oxygen delivery, and waste removal. In addition, every myofiber in a skeletal muscle is supplied by the axon branch of a somatic motor neuron, which signals the myofiber to contract. Each skeletal muscle has three layers of connective tissue (i.e. epimysium, perimysium and endomysium) that enclose it and provide structure to the muscle as a whole, and also compartmentalize the myofiber within the muscle. The endomysium is the connective tissue that surrounds each skeletal myofiber together with blood vessels, lymphatic vessels, and nerves. The perimysium encircles a group of myofibers, forming a fascicle. The epimysium encircles all the fascicles to build a complete skeletal muscle. The epimysium allows a muscle to contract and move powerfully while maintaining its structural integrity. The epimysium also separates muscle from other tissues and organs in the area, allowing the muscle to move independently. Each myofiber contains sarcolemma, sarcoplasm, and sarcoplasmic reticulum. The sarcolemma is the plasma membrane of the myofiber. The sarcoplasm is the cytoplasm of the myofiber contains a large number of glycosomes, a large amount of myoglobin, and a specialized smooth endoplasmic reticulum called the sarcoplasmic reticulum (SR), which stores, releases and retrieves calcium ions (Ca^{2+}) which is the foundation for muscle contraction. Transverse tubules (T-tubules) are invaginations of the sarcolemma into muscle fibers, which are tightly associated with SR, in turn, allow the nerve impulses to penetrate the muscle cell and activate the SR, which results into the initiation of the process of muscle contraction. Each myofiber is composed of several myofibrils, and each myofibril is formed by several functional units called sarcomeres. Each sarcomere is a highly organized arrangement of the contractile myofilament's α-actin (thin filament) and myosin (thick filament), along with other support contractile proteins. The presence of alternating thin and thick filaments along the length of myofibrils results in the striated appearance of skeletal muscle (1).

1.2 Myogenesis

Myogenesis is the formation of skeletal muscle tissue, particularly during embryonic development. During embryonic development and growth, the mesoderm skeletal muscle progenitor cells called myoblasts proliferate and coalesce with the formation of a multinucleated, syncytial muscle fiber or myotube. At this stage, the nuclei of the myotube are still centrally located in the muscle fiber. In the course of the synthesis of the myofilaments/myofibrils, the nuclei are gradually displaced to the periphery of the cell. The formed fibers are called primary myofibers (5, 4) (Fig. 4).

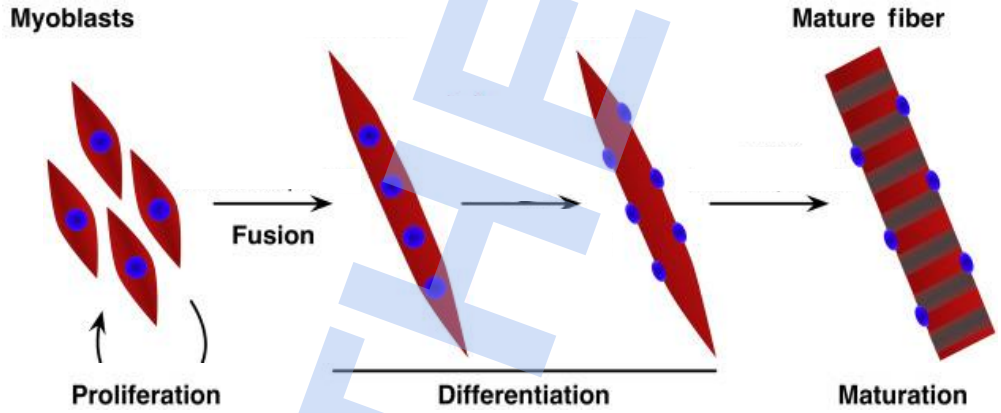


Fig. 4 *Myogenesis. The figure was taken from reference* (6).

Unused myoblasts during embryonic development dedifferentiate into muscle stem cells called satellite cells (SCs), which persist in a quiescent state (5). These cells remain adjacent to muscle fibers, situated between the sarcolemma and the basal lamina. Many cells, including skeletal muscle fibers, are coated by a layer of extracellular matrix material called the basement membrane (BM). The BM, in turn, is composed of two layers: an internal, basal lamina (BL) directly linked to the plasma membrane, and an external, fibrillar reticular lamina. In the postnatal stage, when needed, the SCs can be activated and undergo self-renewal or myogenic differentiation either by fusion to myofibers in order to augment existing muscle fibers or can form completely new muscle fibers called secondary myofibers (4, 5, 7). In neonatal stages, the number of formed primary myofibers remains constant, but each primary myofiber grows in size by fusion of SCs (8).

Adult mammalian skeletal muscle is stable under normal conditions, with the only sporadic fusion of SCs to compensate for muscle turnover caused by daily wear and tear. However, adult skeletal muscle has a unique feature, i. e. remarkable ability to regenerate after exercise and injury. The SCs play the main role in this process (4, 7).

1.3 Human Satellite Cells and Skeletal Muscle Regeneration

Responding to exercise or injury, skeletal muscle undergoes a highly orchestrated regenerative process that takes place at the tissue, cellular, and molecular levels, which results in the reformation of innervated, vascularized contractile muscle system, the process is called skeletal muscle regeneration (8). Satellite cells (SCs) are adult skeletal muscle stem cells which play a principal role in the process of skeletal muscle regeneration (9, 10).

The adult stem cells are undifferentiated cells found among differentiated cells in a tissue or organ. The adult stem cell can renew itself and can differentiate to yield some or all of the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are present (11-13). The history of research on adult stem cells began more than 70 years ago, in the 1950s, when researchers discovered that the bone marrow contains a population of cells called hematopoietic stem cells, which can differentiate into all the types of blood cells in the body. Later, researchers have identified adult stem cells in many organs and tissues, including brain, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium, and testis (12, 14). The features of the adult stem cells are that they are very small in number in each tissue, reside in a specific area of each tissue (called a "stem cell niche"), have self-renewal capacity, remain quiescent (non-dividing) for long periods of time until they are activated by a need for more differentiated cells to maintain tissues by disease or tissue injury (12). The adult stem cells divide as little as possible to minimize the risk of accumulating harmful genetic mutations. The findings of adult stem cells have generated a great deal of excitement as the stem cells offering the possibility of a renewable source of replacement of specific types of cells and, a model to study pathologies and to find therapeutic drug targets or to test the effectiveness of available drugs (14). However, adult stem cells have a limited capacity

to divide, which makes it difficult to generate a large number of stem cells (11,14).

Mesenchymal stem cells (MSCs) are specialized adult stem cells characterized by their trilineage differentiation potential into mesodermal lineage such as bone, cartilage, adipose tissue, tendons, skeletal muscle, blood cells and skin (15). Though the concept of MSCs attributed to Bone Marrow derived Mesenchymal Stem Cells (BM-MSCs) however at present, it encompasses cells from other sources too, such as adipose tissue, peripheral blood, and skeletal muscle (16). MSCs are also capable of differentiating into non-mesenchymal lineages such as neural cells, endothelial cells, astrocytes, cardiomyocytes, etc. MSCs are characterized by properties such as adherence to plastic in culture and expression of cell surface markers such as CD73, CD90 and CD105 (15). MSCs have received significant attention in recent years with regard to their suitability for use in cellular therapies due to their high growth potential and multipotential differentiation capacity (15, 16). The SCs are traditionally considered as committed myogenic precursors cells, but they have also found to possess mesenchymal plasticity, shown by their potential to differentiate into mesenchymal alternatives such as adipocytes and chondrocytes (17, 18).

Alexander Mauro discovered the SCs in 1961. He observed a group of mononuclear cells at the periphery of adult skeletal muscle myofibers by electron microscopy (19). These cells were named satellite cells due to their sublaminar location and intimate association with the plasma membrane of myofibers. The direct juxtaposition of SCs and myofibers immediately raised a hypothesis that these cells may be involved in skeletal muscle regeneration and growth (19). Later on, the studies demonstrated that the SCs are the cells that proliferate and differentiate into myoblasts to form multinucleated myofibers, which contribute to postnatal skeletal muscle growth and repair (20-25).

The studies have shown that the SCs reside in bundles of muscle fibers (myofibers) located between the basal lamina and sarcolemma of muscle fibers, the niche enables SCs to exist in a dormant state until

needed. In the dormant or quiescent state, SCs express satellite cell-specific transcriptional factors PAX7 (9). The exercises or injury to skeletal muscles, cause deterioration of the basal lamina surrounding SCs which cause SCs to exit from the quiescent state to a state called satellite cell activation. At this state, the SCs divide for self-renewal, subsequently undergo myogenic differentiation to regenerated myofibers along a unidirectional, hierarchical pathway. The process of myogenic differentiation of SCs is an irreversible procedure and is driven by the sequential expression of key myogenic regulatory transcriptional factors Myf5, MyoD1, Myogenin and Mrf4 (master regulators), which are destined to transduce gene expression signals to their target genes and finally leading to the expression of muscle-specific structural and contractile genes such as those encoding α-actins, myosin heavy chain (MHC), troponins and other contractile proteins (9, 10)(Fig. 5).

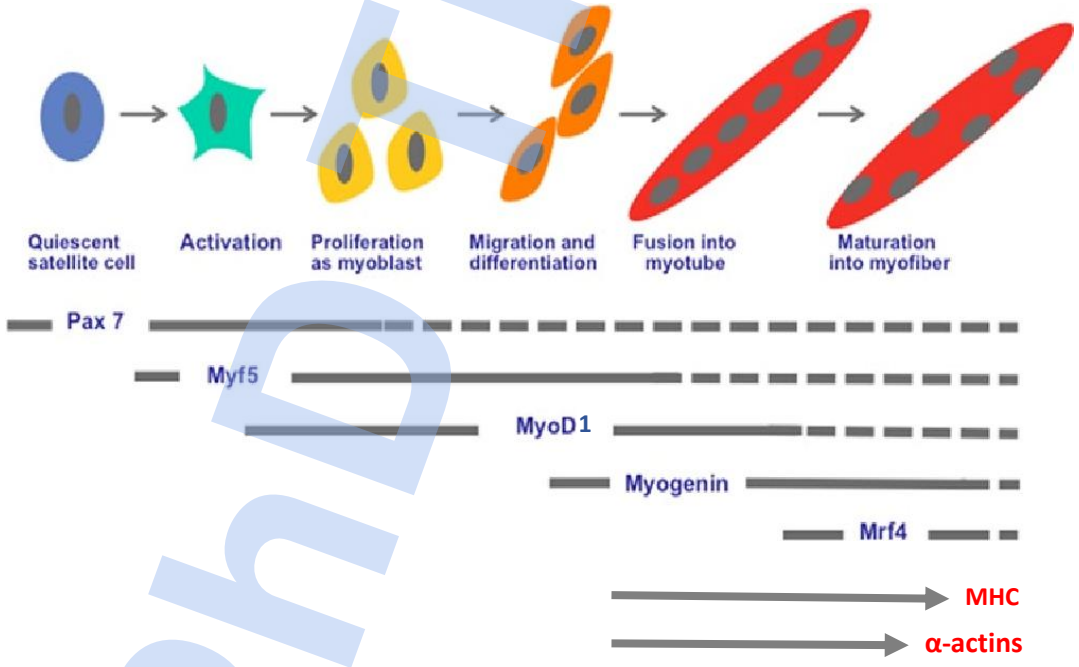


Fig. 5. *Satellite cells activation, proliferation, differentiation and fusion to produce myofibers, is regulated by the sequential expression of transcription factors Myf5, MyoD1, Myogenin and Mrf4 (master regulators), which leads to the expression of muscle-specific structural and contractile genes such as myosin heavy chain (MHC) and a-actins. The figure was taken from reference* (26).

The activated and proliferating SCs and their progeny are referred to as myogenic precursor cells or adult myoblasts. Adult myoblasts express the myogenic transcription factors Myf5 and MyoD1. At this stage, activated SCs have the capability to return to quiescence. This capacity permits to maintain stem cell pool, which is extremely important for long-term muscle integrity. The expression of MyoD1 is proposed to be associated with a subpopulation of committed SCs, which are poised to differentiate without proliferation, thus the expression of MyoD1 is a crucial determinant of myogenic differentiation. In the absence of MyoD1, activated myoblasts have a propensity for proliferation and self-renewal only. This also explains the spectrum of proliferation and differentiation potential observed in different primary myoblast clones cultured *in vitro*, which results in a heterogeneous population. The adult myoblasts co-expressing both Myf5 and MyoD1 begin differentiation by downregulation of Pax7, implying to this, the ratio of PAX7 and MyoD1 expression is considered a determining factor for SCs fate. A high ratio of PAX7 to MyoD1 (as seen in quiescent SCs) keeps SCs in their quiescent state. The initiation of terminal differentiation starts with the expression of Myogenin and Mrf4. The induction of Myogenin expression primarily depends on MyoD1 and is proposed to enhance expression of a subset of muscle-specific structural and contractile genes such as those encoding α-actins, myosin chain, and troponins, previously initiated by MyoD1. The studies have shown that Myf5, MyoD1, and Myogenin activate the expression of a collection of myogenic microRNAs (e.g., miR-1, miR-133, miR-206 together called MyomiRs). These myogenic microRNAs, in conjunction with other microRNAs, modulate the expression levels of other myogenic factors during satellite cell activation/proliferation and differentiation (9,10, 27, 28).

Because satellite cells are committed myogenic precursors cells and also possess the capability of self-renewal, a unique feature of stem cells, they play a central role in the search for therapies for skeletal muscle disorders.

1.4 Skeletal Muscle Regeneration Disorders

Although a robust trait in healthy conditions, the self-repair capacity of skeletal muscle can be severely impaired in different types of congenital or acquired diseases, called myopathies. Among which we can find sarcopenia and muscular dystrophies.

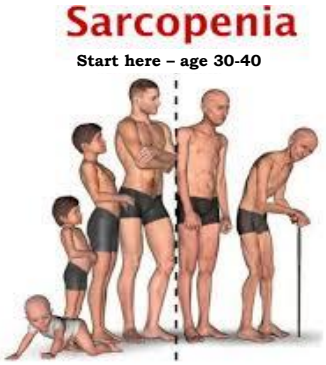


Fig. 6. *Progression of Sarcopenia. The figure was taken from reference* (110).

Sarcopenia is a syndrome characterized by progressive and generalized loss of skeletal muscle mass and strength as a consequence of aging. Loss of muscle can begin as early as 30-40 years of age in sedentary individuals and is responsible for frailty and lack of mobility in the aged (Fig. 6). One of the leading causes of sarcopenia is the failure of Satellite Cells (SCs) activation to undergo myogenesis for skeletal muscle regeneration (29-31). Age induced muscle weakness leading to frailty is a major public health problem that is predicted to escalate in the future as the number and proportion of older adults increase in the general population (32). At present, resistance exercise with protein supplementation is the only effective treatment available for sarcopenia.

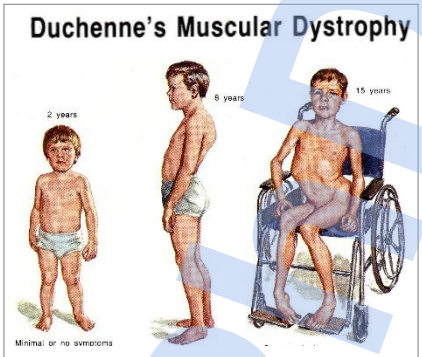


Fig. 7. *Progression of muscular dystrophies. The figure was taken from reference* (111).

The muscular dystrophies are a heterogeneous group of heritable disorders in which progressive muscle degeneration provokes an eventual decrease in muscle mass and extensive fibrosis without a central or peripheral nerve abnormality (Fig. 7). The studies have shown that one of the main causes of muscular dystrophies, is the failure of SCs to enter into the myogenic program (33, 34). There is

currently no cure available except specific physical and medical treatments that can improve symptoms and slow down the progression of the disorder.

The muscle weaknesses are also reported as a secondary symptom in different diseases such as influenza, diabetes, multiple sclerosis, Guillain-Barre syndrome, fibromyalgia syndrome, and hypoparathyroidism. Nowadays, the pathophysiologic mechanism for secondary myopathy is not known.

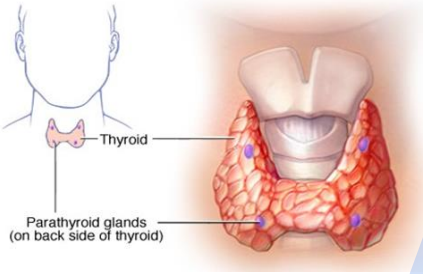


Fig. 8. *Parathyroid glands. The figure was taken from reference* (56).

Hypoparathyroidism is an uncommon condition in which our body secretes abnormally low levels of Parathyroid Hormone (PTH). PTH is a key regulator of calcium homeostasis (Fig. 8). The majorly affected organs in this disorder are skeletal muscle, nervous system, and cardiac system due to hypocalcemia. However, it has been observed that even after normalizing the level of calcium in the blood with conventional treatment such as with oral calcium and active vitamin D supplementation, some patients frequently complain about the muscle weakness and poor quality of life and need multiple hospitalizations (35-37). The cause of these kinds of skeletal muscle weaknesses in this disorder has not been understood.

Since the majority of the skeletal muscle disorders have no cure, there is an unmet need for therapeutic strategies that can treat skeletal muscle degenerative disorders.

1.5 Calcium Sensing Receptor (CaSR)

The CaSR is a class C G Protein-Coupled Receptor (GPCR) and is the main systemic regulator of calcium homeostasis (38). GPCRs, which are also known as the seven-transmembrane domain (7TM) receptors, constitute a large protein family of receptors that detect molecules outside the cell and activate internal signal transduction pathways and, ultimately, cellular responses. GPCRs are involved in many diseases and are molecular targets of approximately 40% of all modern medicinal drugs (39). Calcium (Ca^{2+}) is a universal signal carrier for biological information, being one of the most specific and most selective messengers in nature. It is involved in multiple signaling cascades critical for cell survival, growth, differentiation, and death (40, 41). The CaSR is the fundamental tool used by cells to detect subtle changes in extracellular Ca^{2+} and has been the subject of intense research activity since it was first cloned 25 years ago (42).

The CaSR expressed primarily within parathyroid glands where it regulates Parathyroid Hormone (PTH) secretion, which in turn controls calcium homeostasis via acting upon kidney, bone, and intestine to maintain Ca^{2+} within the physiological range (1.1–1.3 mM) (38). Initially, the studies on CaSR were focusing on calciotropic tissues such as parathyroid, kidney, and bone, which have apparent roles in maintaining calcium homeostasis. By now, it has become clear that CaSR is expressed in many other cells and organs, without any evident role in maintaining calcium homeostasis such as in neurons, lungs, skin, placenta, breast and endothelium cells (blood vessels) (40, 43). Several studies have observed that an abnormal CaSR expression and function is implicated not only in calciotropic disorders such as hyper- and hypoparathyroidism but also in diseases linked to non-calciotropic systems, such as of the nervous, reproductive, respiratory system and even in conditions such as chronic inflammation & cancer (40, 41, 43).

1.5.1 CaSR Structure and Signalling Pathways

The human CaSR gene is located on chromosome 3q13. 3–q21 and spans over 50 kb of genomic DNA (44). It has a coding region of 3234 bp, which is contained within six exons. The full-length human CaSR mRNA encodes a protein of 1078 amino acids consisting of a 612 amino acids signal peptide extracellular domain (ECD), a 250 amino acid seven transmembranes (7TM) domain and an unusually long 216 amino acids intracellular tail domain (ICD) (43-45) (Fig. 9).

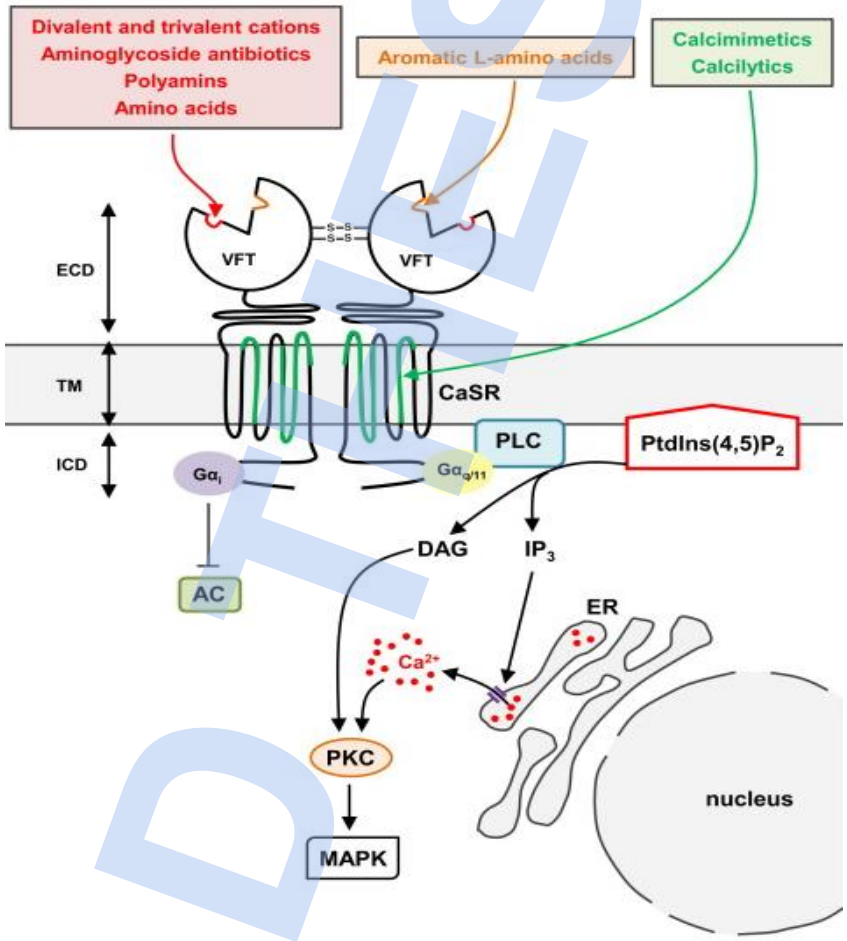


Fig. 9. *Structure and signaling pathways of CaSR.* ***ECD:*** *Extracellular domain,* ***TM:*** *Transmembrane,* ***ICD:*** *Intracellular tail domain,* ***VFT:*** *Venus flytrap,* ***$G_{q/11}$:*** *G protein subtypes,* ***AC:*** *Adenylate cyclase,* ***PLC:*** *Phospholipase C,* ***PtdIns(4,5)P_2:*** *phosphatidylinositol 4,5-bisphosphate,* ***DAG:*** *diacylglycerol,* ***IP_3:*** *inositol 1,4,5-trisphosphate,* ***PKC:*** *Protein kinase C,* ***ER:*** *Endoplasmic reticulum,* ***MAPK:*** *Mitogen-activated protein kinases. The figure was taken from reference (46).*

The NH_2-terminal signal peptide is 19 amino acids in length, which directs the nascent protein to the endoplasmic reticulum (ER), where it

is subsequently removed. The ECD is composed of a bilobed Venus flytrap (VFT) domain followed by a cysteine-rich domain connected via a peptide linker region to the 7TM, which in addition to the membrane α-helices also contains three intracellular and three extracellular loops, the final 7TM α-helix is connected to the ICD, the membrane-proximal domain, which is essential for CaSR expression and intracellular signaling (43). The CaSR protein is processed as a homodimer in the ER via 2 covalent intermolecular disulfide bonds (present in VFT domain) and hydrophobic interactions and functions as a dimer when mature (43). The CaSR contains 11 potential N- linked glycosylated sites in the ECD and is glycosylated in the ER with high mannose carbohydrates (immature receptor) after which it is transported to the Golgi where it is further modulated with complex carbohydrates (mature receptor) (43, 44). Western blot analysis of protein extracted from cells represents two major bands under reducing conditions, corresponding to an incompletely processed, high mannose intracellular form the receptor at ~140 kDa and a fully glycosylated cell surface-expressed form at ~160 kDa. Glycosylation does not appear to be crucial for CaSR function, but rather for proper protein folding and trafficking (43, 44).

The CaSR can be activated not only by Ca^{2+} ions but also by Mg^{2+}, amino acids, polyvalent cations such as spermine, aminoglycoside antibiotics such as neomycin and polypeptides such as amyloid β-peptide (43) (Fig. 9). The CaSR activity is also modulated by pH and salinity and can be potentiated in the presence of allosteric activators such as L-amino acids and type II calcimimetics (interact with the CaSR at transmembrane domains) (43) (Fig. 9). Essentially, agonist Ca^{2+} binding to a cleft in the VFT lobes of each monomer leads to their closure and rotation about the VFT dimer interface, thus transmitting a conformational change to the cysteine-rich domain which in turn allows movement of the 7TM α-helices with respect to one another resulting in connection of G proteins to the intracellular loops and initiation of signaling pathways (43, 44, 47). Depend on the ligand binding and cellular context, CaSR interacts with multiple

heterotrimeric G protein subtypes (Gq/11, Gi/0, G12/13), stimulating diverse secondary messengers, thereby regulating highly divergent downstream signaling pathways (43). Thus, a broad understanding of the genetic, molecular, and cellular regulation of CaSR expression and signaling is crucial to comprehend both its importance in normal physiology and pathophysiology to devise unique, targeted drugs to treat diseases linked to impaired CaSR expression or function.

1.5.2 CaSR Drugs

The drugs NPS R-568, cinacalcet (a modified NPS R-568), and etelcalcetide are positive allosteric modifiers of CaSR. They are called calcimimetics, which potentiate the activity CaSR and decreasing parathyroid hormone release (48, 49). The cinacalcet and Etelcalcetide have been approved for treatment for secondary hyperparathyroidism caused by chronic kidney disease (49, 50). On the other hand, NPS 2143 is a negative allosteric modifier of CaSR called calcilytics, which blocks CaSR, and permit an increase in bone density. Hence, it has been researched as a possible therapy for osteoporosis (51). Unfortunately, clinical trial results in humans have proven disappointing, with sustained changes in bone density not observed, despite the drug being well tolerated. More recent research has shown the CaSR receptor to be involved in numerous other diseased conditions, including Alzheimer's, asthma, and some forms of cancer, and calcilytic drugs are being researched as potential treatments for these diseases (40). The CaSR pharmacological drugs, the phenylalkylamines; NPS R-568 and NPS 2143 are frequently used in research as an agonist and an antagonist of CaSR, respectively (48, 52). Both these drugs bind in the 7TM Domain of CaSR (53, 54) (Fig. 9).

1.6 Hypoparathyroidism

Hypoparathyroidism is one of the disorders which is characterized by the presence of skeletal muscle dysfunctions, is an endocrine deficiency disease identified by the absence or inappropriately low concentrations of circulating Parathyroid Hormone (PTH) (36, 37, 55). In hypoparathyroidism the absent or low circulating PTH levels lead to hypocalcemia by impairing its function in the efflux of calcium from bone; enhancing urinary calcium excretion, and inhibiting the renal synthesis of an active form of vitamin D called calcitriol or 1,25-dihydroxyvitamin D (1,25 (OH)2 D), which impairs the intestinal absorption of dietary calcium (Fig. 10). The deficiency of PTH also causes hyperphosphatemia due to an increase in the renal tubular reabsorption of phosphate. The typically low 1, 25 (OH) 2 D, hyperphosphatemia, and absent or low PTH levels at the time of hypocalcemia is the hallmark of hypoparathyroidism and helps to differentiate hypoparathyroidism from other disorders associated with hypocalcemia (36, 37, 55).

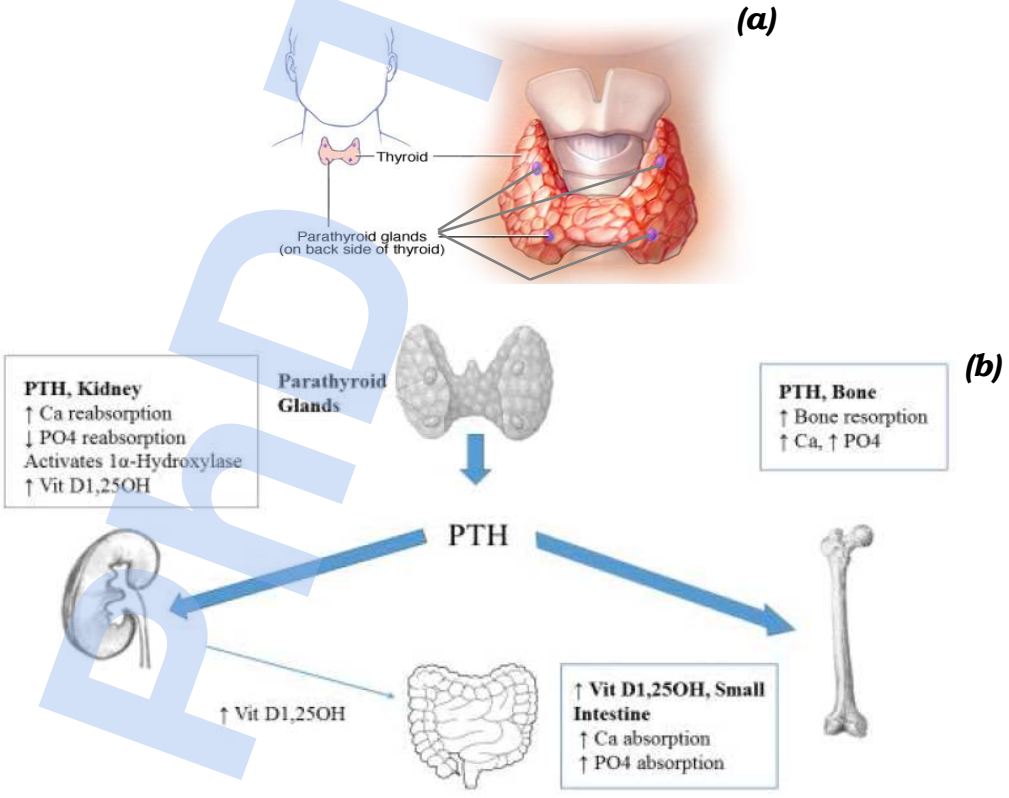


Fig. 10. *Calcium homeostasis. Figure (a) was taken from reference* (56) *and figure (b) was taken from reference* (57).

Several studies have shown that overall higher prevalence of hypoparathyroidism in women (71–88% depending on studies) in the fifth decade of life (58). Epidemiologic studies estimate that the incidence of hypoparathyroidism is 37 per 100 000 person-years in the United States (58) and 22 per 100 000 person-years in Denmark (58) while lower prevalence found in Norway (59). The mean hospitalization rate for hypoparathyroidism in Italy was 5.9 per 100,000 person-year (60). The most common cause of hypoparathyroidism is inadvertent removal or irreversible damage to the parathyroid glands during neck surgery (36). The clinical presentation of hypoparathyroidism correlates strongly with the levels of serum calcium (less than 7.5–8.0 mg/dl) (36). Hypocalcemia can affect the function of most organs, but the particular clinical manifestation in hypoparathyroidism are neuromuscular irritability (e.g. tetany, muscle cramping, paraesthesia, laryngospasm, and bronchospasm) leading to central nervous system alterations (seizures and altered mental status), cognitive symptoms (inability to concentrate, depression and anxiety), skeletal muscle symptoms (fatigue and weakness) and cardiac complications (e.g. congestive heart failure and prolonged QT interval) (35, 36, 55, 61). Conventional therapy of hypoparathyroidism consists of oral calcium (Calcium carbonate) or activated vitamin D (calcitriol or alfacalcidol) supplements at doses adjusted to meet the needs of the patient, depending on the severity of hypocalcemia (62). However, there are complications related to conventional therapy as the high doses result in higher calcium and phosphate product, which results in calcification of the tissues. The therapeutic goals of conventional therapy are to prevent symptoms and signs of hypocalcemia; to maintain low normal calcium and normal phosphate levels and a calcium-phosphate product less than 55 mg2/dL2 in order to avoid hypercalcemia and complications such as extraskeletal calcifications (37). It has been observed that patients on conventional therapy often have complaints suggestive of reduced quality of life, fatigue, and muscle weakness, and even some patients remain symptomatic, requiring multiple hospitalizations (35, 37, 62). The failure of conventional therapies is likely related to the deficiency of

PTH, as the PTH receptor is expressed in many tissues (63, 64). Thus, to establish a more physiologic alternative to conventional therapy, studies aimed at PTH replacement, which was first initiated with synthetic human PTH (hPTH 1-34; Teriparatide), the biologically active amino-terminal fragment of the full-length PTH peptide (65). Later, the more stable full-length form of recombinant human PTH (rhPTH 1-84; Natpara), which was the first form of PTH approved by the FDA in January 2015 for adjunctive therapy in the management of hypoparathyroidism (66). Both the hPTH 1-34 and rhPTH 1-84, have been shown to bind and to activate the PTH type-1 Receptor (PTH1R), the main receptor of PTH. Both the form of PTH have shown improvement in patients as compared to conventional therapy (66). The clinical trials for hypoparathyroidism using PTH (1-84) for 6 years have shown that this treatment was safe and effective for up to six years (67). Given the lack of such data beyond 6 years, it is recommended that rhPTH 1-84 can be prescribed only for those patients who cannot be well controlled on conventional treatment (35, 66). However, the standard precise physiological minute-to-minute control of calcium homeostasis is not regained in response to any therapy, and the homeostasis of other minerals remains affected, including relatively high phosphate levels.

1.6.1 Role and Function of Parathyroid Hormone type -1 Receptor (PTH1R)

PTH type-1 Receptor (PTH1R) is the main receptor of PTH through which it executes its functions on different cellular systems (63, 68-70). The organization of the PTH1R gene is highly homologous in three mammalian species, namely rat, human, and mouse (71). This gene extends over 22 kb and contains at least 15 exons and 14 introns. The human gene was mapped to chromosome 3p22–p21.1 (71). PTH1R is also the main receptor for PTH-related peptide (PTHrP), a product of a distinct gene, shares 8 of 13 amino-terminal residues with mature PTH, and was first identified as the product released from tumors that cause

the hypercalcemia of malignancy syndrome (63, 70). There is another PTH receptor that exists in humans called PTH type-2 Receptor (PTH2R). In contrast to PTH1R, PTH2R is not activated by PTHrP but by PTH and the tuberoinfundibular peptide of 39 amino acids (TIP39). TIP39 is known as the main ligand of PTH2R, although the similarity of sequence between TIP39 and PTH is low (71). In addition to PTH1R and PTH2R, zebrafish and other teleosts possess a third receptor, PTH3R, which derived by the duplication of PTH1R (71). PTHRs share the same basic structure (seven transmembrane domains) with that of the class B G Protein-Coupled Receptor (GPCR) (63, 71). A defining feature of the class B receptors is the relatively long extracellular N-terminal domain, which is essential for peptide ligand binding. The presence of six cysteine residues that are strictly conserved within the N-terminal domain of all class B GPCRs suggests that three disulfide linkages are of critical importance for peptide binding and signaling (71).

PTH1R is expressed at high levels in bones and kidneys, where it mediates the classical effects of PTH on Ca^{2+} and P_i homeostasis. Furthermore, PTH1R has been reported to have a role in the proliferation and differentiation of chondrocytes (71). PTH1R mRNA expression has been reported in various human tissues (64), but the physiology of the PTH in respective tissues has not been explored.

PTH1R upon activation in bone and kidney reported to couple with heterotrimeric G protein subtypes Gq and Gs and stimulate secondary messengers Inositol trisphosphate (IP_3) and cyclic adenosine monophosphate (cAMP), respectively, which results in the downstream signaling in the cells (72)(Fig. 11).

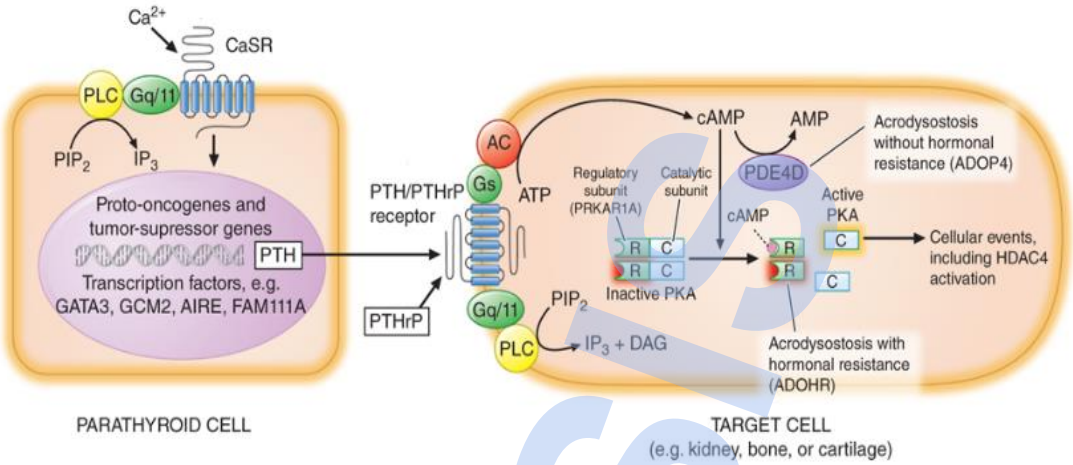


Fig. 11. *PTH1R signaling pathways. The figure was taken from reference* (72).

1.6.2 Hypoparathyroidism and Skeletal Muscle Disorders

The skeletal muscles are severely affected in hypoparathyroidism (35, 36). The studies have correlated the skeletal muscle myopathies in hypoparathyroidism to hypocalcemia, as calcium is one of the main components required for muscle contraction (73). However, it has been reported in many studies that even after normalization of calcium in hypoparathyroidism, patients still complain about fatigue and muscle weakness (35, 37). However, these kinds of hypoparathyroidism-associated myopathies can occur with a greater frequency than the one described in the literature. This is probably due to the fact that myopathy is rarely diagnosed. (35, 74). The effect of PTH in skeletal muscle has not been understood. In a recent randomized clinical trial of six months of PTH replacement treatment provided in 62 patients with chronic hypoparathyroidism, as one daily injection of a fixed dose of 100 µg added to conventional therapy does not improve muscle function, instead, muscle strength is slightly reduced (75). However, in the same study, it was found that the dose of PTH used in the study was too high as the patients developed hypercalcemia in response to therapy; this may have blunted a potential beneficial effect of PTH replacement therapy on skeletal muscle. Thus, further clinical and basic research studies are needed to understand the impact of PTH therapy on skeletal muscle function.

2 AIMS AND SCOPES

Skeletal muscle regeneration is impaired in muscular diseases such as sarcopenia, muscular dystrophies, and other pathologies, including hypoparathyroidism (30, 31, 33-35, 37). Pharmacological treatments are not available for these myopathies. Hence, there is an unmet need for therapeutic strategies that can treat skeletal muscle degenerative disorders. Identifying suitable therapeutic targets and testing candidate drugs for the ability to improve muscle regeneration require cell-based model systems that reliably predict *in vivo* effects in both pre-clinical rodent models and human patients. The satellite cells are the postnatal skeletal muscle stem cells responsible for skeletal muscle regeneration during a lifespan. Thus, one main solution to find suitable drug targets for skeletal muscle degenerative pathologies is the establishment of the reliable human cellular model of myogenesis *in vitro* using satellite cells, which could be later useful for finding therapeutic drugs target for skeletal muscle degenerative myopathies.

In the last years, a number of techniques have been developed to isolate single muscle fiber from small rodents allowing detailed investigations of the functional properties at the biochemical and cellular levels in normal and pathological conditions (76-80). Because of their high degree of differentiation and specialization, it is difficult to maintain differentiated muscle fibers in culture for more than a few days (78, 80). In the last decade, it has become possible to isolate and culture the population of satellite cells. However, the use of primary cultures of satellite cells also has some inherent drawbacks, mainly relating to the fact that they are generally slow-growing and can undergo a limited number of divisions, but this is probably the best possible way to understand skeletal muscle physiology and pathology. Few studies have been reported about *in vitro* myogenic differentiation of primary cultured satellite cells obtained from mice (81-85). However, the studies have shown that not all mechanisms regulating mouse satellite cell activation and myogenic differentiation are conserved in

human satellite cells and that such differences may impact the clinical translation of therapeutics validated in mouse models (86). As to human muscle cells, the only limited studies have been reported about *in vitro* myogenic differentiation of primary cultured human satellite cells (82, 86-91). However, there are methodological variations reported at almost every stage of primary myotube culture, especially in regards to medium composition and the differentiation protocol, which could potentially affect the reproducibility of the therapeutic studies. Our aim was to establish a reliable cellular model of *in vitro* myogenesis using primary culture of human satellite cells, that could be useful to understand the origin of skeletal muscle pathologies and suitable for drug screening, which could reliably predict the effect of the drugs *in vivo*.

To find the therapeutic drug target for skeletal muscle disorders, we thought of Calcium Sensing Receptor (CaSR). CaSR acts as a modulator of extracellular calcium (Ca^{2+}) and proved to be an essential molecule in physiology and pathologies (40). The drugs targeting CaSR in related pathologies are already available (51). Moreover, few recent scientific studies have observed the potential role of CaSR, specifically in the differentiation of cells such as keratinocytes, cardiomyocytes, and neurons (92-95). On the other hand, the Ca^{2+} is essential not only for skeletal muscle contraction, but many studies have shown its importance in myogenic differentiation too (96-100). The expression and function of CaSR in skeletal muscle has not been explored. Thus, in our studies, we hypothesize the presence of CaSR in human skeletal muscle and its possible role in skeletal muscle myogenic differentiation, which could be later used as a therapeutic target for skeletal muscle degenerative disorders.

The skeletal muscles are impaired in hypoparathyroidism, an endocrine deficiency disease identified by an absence or inappropriately low concentrations of circulating Parathyroid Hormone (PTH), which results in an imbalance of Ca^{2+} in our body (36, 55). It has been reported that even after normalizing the level of calcium in the blood

with conventional treatment such as with oral calcium and active vitamin D supplementation, some patients frequently complain about the muscle weakness (35, 37, 61). Even, after the decades of the discovery of PTH physiology, the direct role of PTH in skeletal muscle myopathies in hypoparathyroidism is not clear. Since we had already aim of developing the reliable human cellular model of *in vitro* skeletal muscle myogenic differentiation, we thought to use this opportunity to analyze the role of PTH in skeletal muscle myogenic differentiation, which could contribute to our understanding of the hypoparathyroidism related myopathies. Thus, we hypothesize the possible role of PTH and the presence of its main receptor, PTH type-1 Receptor (PTH1R) in human skeletal muscle myogenic differentiation.

3. MATERIALS AND METHODS

3.1 Primary Culture and Development of Lines of Human Satellite Cells (hSCs)

3.1.1 Primary Culture of hSCs

Human skeletal muscle biopsies were obtained from the discarded tissue of patients undergoing plastic surgery from SOD of Prof. Marco Innocenti after signed an informed consent approved by the Local Ethical Committees of the University Hospital of Florence (AOUC), (Reference No. BIO.16.022). The biopsies were poured in Dulbecco's Modified Eagle Medium (DMEM) culture medium, and within 24 hours of collection, they were processed in the laboratory. The information of all 11 collected biopsies such as age, gender, and type of skeletal muscle of humans was recorded. All the processing steps of the biopsies and cell culture was performed in the sterile condition in class II, vertical biosafety laminar flow cabinets at Room Temperature (RT). In order to proceed, the biopsies were poured in a 100 mm tissue culture Petri dish, washed in sterile Dulbecco's Phosphate Buffer Saline (DPBS). All burn and fibrous parts were removed from the biopsies with sterile forceps and scalpels. Subsequently, the biopsies were cut into two pieces. One-piece was stored in RNA later (Sigma, R0901-100ML) in a 1.5 ml vial, at -20°C for mRNA isolation, and the other part was taken for isolation of hSCs. To isolate hSCs, the biopsies were minced and incubated with collagenase type II 0.3 mg/ml overnight or 3 mg/ml for 3-4 hours at 37°C in a modified atmosphere with 5% CO_2 in the air. Following incubation, the minced biopsy was mechanically dispersed with a rubber pump in order to separate the hSCs from the tissue. First, the solution formed by the minced biopsy with collagenase type II was transferred to a 15 ml centrifuge tube and centrifuged at 300 rcf/RT/5 minutes in order to remove collagenase. The supernatant was removed, and the pellet was re-suspended in DMEM. Following the

re-suspension of cells, the mechanical dispersion was performed with a rubber pump (10 times in and out), and then tubes were centrifuged at 300 rcf/RT/5 minutes. The supernatant was removed, and the obtained pellet was re-suspended in DMEM. The mechanical dispersion in DMEM and centrifugation step, as mentioned above, was repeated for one more time. Finally, the pellet obtained was re-suspended in 10 ml Skeletal Muscle Cell Growth Medium (SMCGM). The SMCGM was prepared by supplementing the Skeletal Muscle Cell Basal Medium (SMCBM) (PromoCell, C-23260) with skeletal muscle cell growth medium supplement mix (PromoCell, C-39365) according to manufacturer's protocol and with 100 IU/ml penicillin and 100 µg/ml streptomycin (Penicillin-Streptomycin, Sigma, P0781) (Table 1 & 2). The prepared SMCGM was sterilized by filer sterilization method with a 0.22 µm PVDF filter membrane.

Preparation of Skeletal Muscle Cell Growth Medium (SMCGM) (According to PromoCell Instructions)	
Skeletal Muscle Cell Basal Medium (SMCBM) (PromoCell, C-23260)	500 ml
Skeletal Muscle Cell Growth Medium Supplement Mix (PromoCell, C-39365)	1 Pack for 500 ml of SMCBM
100 IU/ml penicillin and 100 µg/ml streptomycin (Penicillin-Streptomycin, Sigma, P0781, 10,000 IU penicillin/mL and 10 mg streptomycin/mL.)	5 ml

***Table 1**. The preparation of the growth medium used for the proliferation of primary cultured human satellite cells.*

Final concentrations of main supplements in SMCGM (According to PromoCell Information) *https://www.promocell.com/f/product-information/manual/C-23260.pdf*	
Fetal Calf Serum	0.05 ml / ml
Fetuin (bovine)	50 µg / ml
Epidermal Growth Factor (recombinant human)	10 ng / ml
Basic Fibroblast Growth Factor (recombinant human)	1 ng / ml
Insulin (recombinant human)	10 µg / ml
Dexamethasone	0.4 µg / ml

***Table 2.** The final concentrations of main components of the growth medium used for the proliferation of primary cultured human satellite cells.*

The matrigel coated plates were used for culturing of hSCs, to promote the cellular adhesion to the surface of the tissue culture dishes. The matrigel coated plates used for culturing hSCs were prepared freshly before every use. For coating the bottom upper surface of the tissue culture dishes, the matrigel (Corning, 354234) was diluted to 1:20 with Ham's F12 Coon's modification medium, and 5 ml of that was added to 100 mm tissue culture dish and incubated at RT for 2 hours. After the incubation, the matrigel solution with Ham's F-12 Coon's modification medium was removed, and the bottom upper surface of the tissue culture dishes was washed two times in Ham's F-12 Coon's modification medium and then for two times with SMCBM. The cellular suspension obtained by mechanical dispersion was seeded in matrigel coated tissue culture dish and cultured in a modified air with 5% CO_2 at 37°C, and was observed by compound microscope periodically for 2 to 10 days, to control the adhesion and confluency of hSCs on the bottom surface of the culture dish. The obtained attached cells were called primary cell cultures of hSCs. After obtaining the primary cell culture of hSCs, the SMCGM was changed every 2-3 days until the cell growing on culture dish reached 60-70 % confluency.

3.1.2 Development of hSCs Lines

When the obtained primary cell culture of hSCs in 100 mm tissue culture dish reached to 60-70 % confluency, then cells were split into multiple tissue culture dishes. This process is called "subculturing". To subculture the cell culture of hSCs growing in 100 mm tissue culture dish, the SMCGM was removed, and then 5 ml of 1X trypsin-EDTA (0.008% (w/v) Trypsin, 0.004% (W/V) EDTA, 0.02 % (w/v) glucose in DPBS without Ca^{2+} & Mg^{2+}) was added to detach the cells from the surface of the culture dish. The trypsin-EDTA was removed immediately, and again 5 ml of 1X trypsin-EDTA was added quickly and removed immediately, leaving few drops of the trypsin-EDTA in the culture dish and then incubated at 37°C in modified air with 5% CO_2 for 2 minutes. After this incubation, the cells were observed in the compound microscope; if the shape of the cells found is round, that

means the cells are adequately detached. Subsequently, the cells were re-suspended in fresh SMCGM, seeded into other matrigel coated tissue culture dishes and incubated at 37℃ in modified air with 5% CO_2. The obtained proliferating hSCs from the primary cell cultures of hSCs are called hSCs line.

3.1.3 Storage of hSCs Lines

To maintain and preserve the established hSCs lines obtained from each biopsy, these were stored in Liquid Nitrogen (N_2) for future uses. For storage, at 60-70% confluent hSCs on 100 mm tissue culture dish were detached with trypsin-EDTA and collected in SMCGM in a 15 ml centrifuge tube as described in the previous section 3.1.2. The obtained cellular suspension was centrifuged at 300 rcf/RT/5 minutes. The supernatant was removed, the cell pellet was re-suspended in 5 ml sterile DPBS and centrifuged at 300 rcf/RT/5 minutes. The supernatant was removed, and the obtained cell pellet was re-suspended in 4 ml of Skeletal Muscle Cell Freezing Medium (SMCFM). The SMCFM was prepared by supplementing the SMCBM (PromoCell, C-23260) with 40% Fetal Bovine Serum (FBS) (Sigma Aldrich F7524), 100 IU/ml penicillin and 100 µg/ml streptomycin (Penicillin-Streptomycin, Sigma, P0781) and 6.5 % Dimethyl Sulfoxide, subsequently sterilized by filer sterilization method with 0.2µm PVDF membrane (Table 3).

Preparation of Skeletal Muscle Cell Freezing Medium (SMCFM)	
Skeletal Muscle Cell Basal Medium (SMCBM) (PromoCell, C-23260)	52.5 ml
40 % Fetal Bovine Serum (FBS) (Sigma Aldrich F7524),	40 ml
100 IU/ml penicillin and 100 µg/ml streptomycin (Penicillin-Streptomycin, Sigma, P0781, 10,000 IU penicillin/mL and 10 mg streptomycin/mL)	1 ml
6.5 % Dimethyl Sulfoxide	6.5 ml

Table 3*. The preparation of the freezing medium used for the cryopreservation of primary cultured human satellite cells.*

The cellular suspension in SMCFM was poured 1 ml per each 1.5 ml cryovial. The cryovials with the cellular suspension were immediately stored at -80℃ in an isopropanol chamber. The isopropanol results in a controlled decrease in temperature of 1 ℃ per minute while freezing, thus gradually decreasing the temperature during the freezing process, which aids in preserving cell viability, and hence it is critical for successful cryopreservation of a viable cell population. However, it is also important to note that dimethyl sulfoxide present in the freezing medium is cytotoxic. Therefore, once cells are combined with the freezing medium, it is crucial to progress to the freezing stage as rapidly as possible. Following 24-72 hours of freezing at -80℃ in the isopropanol chamber, the cryovials were transferred into liquid N_2 for the storage of a long duration.

3.1.4 Thawing of hSCs Lines

The cryovials of hSCs stored in liquid N_2 were taken and thawed in the water bath at 37℃ for 2 minutes. The thawing procedure must take place as rapidly as possible due to the toxic nature of dimethyl sulfoxide. Following thawing, the hSCs with freezing medium was transferred from the cryovial to a 15 ml centrifuge tube, mixed with 10 ml of the SMCGM and centrifuged at 300 rcf/RT/5 minutes. The supernatant was removed, and 10 ml of the SMCGM was added to the tube. The cells were gently mixed and seeded on a 100 mm matrigel coated tissue culture dish and cultured at 37℃ in modified air with 5% CO_2. After 24 hours, the medium was replaced with fresh SMCGM, and the cells were cultured at 37℃ in modified air with 5% CO_2.

3.2 Myogenic Differentiation *In Vitro*

The Myogenic Differentiation Medium (MDM) was prepared by supplementing the SMCBM (PromoCell, C-23260) with skeletal muscle differentiation medium supplement mix (PromoCell, C-39366) according to manufacturer's protocol and with 100 IU/ml penicillin and 100 µg/ml streptomycin (Penicillin-Streptomycin, Sigma, P0781) (Table 4). The MDM was sterilized by filer sterilization method with a 0.22µm PVDF filter membrane. When the cultured hSCs have reached 60-70% confluent in a 60 mm matrigel coated culture dish, the hSCs were washed in sterile DPBS. Then the MDM was added, and hSCs were cultured at 37℃ in modified air with 5% CO_2. The observation of cultured hSCs in MDM was taken periodically for 2 to 10 days in the compound microscope to monitor the differentiation of hSCs into multinucleated cells. The MDM was refreshed every 2 days for the 7-9 days. Myogenic differentiation was stopped at 7 or 9 days concerning the progression of the myogenesis.

Preparation of Myogenic Differentiation Medium (MDM) (According to PromoCell Instructions)	
Skeletal Muscle Cell Basal Medium (SMCBM) (PromoCell, C-23260)	500 ml
Skeletal Muscle Cell Differentiation Medium Supplement mix (PromoCell, C-39366)	1 Pack for 500 ml of SMCBM
100 IU/ml penicillin and 100 µg/ml streptomycin (Penicillin-Streptomycin, Sigma, P0781, 10,000 IU penicillin/mL and 10 mg streptomycin/mL)	5 ml

***Table 4**. The preparation of the differentiation medium used for myogenic differentiation of primary cultured human satellite cell lines.*

3.3 Immunofluorescence Analysis

The immunofluorescence staining was performed to characterize the obtained myotubes for the presence of one of the main terminal myogenic differentiation marker protein MHC. The immunofluorescence staining was also performed to analyze the presence of CaSR protein in human skeletal muscle tissue sections and cultured hSCs lines. Furthermore, immunofluorescence staining was performed to investigate the presence of PTH1R protein in human skeletal muscle tissue sections.

3.3.1 TissueFAXS Analysis

The TissueFAXS immunofluorescence staining was used to analyze the presence of CaSR and PTH1R proteins in human skeletal muscle tissue sections using mouse monoclonal primary antibodies anti-CaSR, (ADD) (Thermo Fisher Scientific, MA1-934), dilution 1:500 and anti-PTH1R 4D2 antibodies (Thermo Fisher Scientific, MA5-15676), dilution 1:50, respectively.

For this analysis, the paraffin-embedded skeletal muscle tissue sections present on a glass slide were analyzed. The ideal thickness of the paraffin-embedded tissue sections for this type of analysis is 4-13 µM and these sections must be prepared on specific coated/charged (for example Poly L Lysine coated) slides; otherwise, the tissue section might detach from glass slide while heating during antigen retrieval. In order to start the immunofluorescence analysis, the paraffin of the tissue sections was melted by heat in the oven at 60℃ for 25 minutes. Subsequently, the tissue section was serially rehydrated at RT 3 times in Xylol for 10 minutes each, 2 times in 100% Ethanol (EtOH) for 5 minutes each, in 96% EtOH for 5 minutes, in 70% EtOH for 5 minutes, in 50% EtOH for 5 minutes, in 30% EtOH for 5 minutes and finally 3 times in DPBS for 5 minutes each. After that, antigen retrieval was performed, formalin-fixed tissues require an antigen retrieval step before immunostaining. Methylene bridges formed during fixation which cross-link proteins and mask antigenic sites, antigen retrieval methods break these methylene bridges and expose antigenic sites,

allowing antibodies to bind. For antigen retrieval, 0.05% citraconic anhydride pH 7.4 hot buffer was used. The slide was added into the hot buffer and boiled in that for 20 minutes, followed by cooling for 20 minutes at RT outside the buffer. Then, the slide was washed two times in DPBS for 5 minutes each, and the tissue area were marked with Dako pen. Subsequently, the tissue was permeabilized, and nonspecific sites were blocked by incubating the tissue sections in a solution (0.05% saponin and 5% goat serum in DPBS) for 30 minutes at RT. After that, the tissue sections were stained with mouse monoclonal (anti-CaSR/anti-PTH1R) primary antibodies. The primary antibodies diluted in blocking buffer (5% goat serum in DPBS) were applied to one of the areas of the tissue section marked with the Dako pen. For CaSR analysis, in another selected area of the tissue section marked with Dako pen, anti-CaSR antibodies along with 5X blocking peptide (ADDDYGRPGIEKFREEAEERDI, Thermo Fisher Scientific, project: BC101281.1, solubilized in H_2O) in blocking buffer was applied. The blocking peptide is the peptide sequence against which the antibodies are raised. Before using, the diluted primary antibodies were centrifuged at 14000 rcf at 4℃ for 10 minutes to remove the possible aggregates. As a negative internal control, only a blocking buffer was applied in another area of the tissue section marked with the Dako pen. After that, the tissue sections were incubated in a humid environment at RT for two hours or at 4°C overnight. After that, the primary antibody from tissue sections was removed and then washed three times thoroughly with distilled water, and then were washed in DPBS for 5 minutes. After that, the tissue sections were stained with the secondary antibody goat anti-mouse IgG Alexa Fluor 647 (Abcam, ab150115) 1:1000 diluted with blocking buffer and incubated for 1 hour at RT in a humid environment. Before applying the diluted secondary antibodies were centrifuged at 14000 rcf at 4℃ for 10 minutes, to remove the possible aggregates. After the staining with secondary antibodies, the tissue sections were washed three times in DPBS for 5 minutes each, and then the nuclei were counterstained with DAPI (1:1000 diluted in DPBS). Then, the stained tissue sections

were washed three times in DPBS for 5 minutes each and one time in distilled water. In the analysis, human parathyroid tissue sections and human kidney tissue sections were used as a positive control for CaSR and PTH1R, respectively. Finally, all the slides with stained tissue sections were mounted with Fluoromount-G medium (SouthernBiotech, 0100-01) and examined under wide-field fluorescence microscopy using TisssueFAXS (TissueGnostics).

3.3.2 Immunofluorescence Staining of Cells

The immunofluorescence staining was performed to characterize the cultured myotubes fixed in 4% paraformaldehyde (PFA)/DPBS, for the presence of one of the main terminal myogenic differentiation marker MHC protein using primary antibody mouse monoclonal anti-MHC antibodies (Millipore, A4.1024) dilution 1:20. Moreover, immunofluorescence staining was performed to analyze the presence of CaSR protein in hSCs lines, fixed in 4% paraformaldehyde (PFA)/DPBS using primary antibody mouse monoclonal anti-CaSR, (ADD) antibodies (Thermo Fisher Scientific, MA1-934) dilution 1:500.

For the analysis, the cells were cultured in the specific medium on 24 multi-well plates specific for immunofluorescence staining (Eppendorf Cell Imaging Plates). When the cells reached enough number (50 to 70% confluency), the cells were washed two times in DPBS, fixed in 4% PFA/DPBS for 15 minutes at RT, and washed three times with ultrapure water. After that, the fixed cells were permeabilized by 0.2% Triton X-100/DPBS at 37°C for 15 minutes. Subsequently, the cells were washed three times in DPBS and were treated by RNase (Stock solution: 0.02% (w/v) RNase-A (Sigma-Aldrich, R6513) prepared in 2% Bovine Serum Albumin (BSA)/DPBS) diluted 1:1000 with 2% BSA/DPBS at 37°C for 30 minutes. After that, the cells were washed three times in 2% BSA/DPBS to block the non-specific binding sites, and then stained with primary mouse monoclonal antibodies (anti-MHC/anti-CaSR) diluted in 2% BSA/DPBS in the humidified environment at 4°C overnight. After that, the cells were washed three

times in DPBS and two times in 2% BSA/DPBS. Then, cells were stained with the secondary antibody goat anti-mouse Alexa Fluor 488 (Invitrogen, A28175) diluted 1:500 in 2% BSA/DPBS and incubated in the dark and in a humid environment at RT for 45 minutes. Then stained cells were washed several times (5-6) in DPBS and counterstained for nuclei with propidium iodide (1:1000 diluted in DPBS). After that, stained cells were washed three times in ultrapure water. As negative internal control cells were stained with the secondary antibody only. Stained cells were examined on a Laser-Scanning Confocal Microscopy (LSM510 META, Zeiss) Microscope equipped with Ar/ML458/477/488/514, HeNe543, and HeNe633 Laser Lines.

3.4 Flow Cytometry Analysis

The flow cytometry was performed to characterize the cultured hSCs lines for the presence of main marker protein PAX7 using primary antibody mouse monoclonal anti-PAX7 antibody (Sigma Aldrich, SAB1412356-100UG) dilution 1:10. Moreover, the flow cytometry was performed to analyze the presence of CaSR protein in cultured hSCs lines using primary antibody mouse monoclonal anti-CaSR, (ADD) antibody (Thermo Fisher Scientific, MA1-934) at two different dilutions 1:500 and 1:100.

For flow cytometry, hSCs were detached from 100 mm tissue culture dish with trypsin-EDTA, suspended in growth medium (SMCGM) at a density of 3 x 10^6 cells/ml and centrifuged at 250 rcf/RT/5 minutes. After that, the cells were collected and re-suspended with required volume (3 x 10^6 cells in 1 ml for each vial) of cold MACS buffer (Miltenyi Biotec S.r.l.130-091-221) then mixed to achieve a homogenous distribution of cells, poured 3 x 10^6 cells (1 ml) in each 1.5 ml vial and centrifuged at 100 rcf/4℃/5 minutes. After that, the supernatant was carefully removed, leaving a little volume to avoid aspiration of cells. Subsequently, cells were re-suspended in 25 µl Invitrogen Fix and Perm solution containing formaldehyde (Invitrogen GAS001), vortexed for 30 seconds, and incubated at RT for 15 minutes. After this incubation, the volume into each vial was made up to 1 ml by adding cold MACS buffer, and the cells were centrifuged at 100 rcf/4℃/5 minutes. At this point, cells were re-suspended with the mouse monoclonal primary antibodies (anti-PAX7/anti-CaSR) diluted with Invitrogen Fix and Perm solution (Invitrogen GAS002). As a negative internal control, hSCs present in one of the vials were re-suspended only in the Fix and Perm solution. The vials containing stained cells by primary antibody and not stained cells (negative control) were vortexed for a few seconds and incubated for 30 minutes at RT. After incubation, the volume was made up to 1 ml with cold MACS buffer, and cells were centrifuged at 100 rcf/4℃/5 minutes. After that, cells were washed in 1ml of cold MACS buffer and centrifuged at 100 rcf/4℃/5 minutes. Subsequently, the cells were re-suspended in anti-mouse fluorescent

dye Alexa Fluor 488 conjugated secondary antibody (Invitrogen A28175) 1: 1000 diluted with MACS buffer, gently mixed for few seconds and incubated for 30 minutes at RT in the dark. After incubation, the volume into each vial was made up to 1 ml with cold MACS buffer, and cells were centrifuged at 100 rcf/ 4℃/5 minutes. The stained cells were re-suspended in 800 µl of MACS buffer and were transferred into 5 ml tubes and were analyzed in a flow cytometer (CyFlowSpace Partec) equipped with the FCS Express 6 software (De novo software, USA).

3.5 Molecular Analysis Techniques

3.5.1 Gene Expression Analysis by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The RT-PCR was performed to characterize the hSCs lines by analyzing the presence of the expression of the *PAX7* gene. The RT-PCR analysis was also performed to characterize the *in vitro* myogenic differentiation by analyzing the presence of the myogenic regulatory factor's genes *Myf5, MyoD1, Myogenin,* and of terminal differentiation marker gene *MHC*. Moreover, the RT-PCR was used to analyze the expression of the *CaSR* gene in human skeletal muscle tissue, in established hSCs lines and cultured myotubes, using couples of primers targeting the various exons 2/3, 4/5 and 6/7 of human CaSR. Finally, the RT-PCR was performed to analyze the expression of *GPRC6A* gene in human skeletal muscle tissue and established hSCs lines using human GPRC6A specific primers. The information of the primers used in the analysis is given in Table 5.

To proceed with RT-PCR, first the cells were cultured. For the analysis of *PAX7, CaSR,* and *GPRC6A* genes in hSCs lines, the hSCs were cultured with growth medium (SMCGM) in 100 mm Matrigel-coated tissue culture dish. When cells reached 60-70% confluency, the cells were detached with trypsin-EDTA, washed with PBS, and collected in 1.5 ml vial by centrifugation at 13400 rcf/4℃/2 minutes. To perform RT-PCR analysis of myogenic differentiation markers genes Myf5, MyoD1, Myogenin, and MHC in obtained differentiated myotubes, the hSCs were seeded in 60 mm matrigel coated tissue culture dishes for 2 different experimental points 0 days (undifferentiated hSCs) and 7 days (differentiated multinucleated cells called myotubes) of *in vitro* myogenic differentiation. Upon reaching the 60-70% confluency, the cells were induced for myogenic differentiation medium. The cells from 60 mm culture dishes were collected from both experimental points (0

days and 7 days) in a 1.5 ml vial using trypsin-EDTA, as described above. The obtained cell pellets were stored at -80℃ until use.

RNA was isolated from the cellular pellets using QIAzol Lysis Reagent (Qiagen, 79306) according to the manufacturer's protocol. To isolate the RNA from the human skeletal muscle tissue stored in RNA later, the amount of the tissue was determined. The tissue was then minced with a sterile scalpel and transferred to 1.5 ml vial with metal beads in 1 ml QIAzol lysis reagent and lysed in TissueLyser according to the manufacture's protocol. Then, the RNA was isolated from the tissue samples by the QIAzol Lysis Reagent kit according to the manufacturer's instructions. The isolated RNA was stored at -20℃ until use. The isolated RNA was quantified using NanoDrop ND-1000 Spectrophotometer v3.3, (Thermo Fisher Scientific, Delaware, USA) according to the manufacturer's protocol. The integrity of the isolated RNA was analyzed using 500 ng of isolated RNA by electrophoresis in Tris-Borate-EDTA (TBE) running buffer at 120 V for 1 hour on 1% agarose gel prepared in TBE buffer stained with ethidium bromide. Total RNA isolated run on agarose gel displays three distinct ribosomal fragments corresponding to 18S, 28S and 5S for eukaryotic RNA. After the verification of the integrity of isolated RNA, the 500 ng of the isolated RNA was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen, 205310) according to the manufacturer's protocol. The remaining isolated RNA was stored at -80℃ for future experiments (long term storage). The cDNA obtained using the reverse transcription kit was stored at -20℃ before performing PCR analyses for the expression of *Pax7, Myf5, MyoD1, Myogenin, MHC, CaSR,* and *GPRC6A* genes.

The primers were designed for amplification of the specific genes. For primers designing, the following protocol was used:

1. Go to NCBI-Gene, type the name of the gene of interest, select organism name- Homo sapiens (human), click-Genomic regions

transcripts and products, click-gene bank, look number of variants of the mRNAs.

2. Take the sequences of the exons of the all mRNA variants from NCBI and align them in word file. Make always the first base of an exon in capital letter to differentiate from next exon. Look for the common exons presents in all variants of mRNA.
3. Then after, go to IDT (Integrated DNA Technologies) website, Products and services, Custom qPCR Probes- PrimerQuest® Design Tool, paste the exons sequence of the mRNA that we have selected, also here make the first base of exon in capital letter to differentiate from next exon, name the sequence, click on PCR two primers
4. Match the obtained sets of primers in word sequence file to verify the binding sites of the primers. Then, using the NCBI-NIH primer designing tool, blast the sequence of the primers in order to confirm further the specificity of the primers in the human genome. If the primers couple in blast results shows that it binds to all mRNA variants, with all residues, and no other unspecific gene around the size of the amplicon, and Tm of the primers in blast results are in range (50 to 65 ℃), length of the primers are in range (18-30 nucleotide), GC content is in range (40-60%), that means the primer couple that we have designed is suitable for PCR.

The PCR was carried out on obtained cDNA using GoTaq DNA Polymerase (Promega, M3001) with 1X Green GoTaq Reaction Buffer, 0.2 mM of the dNTPs mix, 1 µM primers and 1 µl of the obtained cDNA template according to manufacturer's protocol. The PCR cycle was used as follows: initial denaturation at 95°C for 2 minutes, 35 repeated cycles composed of denaturation at 95°C for 30 seconds, annealing at 42-65°C (depend on primers Tm) for 30 seconds and extension at 72°C for 30 seconds (1 min/kb) and a final extension step at 72°C for 5 minutes. *β-ACTIN* house-keeping gene was used as an internal control. In CaSR gene expression analysis, the cDNA isolated from primary

cultured human parathyroid cells and HEK293 cells were used as the positive and negative control, respectively. HEK 293 cells have been reported not to express the CaSR gene and protein; thus, it can be used as a reasonable negative control for CaSR expression analysis (101). The cDNA isolated from human skeletal muscle tissue was used as a positive control in expression analysis of markers genes for characterization of primary cultured hSCs and *in vitro* myogenic differentiation of cultured hSCs. The obtained amplicons were verified by electrophoresis in 2% agarose gel (% of agarose gel used according to the size of amplicon). After that, the pictures of agarose gels were taken with ultraviolet transilluminator. To verify the sequence of the obtained amplicons by RT-PCR, sequencing analysis was performed using BigDye™ Terminator v1.1 Cycle Sequencing Kit based on Sanger sequencing reactions (Thermo Fisher Scientific, 4337451) according to manufacturer's protocol.

Name of Genes	Primer's Sequences	A. T.	Amplicon size (bp)
Human ***ß-actin***	Forward 5'-AGCCTCGCCTTTGCCGA-3' Reverse 5'-CTGGTGCCTGGGGCG-3'	60°C	174
Human ***Pax7***	Forward 5'-GGTACCGAGAATGATGCGG-3' Reverse 5'-CCCATTGATGAAGACCCCTC-3'	55°C	124
Human ***Myf5***	Forward 5'-ATGCCATCCGCTACATCG-3' Reverse 5'-ACAGGACTGTTACATTCGGC-3'	55°C	145
Human ***MyoD1***	Forward 5'-GACGTGCCTTCTGAGTCG-3' Reverse 5'-CTCAGAGCACCTGGTATATCG-3'	55°C	148
Human ***Myogenin***	Forward 5'-AGCGAATGCAGCTCTCAC-3' Reverse 5'-TGTGATGCTGTCCACGATG-3'	55°C	150
Human ***MHC***	Forward 5'-GAGTCCTTTGTGAAAGCAACAG-3' Reverse 5'-GCCATGTCCTCGATCTTGTC-3'	55°C	143
Human ***CaSR 2/3***	Forward 5'- GATCAAGATCTCAAATCAAG -3' Reverse 5'- CCAGCGTCAAGTTGGGAAGA -3'	57°C	139
Human ***CaSR 4/5***	Forward 5'- CTGAGAGGTCACGAAGAAAGTG -3' Reverse 5'- GGTGCCAGTTGATGATGGAATA -3'	58°C	367
Human ***CaSR 6/7***	Forward 5'- CTGCTGCTTTGAGTGTGTGG -3' Reverse 5'- CTTGGCAATGCAGGAGGTGT -3'	60°C	124
Human ***GPRC6A***	Forward 5'- CAGGAGTGTGTTGGCTTTGA -3' Reverse 5'-CTCTTGGCATGTAGCTGGAA -3'	58°C	238

Table 5*. List of the human gene specific primers used in the analysis.*
A. T.: *Annealing temperature.*

3.5.2 Gene Expression Analysis by Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (Real-Time qRT-PCR)

The Real-Time qRT-PCR was performed to analyse the expression of *CaSR* and *PTH1R* genes in human skeletal muscle tissues and hSCs lines. Moreover, the Real-Time qRT-PCR was performed to investigate the expression of *MHC, CaSR,* and *PTH1R* genes during *in vitro* myogenic differentiation. The information of the probe and primers used in the analysis is given in Table 6.

For Real-Time qRT-PCR analysis of *CaSR* and *PTH1R* gene in human skeletal muscle tissues and hSCs lines, the cDNA was isolated according to the protocol described in section 3.5.1. To perform Real-Time qRT-PCR analysis for *MHC, CaSR,* and *PTH1R* genes during *in vitro* myogenic differentiation, the hSCs were subcultured in growth medium (SMCGM) in 100 mm Matrigel-coated tissue culture dish until passage 3 in order to obtain enough number of cells for myogenic differentiation experiment. After that, hSCs were detached and with growth medium (SMCGM) were seeded in 60 mm matrigel coated tissue culture dishes for 4 different experimental points (0 days, 3 days, 6 days, and 9 days) during *in vitro* myogenic differentiation. Upon reaching 60-70% confluency, the cells were induced for myogenic differentiation with the MDM medium. The cells from 60 mm culture dishes were collected from all experimental points in a 1.5 ml vial using trypsin-EDTA, as previously described in section 3.5.1. The RNA was isolated from each cell pellet and reverse transcribed to obtained cDNA. The obtained cDNA samples were verified by qualitative PCR of housekeeping gene *β-ACTIN* as described in section 3.5.1. The obtained cDNA was stored at -20°C before performing Real-Time quantitative PCR (qPCR) to analyze the expression of *MHC, CaSR,* and *PTH1R* genes.

In order to perform Real-Time qPCR, the probe & primers were designed for amplification of the specific gene. For probe & primers designing, the protocol described in previous section 3.5.1 was used except the following consideration:

- In the IDT website, selected “PCR probe + two primers” in place of “two primers” for probe and primers designing.
- The probe & primers were purchased in IDT with the following procedure, In IDT select the chosen primer set, proceed order, assay-give name of specific gene, do not change anything else, add to cart, then go back to the probe & primers set and select for separate vials of forward and reverse primers of the same set, for sequencing analysis, select for/Rev oligos in tube- do not change anything, add to cart, then proceed for purchasing.

In order to begin with Real-Time quantitative PCR (qPCR), the gene of interest was amplified with KAPA PROBE FAST qPCR kit (Kapa Biosystems, KK4703), according to the manufacturer’s instructions in Qiagen Rotor-Gene Q using specific TaqMan probe and primers with 1 µl of cDNA of the sample which is positive for the expression of the gene of interest. The conditions utilized for the PCR reaction were; enzyme activation at 95℃/3 minutes, denaturation at 95℃/10 seconds & cycling condition (annealing/extension/acquire) at 55-65℃/15-30 seconds for 40 cycles (the cycling condition depended on the Tm of specific primers and probe, and amplicon size). The obtained amplicon was run on the agarose gel. The amplicon band was isolated from the agarose gel using the Gel Extraction Kit (Thermo Fisher Scientific, K210012) according to the manufacturer’s protocol. The isolated cDNA amplicon was quantified using NanoDrop ND-1000 Spectrophotometer v3.3, (Thermo Fisher Scientific, Delaware, USA) according to the manufacturer’s protocol. According to the presence of µg/µl concentration and the number of nucleotide bases in an amplicon, the number of amplicons was calculated. The number of amplicons obtained was first diluted with H_2O to get 10^{11} or 10^{10}, subsequently, to prepare the standard curve, the amplicon was serially diluted to 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 & 10^1. Afterward, Real-time qPCR, for the gene of interest was performed with KAPA PROBE FAST qPCR kit in Qiagen Rotor-Gene Q using specific TaqMan probe and primers with 1 µl of the obtained cDNA samples and with 1 µl of standard curve points (10^8, 10^6, 10^4, 10^3, 10^2 & 10^1). The Real-Time qPCR was performed in

three technical replicates. The amplification signal of the gene of interest obtained in the Real-Time qPCR was normalized with the signal obtained in the Real-Time qPCR of the house-keeping gene in the same cDNA samples. In our case, we have used the housekeeping gene, *RPS18,* which encodes 40S ribosomal protein S18. The RPS18 gene was quantified in the samples of different time points of *in vitro* myogenic differentiation and statistically analyzed for significant variation during myogenic differentiation, to prove the suitability of the RPS18 housekeeping gene for normalization. The detailed statistical analysis (One-Way ANOVA Post Hoc test) was performed on obtained results to analyze the significant variation in expression of gene during *in vitro* myogenic differentiation.

Name of Gene	**Probe & Primer's Sequences**	**Cycling Conditions**	**Amplicon size (bp)**
Human ***RPS18***	Forward 5'- GATGGCAAAGGCTATTTTCCG -3' Reverse 5'- TCTTCCACAGGAGGCCTAC-3' Probe 5'-/56-FAM/TTCAGGGAT /ZEN/CACTAG AGACATGGCTGC/31ABkFQ/-3'	58°C/ 15 seconds	132
Human ***MHC***	Forward 5'-GAGTCCTTTGTGAAAGCAACAG-3' Reverse 5'-GCCATGTCCTCGATCTTGTC-3' Probe 5'-/56-FAM/CAAGTCTTC/ZEN/CCCATGAACCCTCCC/31ABkFQ/-3'	58°C/ 15 seconds	143
Human ***CaSR exons 6/7***	Forward 5'- TGCTTTGAGTGTGTGGAGTG -3' Reverse 5'- GGTTCTCATTGGACCAGAAGTC -3' Probe 5'-/56-FAM/AGGCACTGG/ZEN/CATCTGTCTCATCAC/31ABkFQ/-3'	58°C/ 15 seconds	100
Human ***PTH1R***	Forward 5'- GGGAAGCCCAGGAAAGATAAG -3' Reverse 5'- CACAGGATGTGGTCCCATT -3' Probe 5'-/56-FAM/TGCCTCCTT/ZEN/GTCCTCCTCAGACTC/31ABkFQ/-3'	57°C/ 15 seconds	125

Table 6*. List of the human gene specific TaqMan probe and primers used in the analysis*

3.5.3 Protein Expression Analysis by Western Blot

The western blot analysis was performed to characterize the established hSCs lines for the presence of marker protein PAX7 (a nuclear transcriptional factor) using mouse monoclonal primary antibody anti-PAX7 antibody (Sigma Aldrich, SAB1412356-100UG) dilution 1:3000. Moreover, western blot analysis was performed to characterize the *in vitro* myogenic differentiation by analyzing the variation of one of the most significant markers of myogenic differentiation which is Myosin Heavy Chain (MHC) protein during this process using mouse monoclonal primary antibody anti-MHC antibody (Millipore, A4.1024) (dilution 1:3000). Finally, the western blot analysis was performed to analyse the presence of variation of CaSR and PTH1R protein in established hSCs lines and during *in vitro* myogenic differentiation using mouse monoclonal primary antibodies anti-CaSR, (ADD) antibody (Thermo Fisher Scientific, MA1-934) dilution 1:3000 and anti-PTH1R 4D2 antibody (Thermo Fisher Scientific, MA5-15676) dilution 1:5000, respectively.

To perform PAX7, CaSR, and PTH1R protein analysis in established hSCs, the cells were cultured in 100 mm matrigel coated tissue culture dishes, upon reaching 60-70% confluency, the cells detached using trypsin-EDTA and were collected in 1.5 ml using the protocol as described in section 3.5.1. To perform western blot for MHC and CaSR proteins during *in vitro* myogenic differentiation, the hSCs were subcultures in 100 mm matrigel coated tissue culture dish until passage 3 to obtain enough cells for myogenic differentiation experiment. After that, hSCs were seeded in 60 mm Matrigel-coated tissue culture dishes for 4 different experimental points (0 days, 3 days, 6 days, and 9 days) during *in vitro* myogenic differentiation. Upon reaching 60-70% confluency, the cells were induced for myogenic differentiation with the MDM medium. On the other hand, to perform western blot for PTH1R during myogenic differentiation, the hSCs were cultured in the same manner for 3 different experimental points (0 days, 3 days, and 6 days) during *in vitro* myogenic differentiation. The cell pellets were collected from all 60 mm culture dishes in 1.5 ml vials

using trypsin-EDTA, as previously described in section 3.5.1. The cells were lysed in 1.5 ml vials on ice in the following RIPA buffer (~60 µl in a 60 mm culture dish or 100 µl in a 100 mm culture dish). The RIPA Buffer; 12 mM HEPES (pH 7.6), 300 mM mannitol, 1% (v/v) Triton X-100, 0.1% (w/v) sodium dodecyl sulphate (SDS) and supplemented with 1.25 µM pepstatin, 4 µM leupeptin, 4.8 µM phenyl methyl sulfonyl fluoride (PMSF), 1 mM EDTA, 1 mM EGTA, 100 µM vanadate, 1mM Sodium fluoride (NaF), 250 µM sodium pyrophosphate. PMSF to be added fresh. Triton X-100 is widely used to lyse cells to extract protein or organelles. Mannitol stabilizes protein structures by keeping the solution at normal osmolality, plus mannitol is not easily metabolized, so it will help keep contamination in check. SDS solubilize the cell membrane and also denatures most of the proteins in the cells, which helps with the separation of the proteins during SDS-PAGE gel electrophoresis. Pepstatin (inhibitor of aspartyl proteases), leupeptin (inhibitor of cysteine, serine and threonine peptidases), and PMSF (inhibitor of serine proteases) EDTA & EGTA binds to magnesium and calcium, thereby making them unavailable for other reactions. Vanadate, NaF, sodium pyrophosphate inactivates endogenous phosphatases and protects protein from phosphorylation. The cell lysate in RIPA buffer was mechanically disrupted with the pipette (fast in and out) and incubated on ice for 10 minutes. It may be necessary to lyse the cells in higher volumes of RIPA (400-500 µl) if the DNA content makes the lysate "gloopy"; however, this will also dilute the signal. After that, the cell lysate was centrifuged at ~12,000 rcf/4℃/10 minutes. The supernatant was collected and immediately frozen at -80℃. The protein quantity was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23225) according to the manufacture's protocol. Five-fold concentrated Laemmli buffer [0.32 M Tris (pH 6.8), 5% (w/v) SDS, 25% (v/v) glycerol, 1% (w/v) bromophenol blue and reducing agent; 5% (v/v) β-mercaptoethanol or 50 mM DTT] was added in a 1:4 (Protein Sample : Laemmli Buffer) ratio (volume) to 4-8 µg of protein at RT and then fractionated using 1.5 mm thick 5-15% SDS-PAGE gels. 5 or 6% gels to be used for large proteins (>100kDa), as this

improves transfer and resolution. To make SDS-PAGE gel, separating gel [375 mM Tris (pH 8.8), 4.75 % (w/v) Acrylamide, 0.25% (w/v) Bis-acrylamide, 0.1% (w/v) SDS, 0.125 % (v/v) TEMED and 0.3 % (w/v) Ammonium Persulfate (APS), the APS to be added in end] was poured and isopropanol was added to equally align top level of the gel. After ~ 20 minutes, the isopropanol was washed out and stacking gel [125 mM Tris (pH 6.8), 3.99 % (w/v) Acrylamide, 0.21% (w/v) Bis-acrylamide, 0.1% (w/v) SDS, 0.25 % (v/v) TEMED and 0.3 % (w/v) APS] was poured and 10 well comb (each well can accommodate maximum 60 µl volume) immediately inserted. After 15 minutes wait, the comb was removed, and along with prepared gel, the western blot apparatus was filled with running buffer [24.9 mM Tris, 191 mM glycine, 3.46 mM SDS]. The running buffer was removed from wells with a syringe needle to remove the unwanted gel, and then either left empty if large sample volume to be loaded or partially refill if the smaller volume to be loaded. If loading is to be done after 20 minutes of gel preparation, then after removing the comb, refill wells to avoid drying. While loading, it is better to leave empty well between controls and samples, to avoid mixing. Then the protein sample was fractionated in SDS-PAGE Gel at 42 milliamps per gel for 1-2 hours. Proteins are then transferred electrophoretically to a nitrocellulose membrane in blotting buffer [25 mM Tris, 192 mM glycine, 10% (v/v) methanol]. Blotting sandwich comprises; (in order), facing black cathode → white Plastic grid → sponge → 2 sheets pf each 3 mm Whatman paper → gel → membrane → 2 sheets of each 3 mm Whatman paper → sponge → back plastic grid → facing red anode. The blotting sandwich was prepared in a box filled with blotting buffer. Then electrophoresis was performed at 200 milliamps for 1 hour, with constant mixing to dissipate heat and localized ionic concentration. The addition of 0.5% (w/v) SDS to buffer may improve the transfer of the proteins >100kDa (but reduces Ponceau staining). After that, the blotting membrane was stained with Ponceau S for 2 minutes on rocker followed by washing with tap water to indicate the protein samples if required. It was then incubated in 3% (w/v) BSA in TTBS buffer [15 mM Tris, pH 8, 150 mM NaCl, 0.1% (v/v) Tween 20] for 45 minutes at

RT on the rocker, to block non-specific binding sites. After that, the blot was incubated in primary antibody diluted in TTBS buffer for 1 hour at RT or in TTBS Buffer with 0.3% BSA overnight at 4℃ on a rocker. The primary antibody solution can be re-used for 2-3 times. Following primary antibody incubation, the membrane was washed 3 times in TTBS buffer. After that, the blot was exposed to horseradish peroxidase-conjugated anti-(host of primary antibody) secondary antibody diluted in TTBS buffer for 1 hour at RT on the rocker. After secondary antibody incubation, the membrane was washed 3 times in TTBS buffer. The blot was then developed with the ECL kit (Thermo Fisher Scientific, 32209) according to the manufacturer's instructions. The chemiluminescence was detected by machine (The ChemiDoc XRS+ System) equipped with Image Lab software (BIO-RAD, United Kingdon).

The HEK293 cells stably transfected with human CaSR (HEK_{CaSR}) were used as a positive control in the CaSR protein expression analysis. The rat kidney tissue lysate was used as a positive control in the PTH1R protein analysis.

The blotting membrane was stored in TTBS buffer at 4℃ for re-probing if required. For re-probing, the previous antibody and developed blot from the membrane were stripped. To stripe, the membrane was washed with TTBS buffer, followed by incubation in stripping buffer (Thermo Fisher Scientific, 21059) for 15 minutes at 60℃ and then washed 3 times in TTBS buffer. Then, re-probing of the membrane was started from the step of blocking. All the washing in TTBS was done for 10 minutes incubation period at RT on the rocker. For normalization of the expression of a protein of interest during different treatment (different time points of myogenic differentiation), the same amount of protein from same vial of samples was fractionated using 9% SDS-PAGE and the obtained blot was immunostained with house-keeping protein β-ACTIN antibody according to above-described protocol.

3.6 Secondary Messengers Analysis

The secondary messengers Gq-coupled Inositol Monophosphate (IP_3), Gq-IP_3-Intracellular Calcium Mobilization and Gi-coupled Cyclic Adenosine Monophosphate (cAMP) were analyzed in hSCs lines to investigate the effects of Ca^{2+} and of CaSR drugs, calcimimetic (NPS R568, TOCRIS 3815) and calcilytic (NPS 2143, TOCRIS 3626), in order to verify the expression analysis of CaSR in this cellular model. The secondary messenger Gs-cAMP was analyzed in hSCs lines to investigate the effects of PTH to verify the expression analysis of PTH1R in this cellular model.

3.6.1 Inositol Monophosphate (IP_1) ELISA Assay

IP_1 ELISA assay kit (Cisbio, 72IP1PEA/D rev03) was used to analyze IP_1 response in hSCs lines upon stimulation with different Ca^{2+} concentrations (CaSR ligand). The experiment was performed in 3 primary hSCs lines.

For this analysis, the hSCs were cultured in matrigel coated 24 multiwell plates in growth medium (SMCGM). Upon reaching 60-70% confluency of the cells in wells of 24 multi-well plate, the cells were washed once with DPBS and then stimulated independently in quadruplicates of each stimulation with 250 µl of 0.5 mM Ca^{2+}, 250 µl of 1.2 mM Ca^{2+} and 250 µl of 3.5 mM Ca^{2+} prepared in the experimental buffer (0.5 mM $CaCl_2$, 125 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, 20 mM HEPES, 0.1 % (w/v) glucose) for 1 hour at 37°C in modified air with 5% CO_2. Every buffer was prepared freshly and was supplemented with 50 mM LiCl then pH was adjusted to 7.4 using NaOH and HCl. The Inositol triphosphate (IP_3) lifetime within the cell is very short (less than 30 seconds) before it is transformed into Inositol diphosphate (IP_2) and Inositol monophosphate (IP_1). When LiCl was added to the stimulation buffers, the degradation of IP_1 into Myo-inositol is inhibited. Therefore, the IP_1 can be accumulated in the cells and can be measured. Following incubation, 250 µl of absolute EtOH (frozen to -80°C) was added in each well and subsequently was incubated overnight at -20°C. The cold

EtOH permits to stop the reaction with cold temperature and solubilize the cells membrane to remove IP_1 in buffer solution. The next day, the cell lysate from each well was transferred to 1.5 ml centrifuge tubes. The pipetting should be slow in order to have all the volume and to avoid the aspiration of the precipitated cell debris and proteins on the surface of the wells. The solution in each vial was lyophilized and dissolved in 50 µl of diluent provided with IP_1 ELISA assay kit (Cisbio, 72IP1PEA/D rev03). The lyophilization step was performed to concentrate the low amount of IP_1 response, that could be present in the cells. Then IP_1 ELISA assay was performed according to the kit instructions. The detailed statical analysis (ANOVA) was performed on obtained data to analyse the significant difference in IP_1 response in the presence of the different concentrations of Ca^{2+}.

3.6.2 Intracellular Calcium Mobilization Imaging

The intracellular calcium mobilization imaging analysis was performed to analyze the Gq-IP_3-Intracellular Calcium Mobilization, the secondary messenger response in 3 live primary hSCs lines, upon stimulation with various Ca^{2+} concentration and CaSR drugs: the calcilytic (NPS 2143) and the calcimimetic (NPS R-568) along with Ca^{2+}.

For this analysis, the primary hSCs were cultured in growth medium (SMCGM) on 13 mm matrigel coated glass coverslips for 48 hours. In order to use as a positive control in the analysis, the HEK_{CaSR} (HEK239 transfected with human CaSR) cells were cultured on 13 mm glass coverslips in DMEM with 10% FBS for 48 hours. The experimental buffer (0.5 mM $CaCl_2$, 125 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, 20 mM HEPES, 0.1 % (w/v) glucose) was prepared freshly and pH was adjusted to 7.4 using NaOH and HCl. The cells were loaded with 1 µM FURA2-AM in 1.2 mM Ca^{2+} experimental buffer supplemented with 0.1 % (w/v) BSA for 1-2 hours at RT in the dark. Then, cells on a coverslip were mounted in a perfusion chamber and observed through a 40X oil immersion lens on a Nikon Diaphot inverted microscope equipped with MetaFluor® Fluorescence Ratio Imaging Software and then the dual -

excitation-wavelength microfluorometry was performed. The baseline free ionized Ca^{2+} in cells was measured in 0.5 mM Ca^{2+} experimental buffer. Subsequently the cells were stimulated serially for 3 minutes each with 3 mM Ca^{2+}, 3 mM Ca^{2+} + 1 µM R568 (calcimimetic), 0.5 mM Ca^{2+}, 5 mM Ca^{2+}, 5 mM Ca^{2+} + 1 µM NPS2143 (calcilytic), 0.5 mM Ca^{2+} prepared in experimental buffer and free ionized Ca^{2+} in cells were measured. Each simulation was performed in technical triplicates. The obtained results were analyzed by GraphPad Prism software.

3.6.3 Cyclic Adenosine Monophosphate (cAMP) ELISA Assay

The secondary messenger cAMP was analyzed using the cAMP complete ELISA kit (Abcam, ab133051) to evaluate the functionality of Ca^{2+}/CaSR in primary hSCs lines upon stimulation with Ca^{2+} and calcilytic (NPS R-568) and calcimimetic (NPS2143) drugs. This cAMP ELISA assay was also performed to analyse the functionality of PTH/PTH1R in primary hSCs lines upon stimulation with a wide range of hPTH 1-34. Both the experiment were performed in 3 primary hSCs lines.

For the assay, the hSCs were cultured in growth medium (SMCGM) in matrigel coated 24-multiwell plates. Upon reaching 60-70% confluency, the cells were washed once in DPBS and then were stimulated. Since Ca^{2+}/ CaSR inhibit the cAMP response in healthy cells (45), the cells for this assay was stimulated with experimental buffers supplemented with Forskolin (FSK). FSK is a potent activator of stimulatory G protein (Gs), and stimulate the maximum level of cAMP in the cells. So FSK was used as an internal positive control for the assay. Thus, for Ca^{2+}/CaSR cAMP assay, the primary hSCs were stimulated independently in triplicates for each stimulation in wells of 24- multiwell tissue dish with 250 µl of following experimental buffers containing; 0.5 mM Ca^{2+} (control), 0.5 mM Ca^{2+} +10 µM FSK (positive control), 3 mM Ca^{2+} +10 µM FSK (Ca^{2+} effect), 3mM Ca^{2+} +10 µM FSK+ 1µM R568 (calcimimetic

effect) and 3mM Ca^{2+} +10 µM FSK+ 1µM NPS2143 (calcilytic effect) for 15 minutes at 37°C in modified air with 5%CO_2.

Since PTH/PTH1R stimulates the cAMP response, the cells for this assay were stimulated with experimental buffers supplemented with 3-isobutyl-1-methylxanthine (IBMX). IBMX is a phosphodiesterase inhibitor which prevents the degradation of cAMP in cells and permits to analyze the basal level cAMP response. Also, in this case, the experimental buffer with FSK was used as an internal positive control. Thus, to perform the PTH/PTH1R cAMP assay, the primary hSCs were stimulated independently in triplicates in wells of 24- multiwell tissue culture dish with 250 µl of following experimental buffers containing; 0.5 mM Ca^{2+} (control), 0.5 mM Ca^{2+} + 1 mM IBMX (control) , 0.5 mM Ca^{2+}+ 1 mM IBMX +10 µM FSK (positive control), 0.5 mM Ca^{2+} + 1 mM IBMX + 1 pM – 1 µM hPTH 1-34 (PTH effect) (Sigma-Aldrich, P3796) for 15 minutes at 37°C in modified air with 5% CO_2.

Following incubation, 250 µl of absolute EtOH (frozen to -80°C) was added in each well and subsequently incubated overnight. The cold EtOH permits to stop the reaction with cold temperature and solubilize the cells membrane so that that cAMP can be removed from cells in the buffer solution. The next day, the cell lysate from each well was transferred to 1.5 ml centrifuge tubes. The pipetting should be slow in order to have all the volume and to avoid the aspiration of the precipitated cell debris and proteins on the surface of the wells. Then, the solution in each vial was lyophilized and dissolved in 100 µl of the assay buffer provided with cAMP complete ELISA kit (Abcam, ab133051). The lyophilization step was performed to concentrate the low amount of cAMP response, that could be present in the cells. Then cAMP ELISA assay was performed according to the kit instructions. The detailed statical analysis Two-Way ANOVA Post Hoc Test for Ca^{2+}/CaSR cAMP and One-Way ANOVA Post Hoc Test for PTH/PTH1R cAMP were performed on obtained results to analyze the statistical difference.

3.7 Semi-Quantitative Assay of Myosin Heavy Chain (MHC) Protein

The semi-quantitative assay of MHC protein was used to analyze the effect of Ca^{2+}/CaSR drugs stimulation on *in vitro* myogenic differentiation. The MHC protein, one of the main markers of myogenesis, was analyzed to see the variation during *in vitro* myogenic differentiation in response to CaSR stimulations. The experiment was performed in 3 primary hSCs lines, and analysis was done for 3 different time points (0 days, 3 days, and 6 days) of *in vitro* myogenic differentiation. For the assay, the primary hSCs were cultured in growth medium (SMCGM) on matrigel coated special dark 24 multiwell plates (Eppendorf Cell Imaging Plates). Upon reaching 60-70% confluency, the cells were washed once by DPBS and then stimulated with Myogenic Differentiation Medium (MDM) alone (control) and, MDM supplemented with 3 mM Ca^{2+}(Ca^{2+} effect), 3 mM Ca^{2+} + 1µ NPS R-568 (calcimimetic effect) and 3 mM Ca^{2+} + 1µ NPS 2143 (calcilytic effect). The medium with supplements was refreshed every alternate day. The cells were fixed with 4% PFA/DPBS at times (0 days, 3 days and 6 days) during myogenic differentiation, permeabilized with 0.2 % Triton, treated with RNAse, stained with monoclonal mouse anti-MHC primary antibody (Millipore, A4.1024) (dilution 1:20) according to the protocol described in section 3.3.2. The fluorescence was measured in a Laser-Scanning Confocal Microscopy (LSM510 META, Zeiss) Microscope equipped with Ar/ML458/477/488/514, HeNe543, and HeNe633 Laser Lines. The detailed statical analysis (ANOVA test) was performed on obtained results to analyze the significant difference.

4. RESULTS

4.1 Establishment of Primary Culture of Human Satellite Cells (hSCs) and Characterization of Established hSCs Lines

The 11 human skeletal muscle biopsies were obtained from the discarded tissue of patients undergoing plastic surgery from SOD of Prof. Marco Innocenti after signed an informed consent approved by the Local Ethical Committees of the University Hospital of Florence (AOUC), (Reference No. BIO.16.022). All the information of these 11 patients and skeletal muscle types are given in Table 7. The Human Satellite Cells (hSCs) were isolated from all the collected human skeletal muscle biopsies, and the primary cell culture of hSCs was established (Fig. 12).

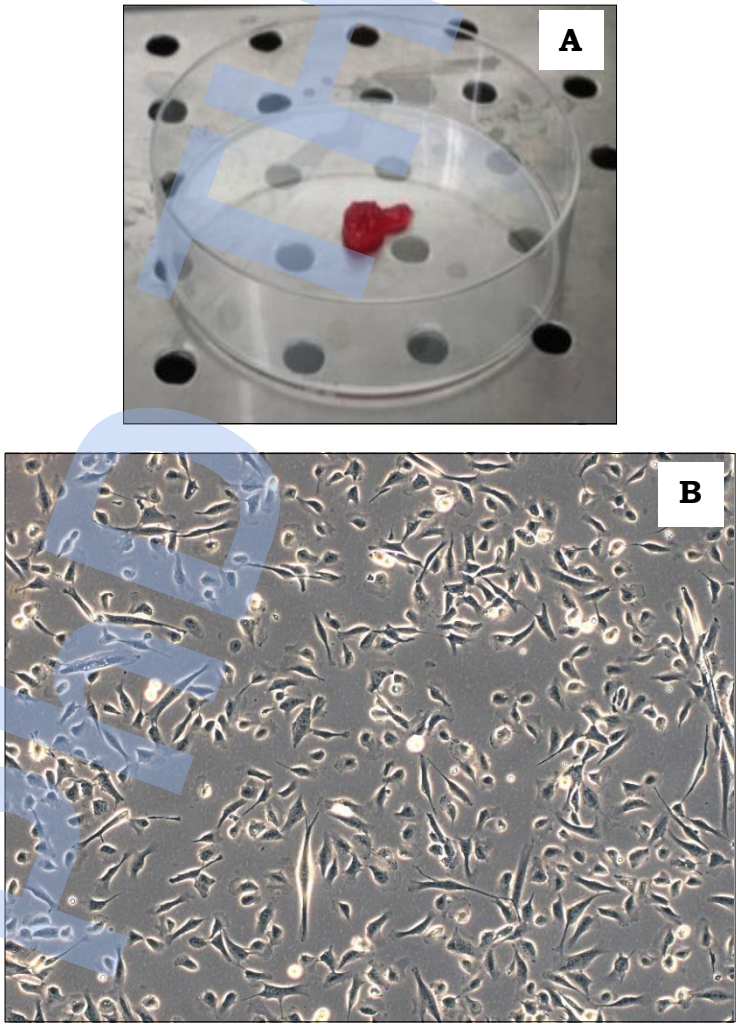


Fig. 12. *Human skeletal muscle biopsy (A) and primary cell culture of hSCs observation in phase contrast, original magnification: 10X (B).*

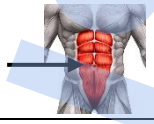
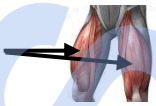
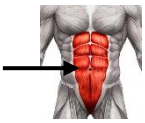
Skeletal Muscle Biopsies	Age	Skeletal Muscle Type	Sex	Derived Satellite Cells
Human Skeletal muscle tissue 1 **(hSMt1)**	50	Rectus abdominis muscle (Located in abdomen) 	Female	Human satellite cell line 1 **(hSCs1)**
Human Skeletal muscle tissue 2 **(hSMt2)**	57	Quadricepes muscle (Located in front of the thigh) 	Male	Human satellite cell line 2 **(hSCs2)**
Human Skeletal muscle tissue 3 **(hSMt3)**	52	Soleo muscle (Located in back part of lower legs) 	Male	Human satellite cell line 3 **(hSCs3)**
Human Skeletal muscle tissue 4 **(hSMt4)**	35	Soleo muscle (Located in back part of lower legs) 	Male	Human satellite cell line 4 **(hSCs4)**
Human Skeletal muscle tissue 5 **(hSMt5)**	50	Vastus medialis (Located medially in the thigh that extends the knee) 	Male	Human satellite cell line 5 **(hSCs5)**
Human Skeletal muscle tissue 6 **(hSMt6)**	63	Soleo muscle (Located in back part of lower legs) 	Male	Human satellite cell line 6 **(hSCs6)**
Human Skeletal muscle tissue 7 **(hSMt7)**	29	Vastus medialis (Located medially in the thigh that extends the knee) 	Female	Human satellite cell line 7 **(hSCs7)**
Human Skeletal muscle tissue 8 **(hSMt8)**	52	Rectus abdominis muscle (Located in abdomen) 	Female	Human satellite cell line 8 **(hSCs8)**
Human Skeletal muscle tissue 9 **(hSMt9)**	45	Tibialis anterior muscle (Located in anterior part of the legs) 	Male	Human satellite cell line 9 **(hSCs9)**
Human Skeletal muscle tissue 10 **(hSMt10)**	80	Soleo muscle (Located in back part of lower legs) 	Female	Human satellite cell line 10 **(hSCs10)**
Human Skeletal muscle tissue 11 **(hSMt11)**	81	Gastrocnemius muscle (Located in back part of legs) 	Male	Human satellite cell line 11 **(hSCs11)**

Table 7. *Information of the collected human skeletal muscle biopsies and respectively their satellite cell lines.*

The primary cultured human satellite cells were subcultured, and human satellite cell lines were obtained. At this point, to characterize the human satellite cell's phenotype of the obtained hSCs lines, we evaluate the expression of PAX7. PAX7 is a nuclear transcriptional factor that is the specific marker of satellite cells. We noticed the expression of PAX7 in all the established hSCs lines by RT-PCR analysis (Fig. 13), by flow cytometry (Fig. 14) and by western blot analysis (Fig. 15).

Fig. 13. RT-PCR analysis of PAX7*-PAX7 gene is expressed in hSCs lines (hSCs1, hSCs2, hSCs3). Negative control: PCR without cDNA template.*

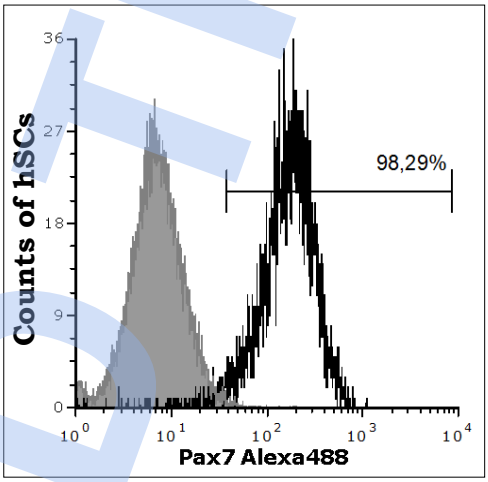


Fig. 14. Flow cytometry analysis of PAX7*-PAX7 protein is expressed in hSCs lines, area in black curve represents the hSCs stained with primary anti-PAX antibody whereas the area under grey shade curve represent the hSCs only stained with secondary antibody.*

Fig. 15. Western blot analysis of PAX7*-confirming the presence of PAX7 protein in hSCs.*

4.2 Establishment of Myogenic Differentiation *In Vitro*

Myogenic differentiation was observed in hSCs lines at 7 days of myogenic induction when several multinucleated cells called myotubes were observed (Fig. 16). The myogenic differentiation was characterized by immunofluorescence staining of the myosin heavy chain (MHC), which is the most important myogenic terminal differentiation marker protein. At 7 days of myogenic induction, the obtained multinucleated cell showed a high positivity of the MHC, observed in laser Scanning Confocal Microscopy (LSCM) (Fig. 17).

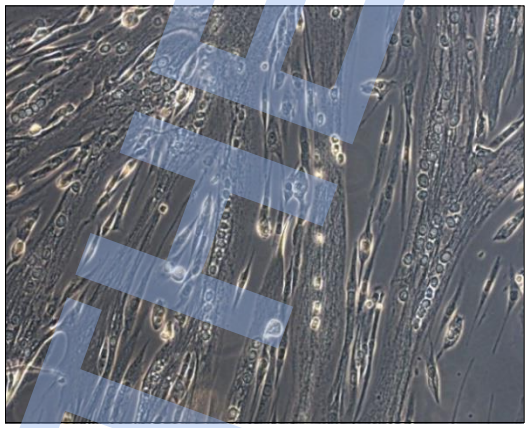


Fig. 16. Myogenic differentiation*-multinucleated cells (myotubes) observed after 7 days of myogenic induction, observation in phase contrast, original magnification: 20X.*

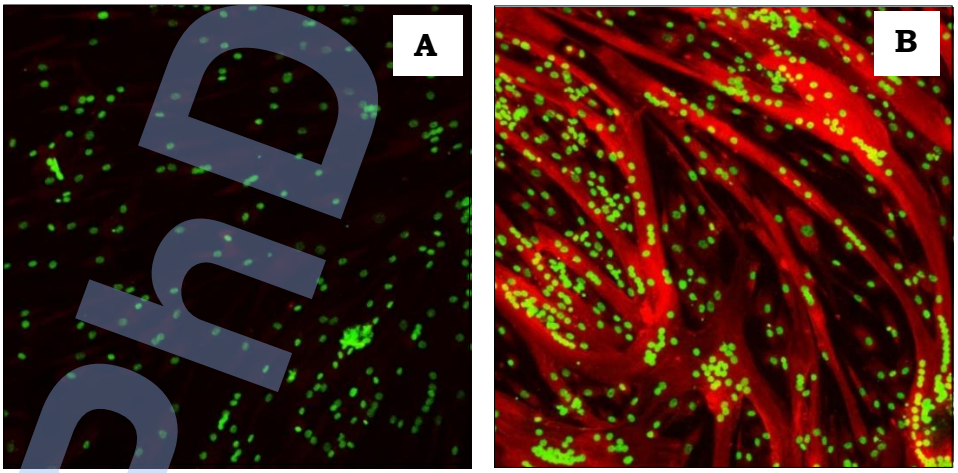


Fig. 17. Immunofluorescence staining of MHC in obtained multinucleated cells*- the obtained myotubes showed a high positivity of MHC protein. A: negative control myotubes stained with secondary antibody only, B: myotubes stained with anti-MHC antibody, LSCM, conventional colors: red for MHC and green for nuclei, original magnification: 10X.*

The myogenic phenotype was also characterized by RT-PCR analysis to evaluate the expression of genes of the myogenic regulatory factors *Myf5, MyoD1, Myogenin,* and of terminal differentiation marker MHC (Fig. 18). We had observed that these genes were contemporary expressed during the last period (7 days) of myogenic differentiation when myotubes were formed, while during the first phase (0 days) of the myogenic differentiation only *Myf5* and *MyoD1* were expressed, and *Myogenin* and *MHC* were completely absent (Table 8).

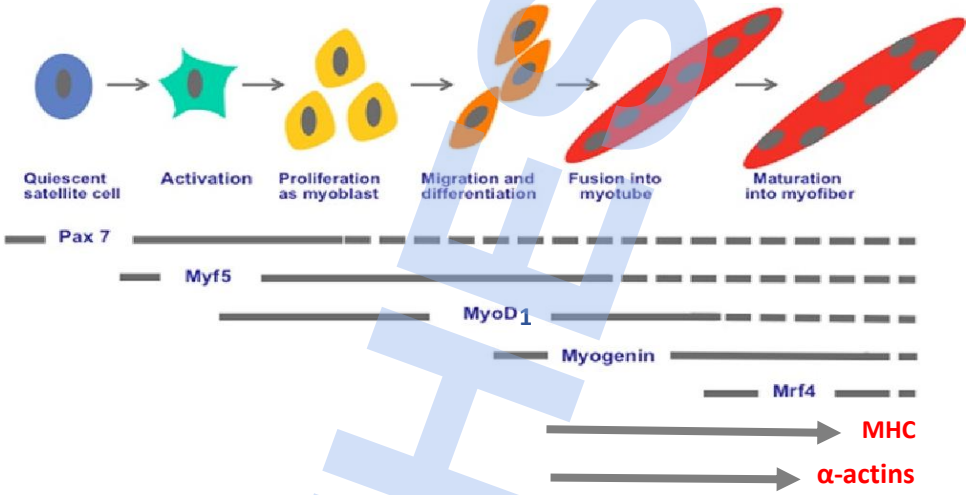


Fig. 18. *Expression profile of myogenic differentiation marker genes. The figure was taken from reference* (26).

Myogenic Differentiation Marker genes	**0 days (hSCsl)**	**0 days (hSCs2)**	**0 days (hSCs3)**	**7 days (hSCs1)**	**7 days (hSCs2)**	**7 days (hSCs3)**
Myf5	+	+	+	+	+	+
MyoD1	+	+	+	+	+	+
Myogenin	-	-	-	+	+	+
MHC	-	-	-	+	+	+

Table 8. RT-PCR analysis of myogenic differentiation marker genes*- RT-PCR analysis showed the different expression of Myf5, MyoD1, Myogenin and of MHC, analysed over the time during myogenic differentiation of three primary hSCs lines (hSCs1, hSCs2, hSCs3).*

To analyse the expression of MHC gene at different time points (0 days, 3 days, 6 days, and 9 days) of myogenic differentiation, we performed a Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (Real-Time qRT-PCR). The data has shown a significant increase of the MHC gene during myogenic differentiation respect to the control group at 0 days ($p < 0.001$) (Fig. 19).

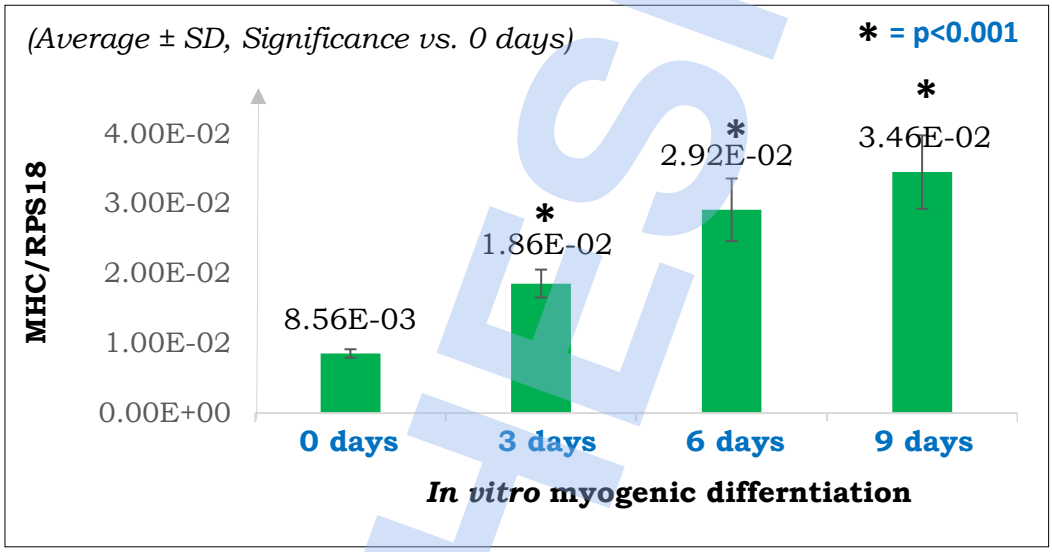


Fig. 19. Real-Time qRT-PCR analysis of MHC during myogenic differentiation- *the analysis showed a significant increase of the MHC gene during myogenic differentiation respect to the control group at 0 days ($p<0.001$). Experiment was carried out in triplicates and is representative of the three established hSCs lines.*

The expression of MHC protein was also evaluated by western blot analysis during different time points (0 days, 3 days, 6 days, and 9 days) of myogenic differentiation. The results obtained have shown an increase of MHC overtime during myogenic differentiation (Fig. 20).

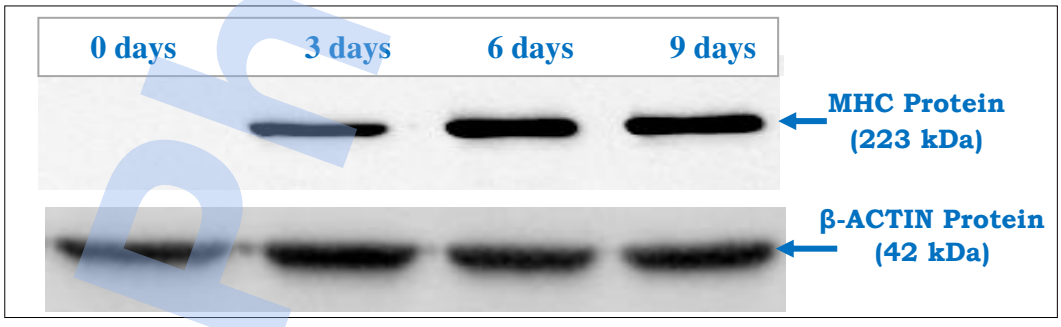


Fig. 20. Western blot analysis of MHC during myogenic differentiation- *the analysis showed an increase of the MHC protein during myogenic differentiation. Experiment was carried out in triplicates and are representative of the three established hSCs lines.*

4.3 Analysis of Calcium Sensing Receptor (CaSR) Expression and Function in Human Skeletal Muscle

4.3.1 CaSR Gene Expression Analysis in Human Skeletal Muscle Tissues (hSMts) and in Established Human Satellite Cells (hSCs) lines

The qualitative RT-PCR revealed that primers which target exon 2/3, 4/5 and 6/7 of human CaSR, have not detected the expression of CaSR in human skeletal muscle tissues (hSMt1, hSMt2, hSMt3) and in respective established human satellite cell lines (hSCs1, hSCs2, hSCs3) (Fig. 21).

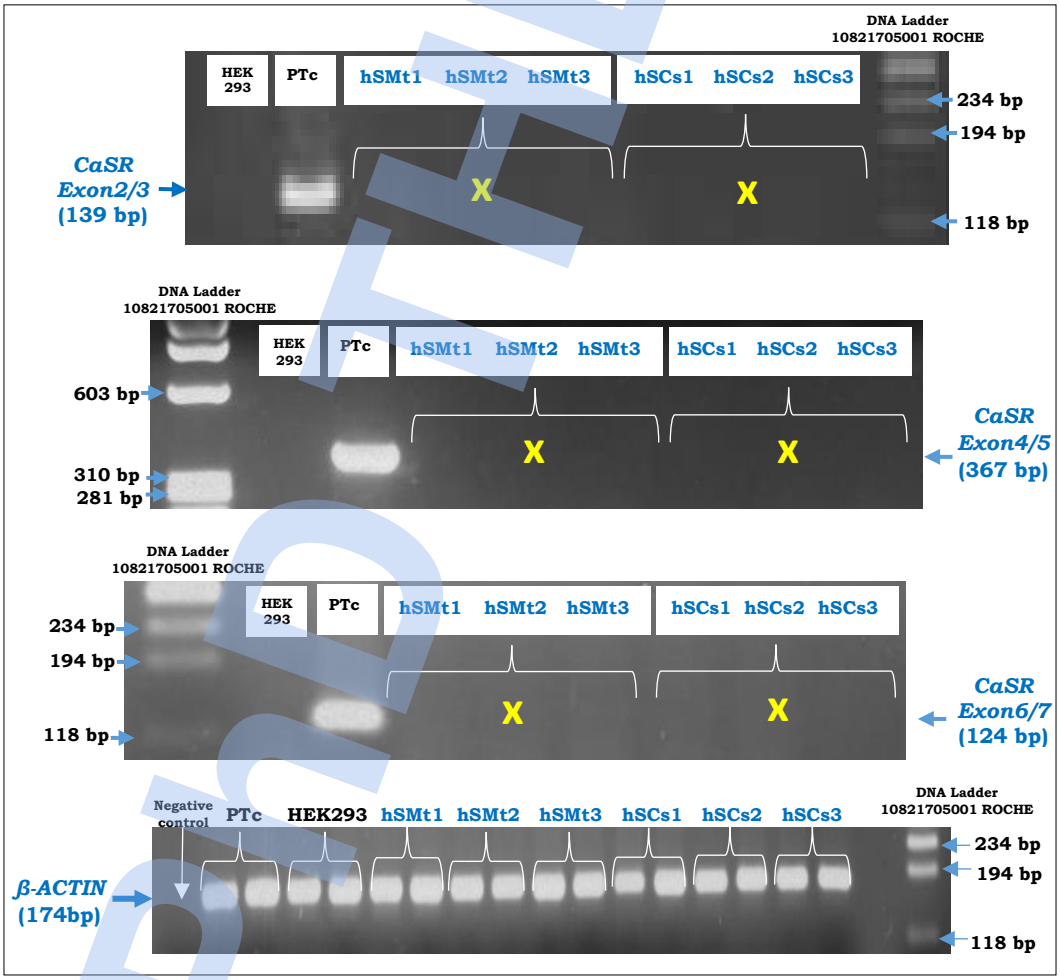


Fig. 21. RT-PCR analysis of CaSR gene in hSMts and established hSCs lines- *the analysis showed the expression of CaSR only in primary cultured human parathyroid cells (PTc) which were used as positive control and the absence of CaSR gene in hSMts and in hSCs lines and in negative control HEK293. Negative control: PCR without template cDNA in β-ACTIN gene analysis.*

To evaluate the expression of CaSR gene in hSMts and established hSCs lines using a highly sensitive method, we have performed a Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (Real-Time qRT-PCR). The obtained results of this analysis showed a very week signal of CaSR expression in hSMts and established hSCs lines, which were not possible to be sequenced (Fig. 22).

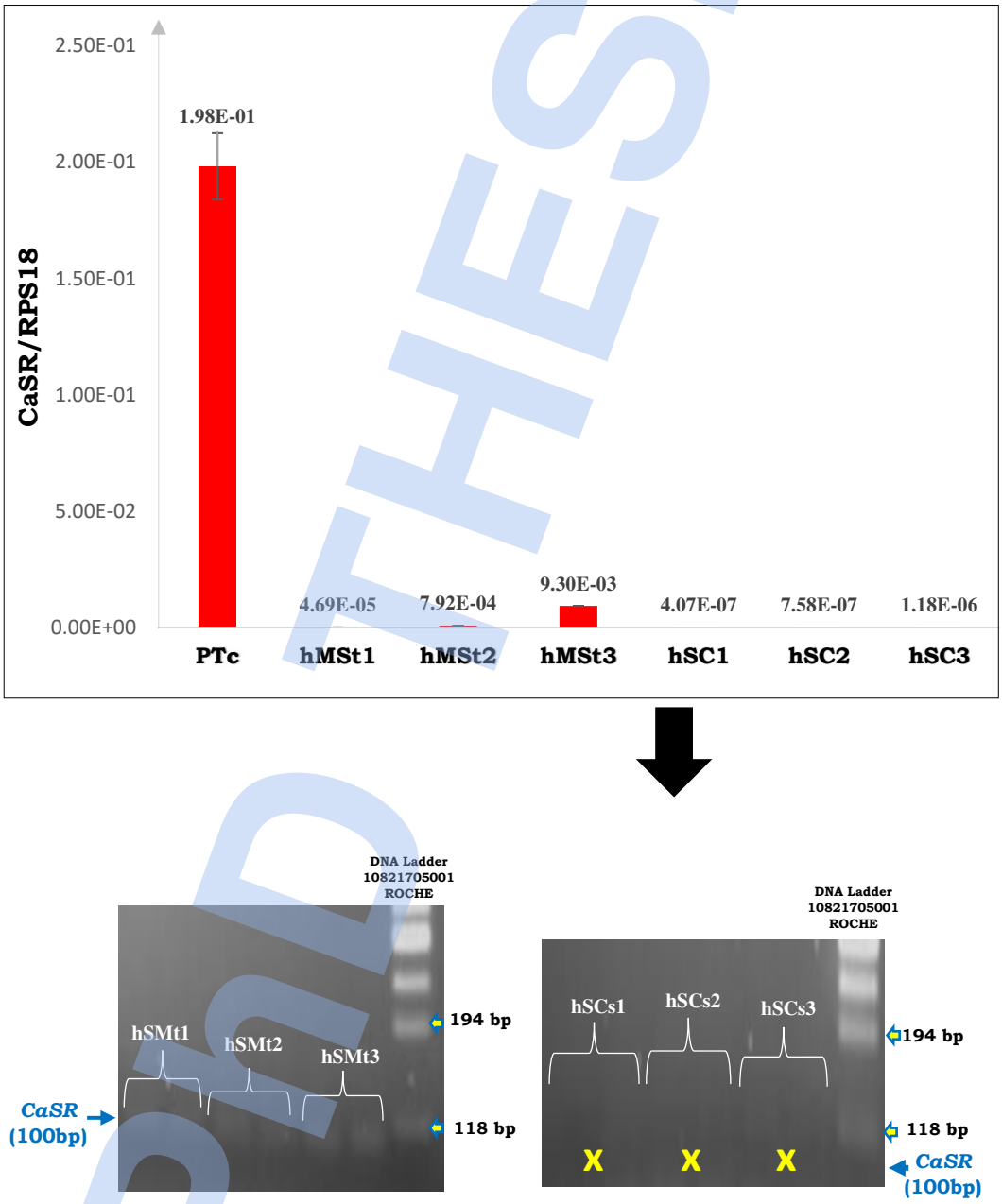


Fig. 22. Real-Time qRT-PCR analysis of CaSR gene in hSMts and in established hSCs lines*- the analysis showed a very low expression of CaSR gene in hSMts and in hSCs lines. The primary cultured human parathyroid cells (PTc) were used as positive control.*

4.3.2 CaSR Gene Expression Analysis during *In Vitro* Myogenic Differentiation

To evaluate the expression of CaSR gene during the myogenic differentiation, we have analyzed the expression of CaSR overtime during the myogenic differentiation *in vitro*. The RT-PCR analysis performed at 0 days and 7 days of myogenic differentiation showed that CaSR gene is not expressed at 0 days and 7 days both (Fig. 23). So, we have noticed the absence of CaSR gene over the time during myogenic differentiation of three primary hSCs lines.

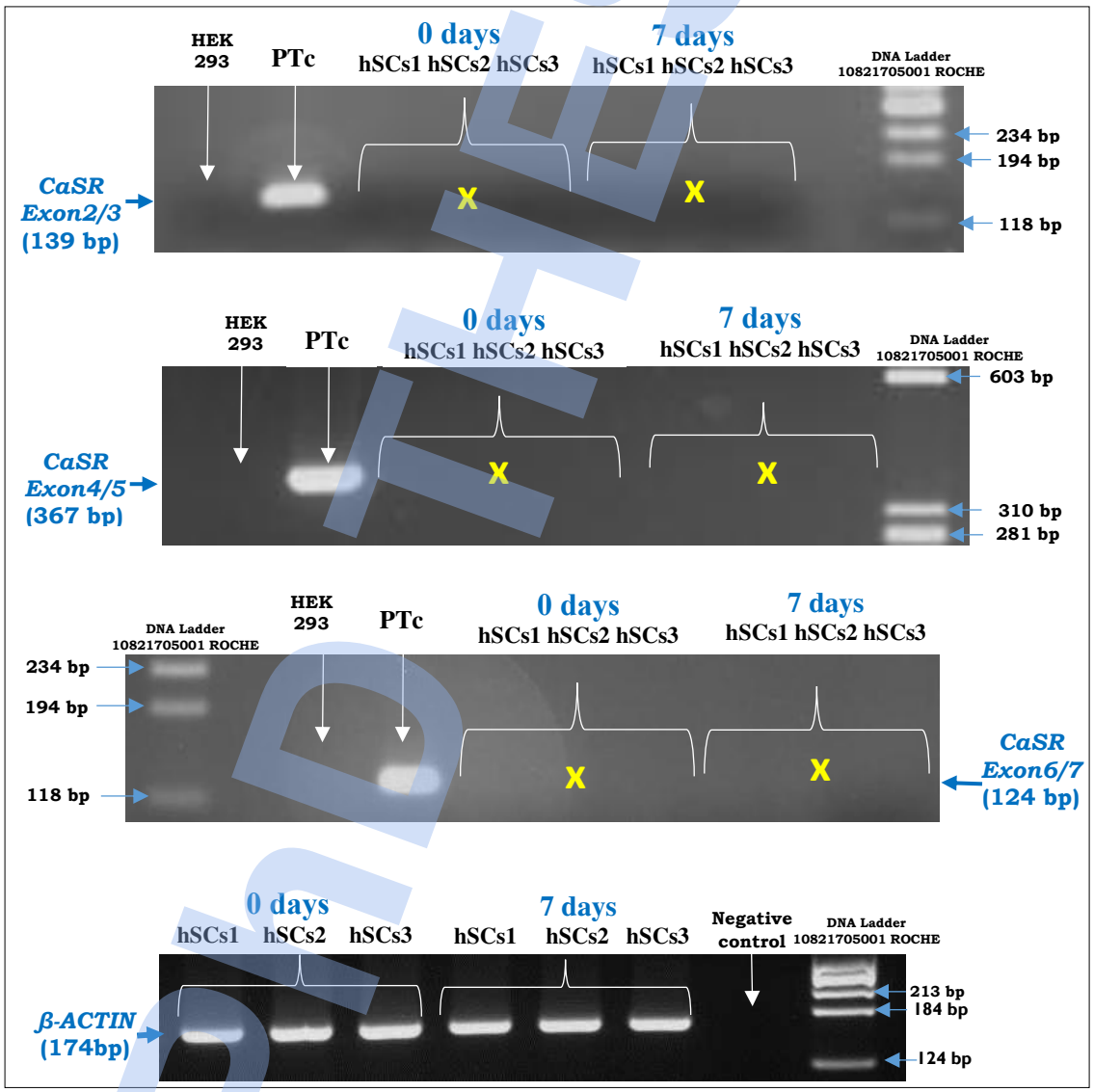


Fig. 23. RT-PCR analysis of CaSR gene over the time during in vitro myogenic differentiation of hSCs lines *- the analysis showed the absence of the CaSR gene at 0 days and at 7 days of myogenic differentiation of 3 primary hSCs lines. The primary cultured human parathyroid cells (PTc) were used as positive control and HEK293 cells were used as negative control. Negative control: PCR without template cDNA in β-ACTIN gene analysis.*

We have also performed a Real-Time qRT-PCR to evaluate the expression of CaSR gene at several points during myogenic differentiation. We have assessed the CaSR gene expression at times (0 days, 3 days, 6 days, and 9 days) during myogenic differentiation of three primary hSCs lines. The data obtained showed the complete absence of CaSR gene expression during myogenic differentiation (Fig. 24).

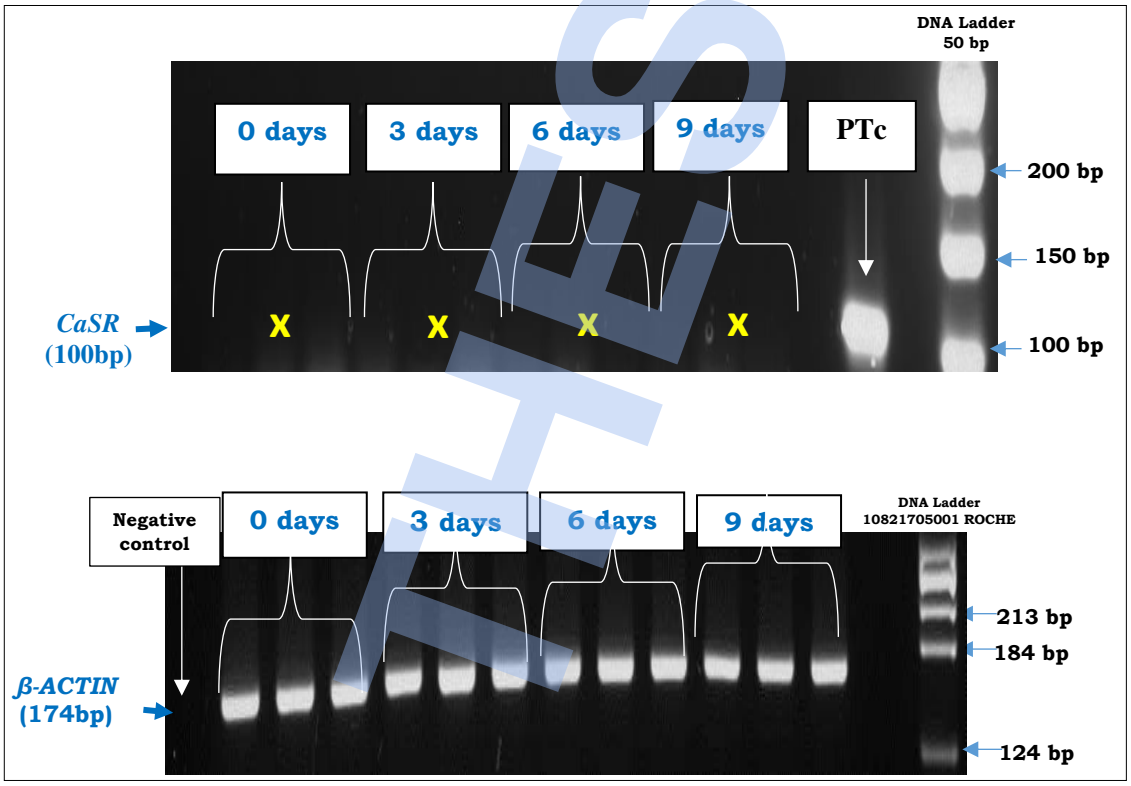


Fig. 24. Real-Time *qRT-PCR of CaSR gene during myogenic differentiation*- *the analysis showed the absence of CaSR gene expression at times 0 days, 3 days, 6 days and 9 days during myogenic differentiation of primary hSCs lines. The primary cultured human parathyroid cells (PTc) were used as positive control. Negative control: PCR without template cDNA in β-ACTIN gene analysis. Experiment was carried out in triplicates and are representative of the three established hSCs lines.*

4.3.3 CaSR Protein Expression Analysis in Human Skeletal Muscle Tissues (hSMts), in hSCs Lines and during *In Vitro* Myogenic Differentiation.

The images of Human Skeletal Muscle Tissues (hSMts) sections observed in phase contrast showed the longitudinal view of the human skeletal muscle fibers (Fig. 25).

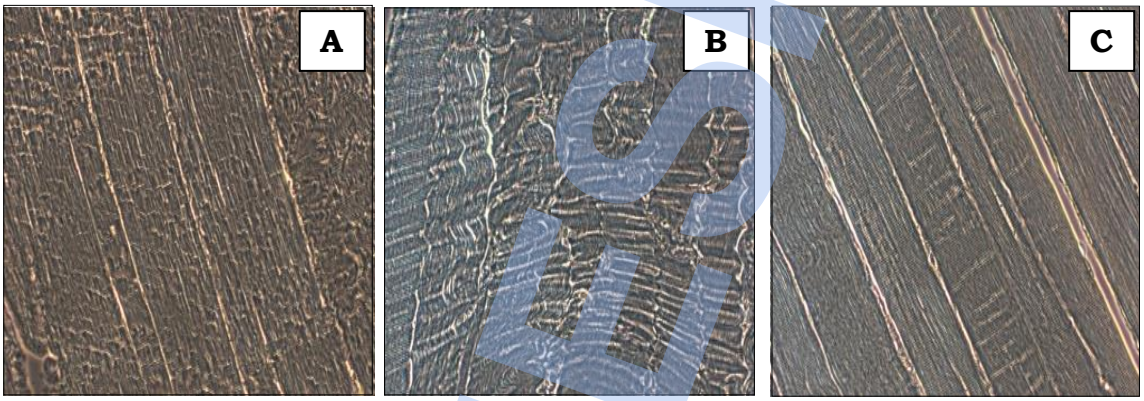


Fig. 25. Human Skeletal Muscle Tissues (hSMts) sections*-observation in phase contrast, original magnification: 10X, A, B, C.*

After the gene expression analysis by RT-PCR and Real-Time qRT-PCR to evaluate the CaSR expression, we have performed a) immunofluorescence staining, b) flow cytometry and c) western blot analysis, to assess a possible expression of CaSR protein in hSMts sections, in established hSCs lines and during myogenic differentiation.

The results of immunofluorescence analysis in hSMts sections for CaSR analysis showed some staining, out of the area of the skeletal muscle tissue section on the glass slide (Fig. 26 (A, B, C)). The observed staining seems like aggregate of the antibodies, or in some parts, it looks like staining of endothelial cells. The positive detection of the CaSR expression in the same analysis in human parathyroid tissue sections has validated our method of analysis (Fig. 26 (D, E, F).). The specificity of the anti-CaSR antibody has been verified by one extra internal negative control in the assay, where the antibody was incubated in the tissue sections along with its blocking peptide (Fig. 26. (B & E)). The results demonstrate the absence of CaSR protein expression in human skeletal muscle tissue.

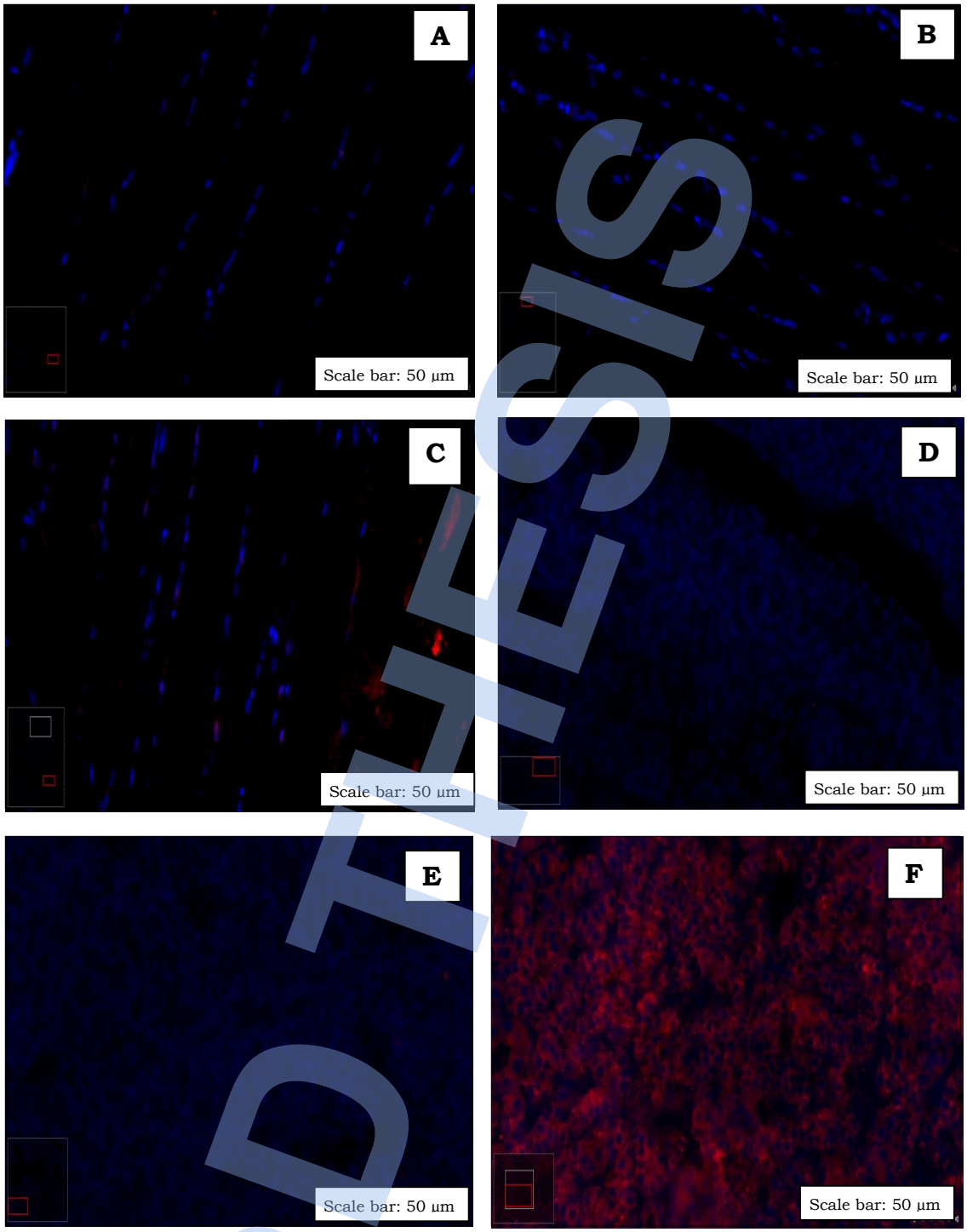


Fig. 26. *Immunostaining of CaSR Protein with TisssueFAXS*- *analysis of CaSR protein in human skeletal muscle tissues sections (A: negative control with secondary antibody only, B: control with anti-CaSR antibody absorbed with blocking peptide and C: with primary anti-CaSR antibody). Analysis of CaSR protein in human parathyroid tissues sections used as positive control. (D: negative control with secondary antibody only, E: control with anti-CaSR antibody absorbed with blocking peptide and F: with primary anti-CaSR antibody). Fluorescent microscopy with TisssueFAXS software, conventional colors: red for CaSR and blue for nuclei, original magnification: 20X, Scale bar: 50 µm. Experiment was carried out in triplicates of hSMts sections from three different human.*

In addition to immunostainings in hSMts to evaluate the CaSR protein, we have performed several analyses in hSCs lines to detect CaSR protein. First of all, we have performed flow cytometry for the CaSR protein expression in hSCs lines using two different dilutions (1:500 and 1:100) of the primary anti-CaSR antibody (ADD). The obtained data showed the absence of CaSR protein in hSCs lines, even at a high concentration of the anti-CaSR antibody (Table 9).

Sample	**Anti-CaSR antibodies**	**Dilution of antibodies (1:500)**	**Dilution of antibodies (1:100)**
hSCs lines	**Monoclonal anti-CaSR ADD antibody** (Mother concentration: 1 mg/ml)	**0.27%**	**0.41%**

Table 9. Flow cytometry analysis of CaSR protein in hSCs lines *- the analysis of CaSR protein performed in hSCs at lower concentration and higher concentration of anti-CaSR antibody showed the absence of CaSR protein in hSCs. Results are the representative of experiment carried out in three established hSCs lines.*

In addition to flow cytometry in hSCs lines, we have also performed the immunostaining, which also showed the absence of CaSR protein in hSCs lines (Fig. 27)

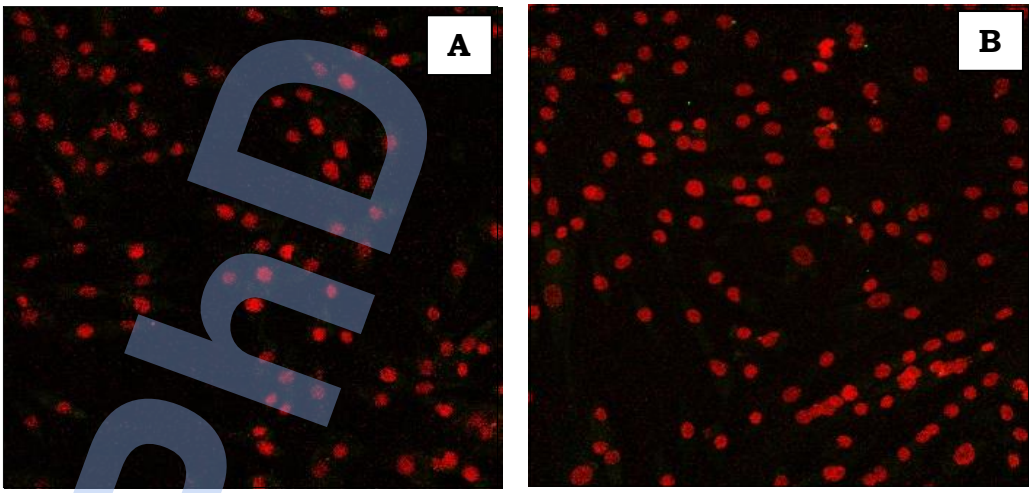


Fig. 27. Immunostaining of CaSR in hSCs lines*- the analysis of CaSR protein in hSCs lines (A: negative control with secondary antibody only and B: with primary anti-CaSR antibody). Laser Scanning Confocal Microscopy (LSCM), conventional colors: green for CaSR protein and red for nuclei, original magnification: 10X. Results are the representative of experiment carried out in three established hSCs lines.*

Finally, the western blot analysis was performed to analyze CaSR protein expression in hSCs lines to verify the results obtained by flow cytometry and by immunofluorescence. The western blot analysis was also performed at different time points (0 days, 3 days, 6 days, and 9 days) of myogenic differentiation of primary hSCs lines. The HEK293 cells stably transfected with human CaSR (HEK_{CaSR}) were used as a positive control in the analysis. The obtained results showed faint bands of about 160 kDa and 140 kDa (size of CaSR monomers) in hSCs lines and during myogenic differentiation. The observed bands were seeming like CaSR (Fig. 28)

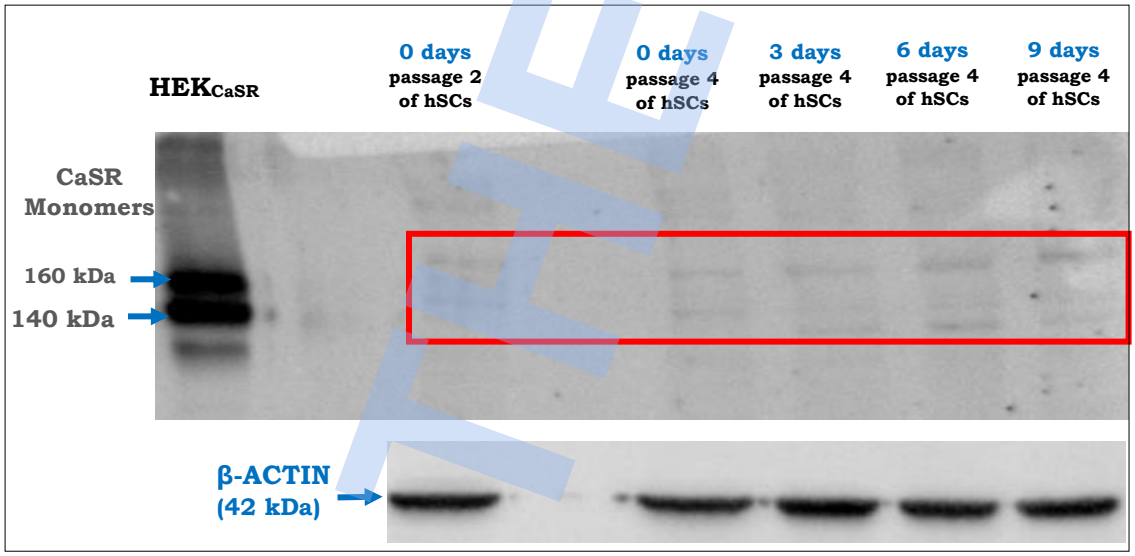


Fig. 28. Western Blot analysis of CaSR protein in reducing condition in hSCs lines and during in vitro myogenic differentiation- *the results showed the presence of faint bands of sizes about 160 kDa and 140 kDa in hSCs lines and at different time points (0 days, 3 days, 6 days and 9 days) of myogenic differentiation of primary hSCs lines. The bands seemed like CaSR. HEK293 cells transfected with human CaSR (HEK_{CaSR}) were used as positive control. Experiment was carried out in triplicates and are representative of the three established hSCs lines.*

Since we have not observed the CaSR gene expression in the established hSCs lines and during myogenic differentiation, but we have observed faint bands in western blot analysis of about the size of CaSR monomer bands in the established hSCs lines and during myogenic differentiation, we thought to verify the specificity of the anti-CaSR antibody. For that, we have performed the western blot in

reducing and non-reducing conditions in the samples of proliferating hSCs lines of passage 2, as the intensity of the bands observed was higher in this sample. In this particular western blot, another native positive control, bovine parathyroid tissue lysate, was also included to verify the binding of the anti-CaSR antibody further. The obtained results showed that the monomers CaSR bands observed in positive control HEK_{CaSR} in reducing condition has shifted to CaSR dimers band in non-reducing condition. While the monomer's bands in the hSCs lines in reducing condition, which seemed like CaSR, did not shift to dimer band in non-reducing condition (Fig. 29). The obtained results conclude that the bands that we have observed in hSCs lines and during myogenic differentiation are due to unspecific reactivity of anti-CaSR antibody, and are false. Thus, western blot analysis also showed the absence of CaSR protein expression in hSCs lines. The analysis showed the absence of CaSR protein expression during myogenic differentiation too.

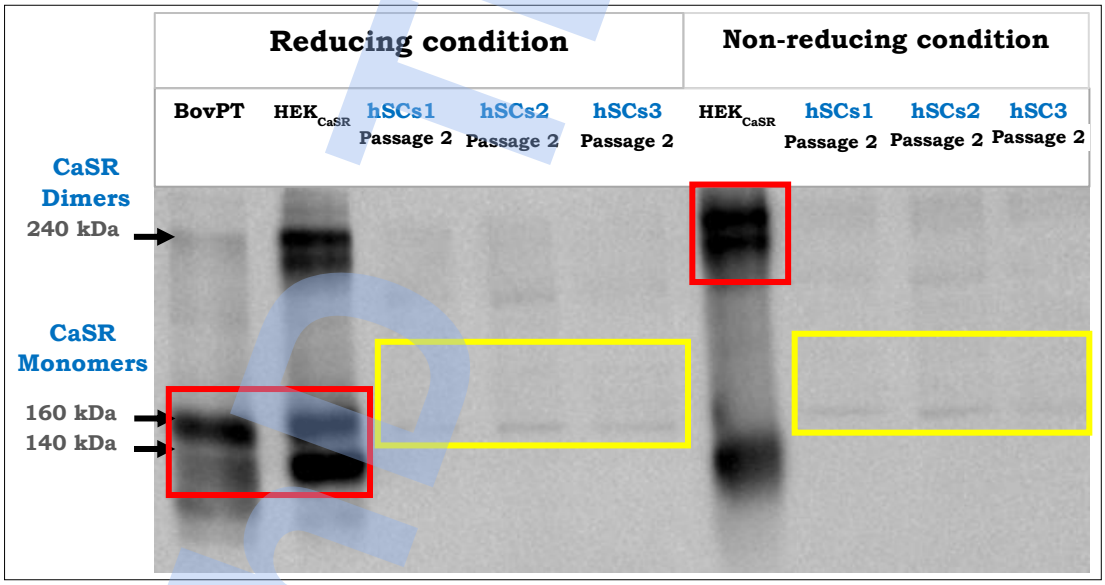


Fig. 29. Western blot analysis of CaSR in reducing and non-reducing conditions- *the analysis for CaSR protein in three hSCs lines showed that the observed bands are not CaSR as they did not shift to dimer form in non-reducing condition. HEK293 cells transfected with human CaSR (HEK_{CaSR}) and bovine parathyroid tissue lysate were used as positive control.*

4.3.4 CaSR Secondary Messengers Analysis in hSCs Lines

To further verify the presence of CaSR in hSCs lines, we performed the analysis of various secondary messengers (Fig. 30) upon inducing the hSCs lines with CaSR ligands Ca^{2+} and CaSR drugs NPS2143 (calcilytic) and NPS R-568(calcimimetic).

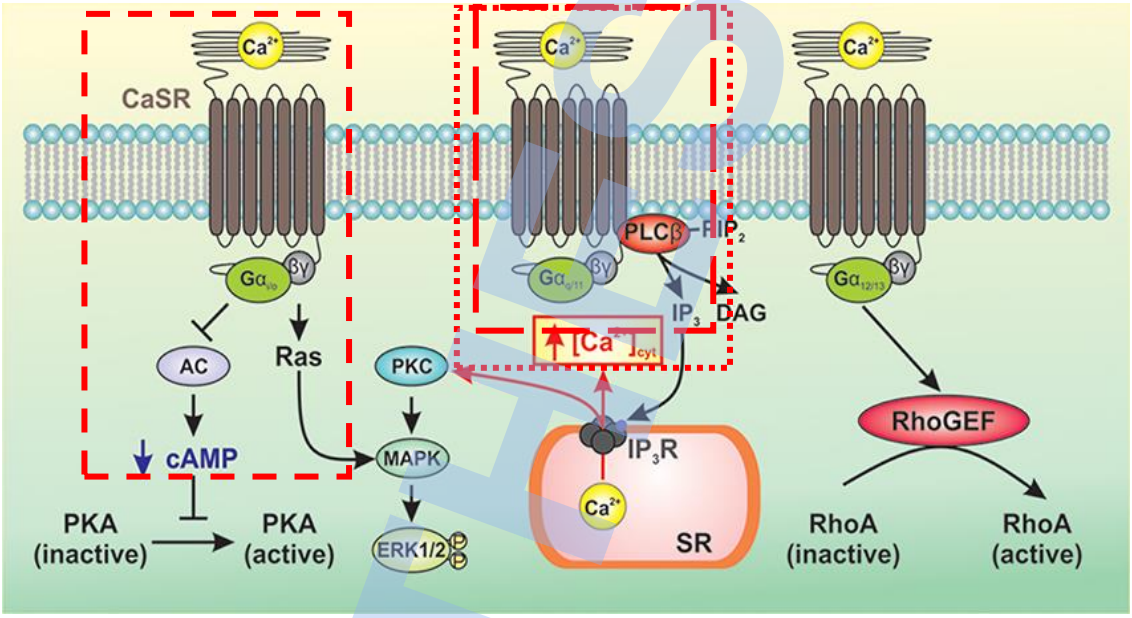


Fig. 30. CaSR secondary messengers in signaling pathways*- the CaSR, upon activation, can couple to Gi/0, Gq/11, and G12/13, which regulate highly divergent downstream signaling pathways, depending on the cellular context. The pathways marked in red rectangles were analyzed in hSCs lines to verify the possibilities of the presence of CaSR in hSCs lines. The figure was taken from* (102).

4.3.4.1 CaSR Secondary Messenger Analysis in hSCs Lines: Gq-Inositol triphosphate (IP_3)

To analyse the possible presence of CaSR in hSCs lines, we performed the analysis of IP_3 secondary messenger using IP_1 ELISA assay, upon inducing hSCs lines independently in technical quadruplicates with 1.2 mM Ca^{2+} and 3.5 mM Ca^{2+}. The obtained results showed no dose-dependent increase in IP_1 response (Fig. 31).

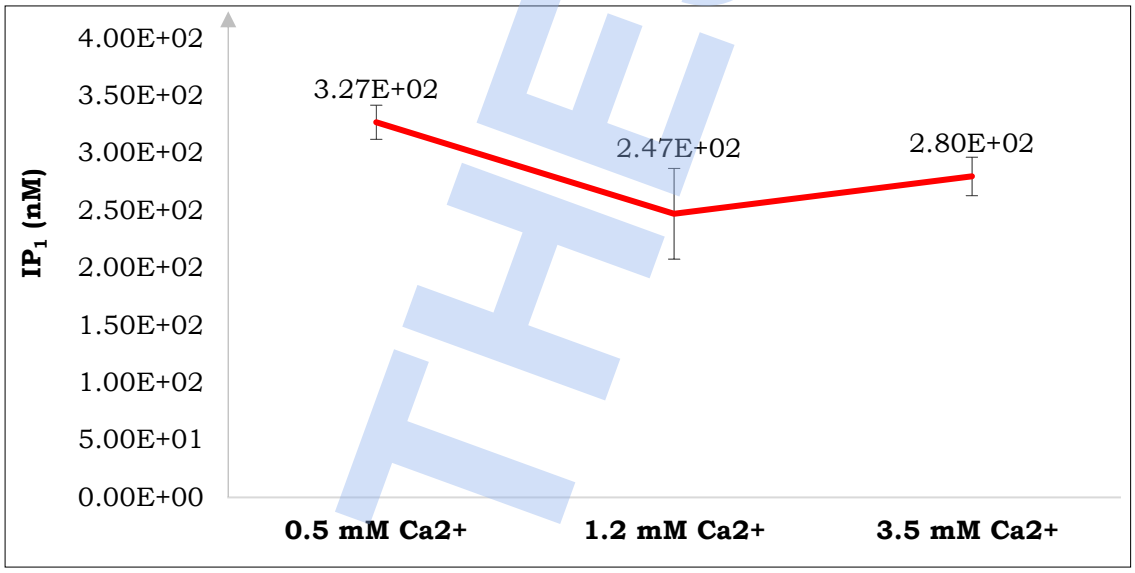


Fig. 31. IP_1-Gq ELISA assay in hSCs lines- *no IP_1 response was detected in Ca^{2+} induced hSCs lines. Experiment was carried out in triplicates and are representative of the three established hSCs lines.*

4.3.4.2 CaSR Secondary Messenger Analysis in hSCs Lines: Gq- IP_3- Intracellular Calcium Mobilization Imaging

After that, we have performed the analysis of the intracellular calcium immobilization assay in three live hSCs lines. The obtained results showed that hSCs do not respond to CaSR stimulations by Ca^{2+} and by CaSR drugs NPS2143 (calcilytic) and by NPS R-568(calcimimetic) in intracellular calcium mobilization assay with Fura2-AM (Fig. 32). The HEK293 transfected with human CaSR (HEK_{CaSR}), were used as a positive control in the assay, responded well to all the CaSR stimulations.

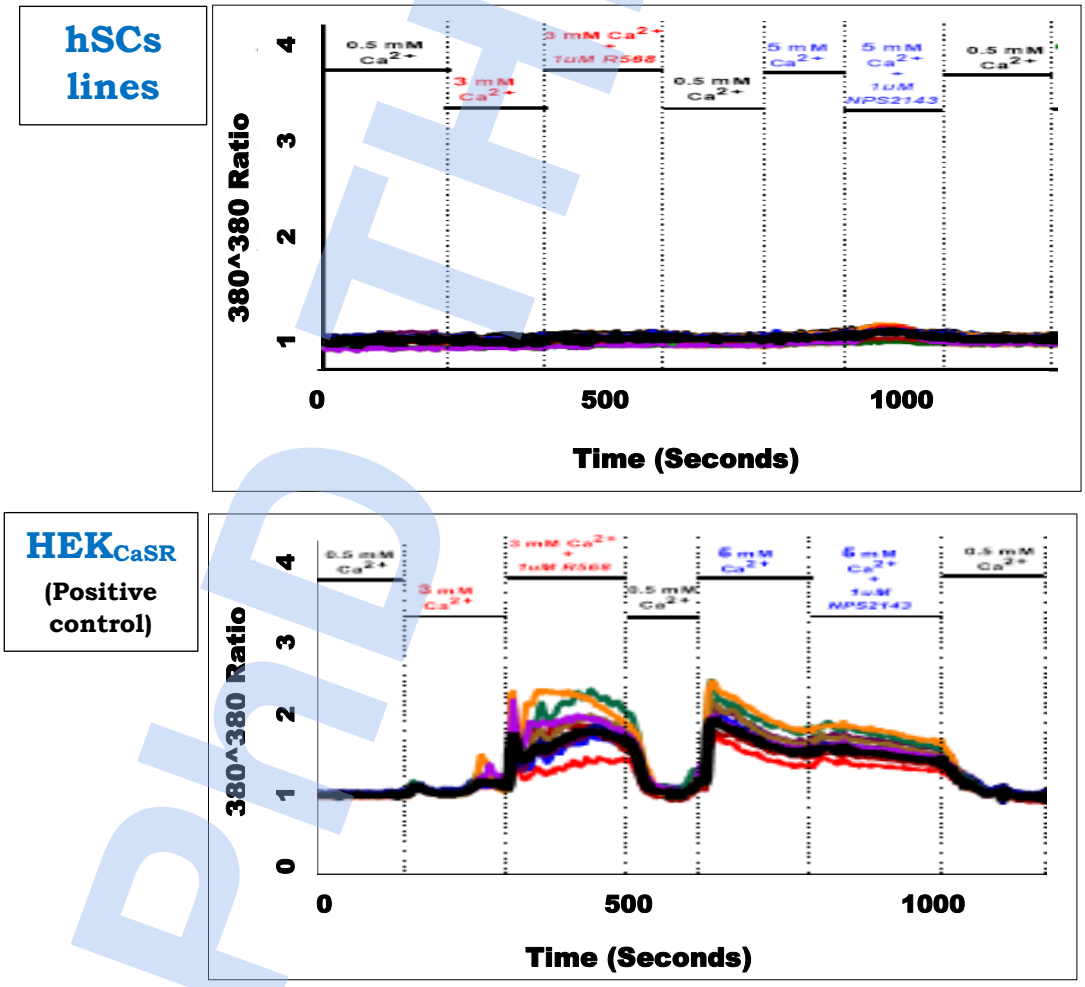


***Fig. 32. IP_3 related intracellular calcium mobilization* assay with Fura2-AM dye in hSCs lines-** *Graphs representing the effect of CaSR stimulations on intracellular calcium mobilization in hSCs lines and positive control (HEK_{CaSR}). Experiment was carried out in triplicates and is representative of the three established hSCs lines.*

4.3.4.3 CaSR Secondary Messenger Analysis in hSCs Lines: Gi- Cyclic Adenosine Monophosphate (cAMP)

We have also performed Gi-cAMP assay using ELISA in hSCs lines upon stimulation with 3 mM Ca^{2+} alone and along with CaSR drugs 1µM NPS2143 (calcilytic) or 1µM NPS R-568 (calcimimetic). The results of cAMP ELISA assay in hSCs lines have shown a slight effect of 3.0 mM Ca^{2+}. The co-treatment of CaSR drugs 1µM NPS2143 (calcilytic) or 1µM NPS R-568 (calcimimetic) with 3.0 mM Ca^{2+} has shown a little effect too. (Fig. 33).

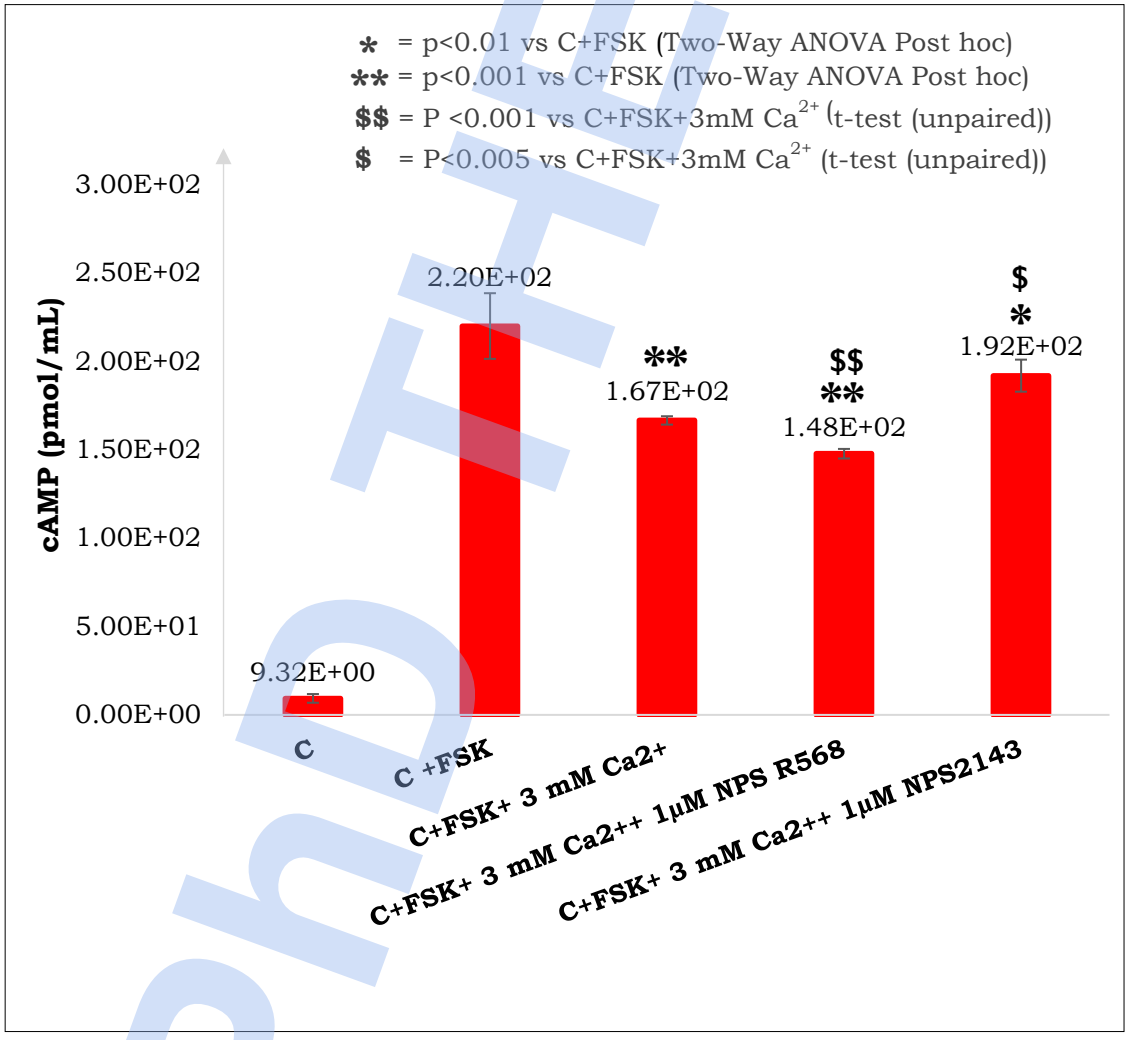


Fig. 33. Gi-cAMP ELISA assay in hSCs lines*- graph representing the effect of CaSR stimulation in cAMP response in hSCs lines. Experiment was carried out in triplicates and is representative of the three established hSCs lines.* ***C:*** *control buffer with 0.5 mM Ca^{2+},* ***FSK:*** *Forskolin (positive control), Stimulation time: 15 min.*

4.3.5 Effect of Ca^{2+} and CaSR Drugs on *In Vitro* Myogenic Differentiation

After the analysis of the expression of CaSR in hSMts, hSCs lines, and during myogenic differentiation, observing the absence of CaSR gene and protein, we have evaluated if there was any effect of Ca^{2+} and CaSR dugs (NPS2143 calcilytic and NPS R-568 calcimimetic) on myogenic differentiation. In order to evaluate this, we have analyzed if these CaSR ligands were able to induce a change in the expression of myosin heavy chain (MHC) protein, which is the crucial myogenic marker during myogenic differentiation. The results showed that no significant difference for CaSR stimulations: 3mM Ca^{2+} and co-treatment of 1 µM NPS2143 or 1 µM NPS R-568 with 3mM Ca^{2+} on the expression of MHC protein during myogenic differentiation (Fig. 34). The obtained results were analyzed with a statistical analysis; ANOVA test.

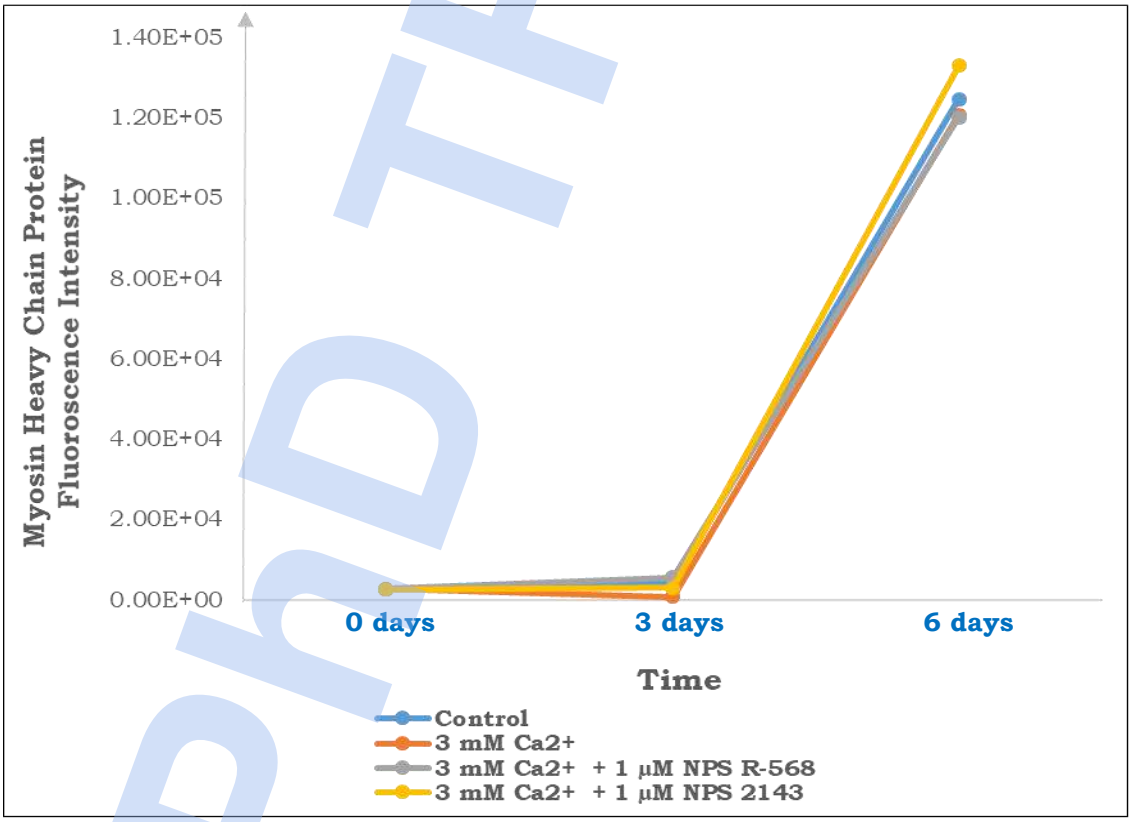


***Fig. 34. Semi-quantitative assay of MHC**- the analysis of MHC during in vitro myogenic differentiation at 0 days, 3 days and 6 days upon stimulation with 3mM Ca^{2+} and co-treatment of 1 µM NPS2143 or 1 µM NPS R-568 with 3mM Ca^{2+}. Experiment was carried out in triplicates and is representative of the three established hSCs lines.*

4.3.6 Possibility of the Presence of a CaSR Homologous Gene/Protein in Human Skeletal Muscle: GPRC6A

Since our results of CaSR gene and protein analysis showed the absence of this receptor in Human Skeletal Muscle Tissues (hSMts) and the Human Satellite Cells (hSCs), but we have got a low response in one of the secondary messenger cAMP in hSCs lines upon stimulation with 3.0 mM Ca^{2+} and cotreatment of CaSR drugs 1µM NPS2143 (calcilytic) or 1µM NPS R-568 (calcimimetic) with 3.0 mM Ca^{2+}. This led us to the thought of the presence of CaSR homologous protein. In search of CaSR homologous protein, we came across GPRC6A, which is the most CaSR homologous protein (Fig. 35). GPRC6A is the physiologically relevant class c family G protein-coupled receptor (GPCR), recently discovered (103, 104). The GPRC6A reported to respond to calcium with low affinity and also with drugs NPS2143 & NPS R-568 and found to produce secondary messenger cAMP in the response; however, the studies have been reported using mouse GPRC6A (104-106). Considering all the reported information about GPRC6A, we hypothesized the presence of GPRC6A receptor in human skeletal muscle as a potential drug target for skeletal muscle disorder.

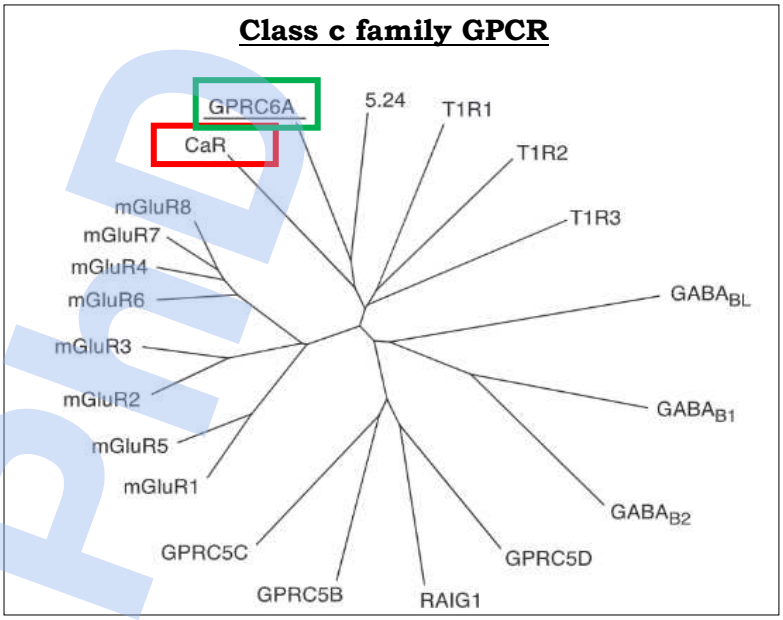


Fig. 35. CaSR homologous Proteins- *GPRC6A is reported as the most CaSR homologous protein (outlined in a green rectangle) in class c family GPCR. The figure was taken from the report* (103)**.** ***CaR:*** *CaSR*

To analyse the expression of GPRC6A gene, we have performed the RT-PCR in the human skeletal muscle tissue (hSMt) and the established Human Satellite Cells (hSCs) lines. The obtained results showed the absence of GPRC6A gene in hSMt and the established hSCs lines (Fig. 36).

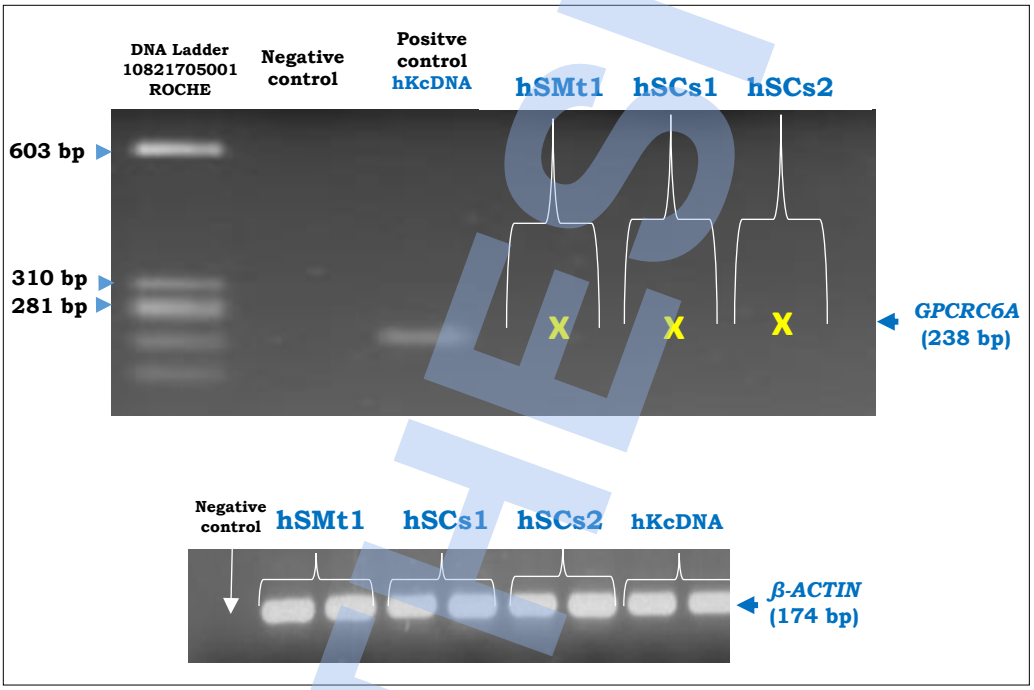


Fig. 36. RT-PCR analysis of GPRC6A gene in hSMt and in established hSCs lines*- the analysis showed the absence of expression of GPRC6A in hSMt and established hSCs lines. The commercial human kidney cDNA (hKcDNA) was used as positive control. The negative control was the PCR without templated cDNA.*

4.4 Analysis of PTH (Parathyroid Hormone) and its Main Receptor PTH1R (Parathyroid Hormone type-1 Receptor) in Human Skeletal Muscle

4.4.1 PTH1R Gene Expression in Human Skeletal Muscle Tissues (hSMts) and in Established Human Satellite Cell (hSCs) Lines

In order to evaluate the expression of PTH1R gene in hSMts and hSCs lines, we performed a Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (Real-Time qRT-PCR). The results showed that there is an expression of PTH1R in hSMts and in the established hSCs lines (Fig. 37).

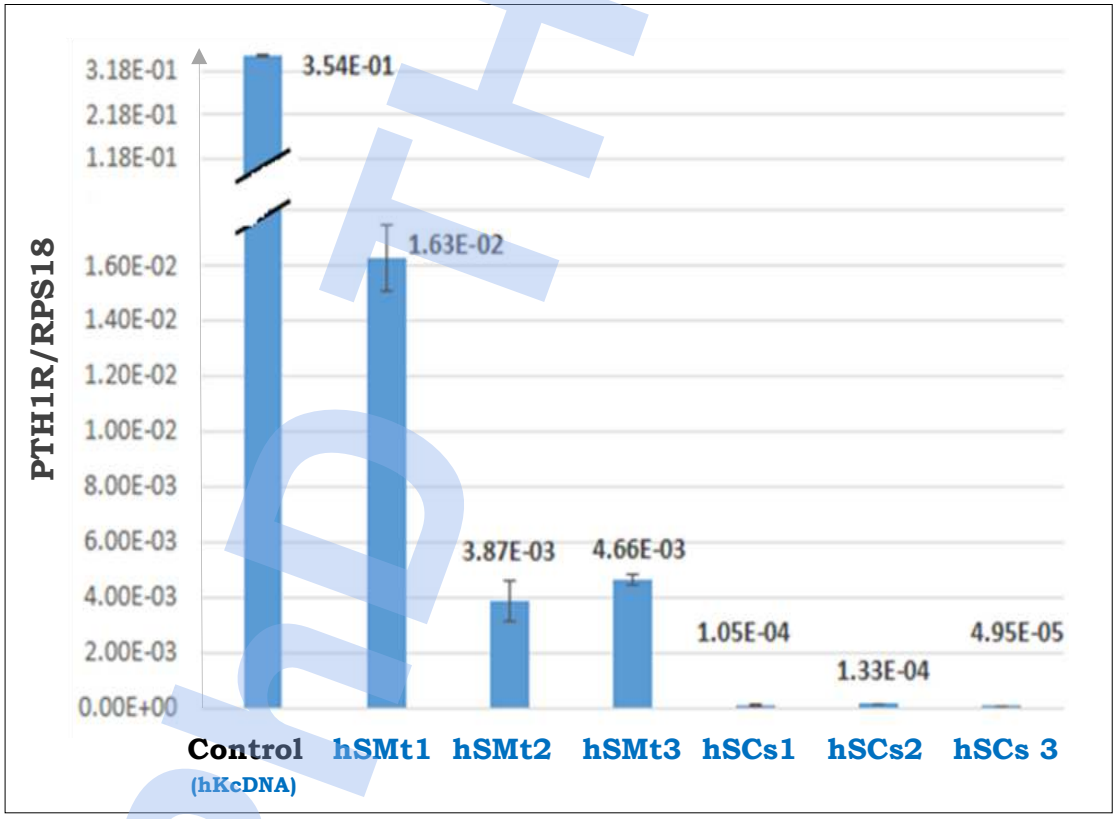


Fig. 37. Real-Time qRT-PCR analysis of PTH1R gene in hSMts and in hSCs lines*- the analysis showed the expression of PTH1R gene in hSMts and in their respectively established hSCs lines. Human commercial kidney cDNA (hKcDNA) was used a positive control. Experiment was performed in technical triplicates.*

4.4.2 PTH1R Gene Expression during *In Vitro* Myogenic Differentiation

The performed a Real-Time qRT-PCR to evaluate the expression of PTH1R during myogenic differentiation has shown a significant increase in PTH1R gene expression over time (0 days, 3 days, 6 days, and 9 days) during myogenic differentiation (Fig. 38).

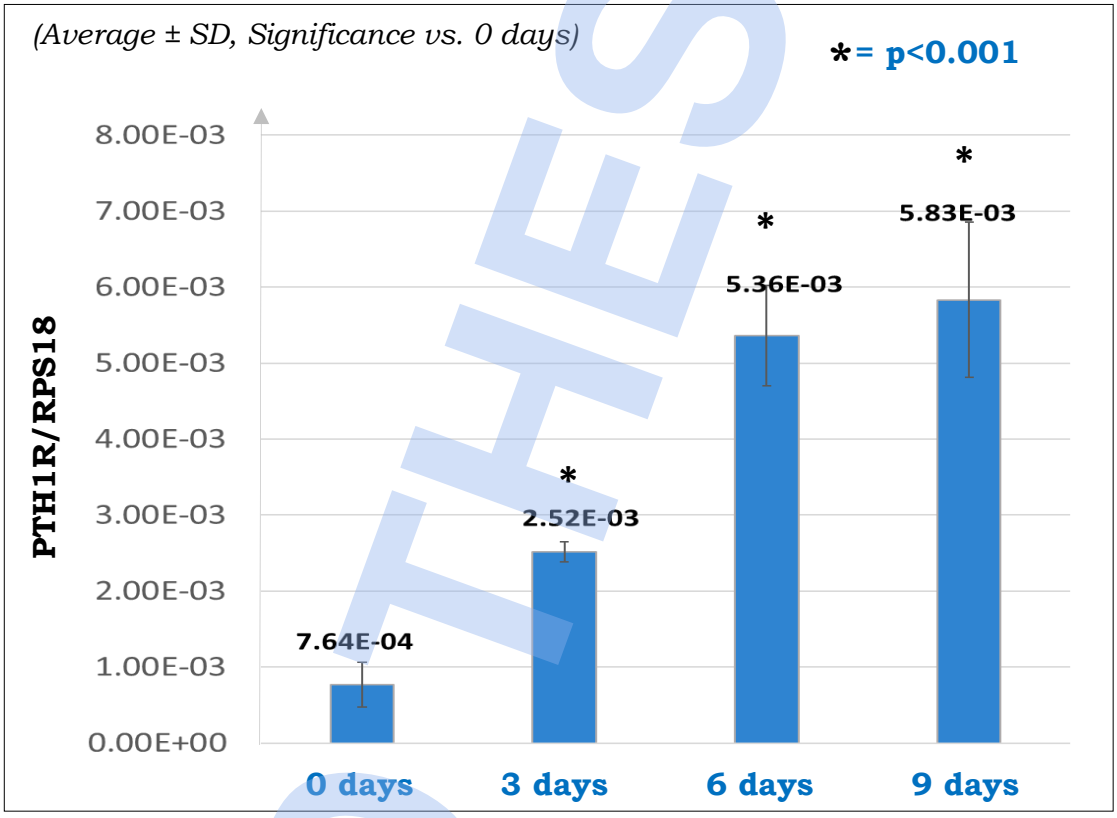


Fig. 38. Real-Time qRT-PCR analysis of PTH1R gene during myogenic differentiation*- the analysis showed that there is an increase of PTH1R expression over the time observed during myogenic differentiation with respect to 0 days (p<0.001). Experiment was carried out in triplicates and is representative of the three established hSCs lines.*

4.4.3 PTH1R Protein Expression in Human Skeletal Muscle Tissues (hSMts)

In order to evaluate the expression of PTH1R protein in hSMts, we have performed an immunofluorescence staining using TissueFAXS technology. The results of immunofluorescence staining showed the presence of PTH1R in human skeletal muscle tissue samples (Fig. 39 (A & B)). The positive detection of the PTH1R protein in the same analysis in human kidney tissue sections has validated our method of analysis (Fig. 39 (C & D)).

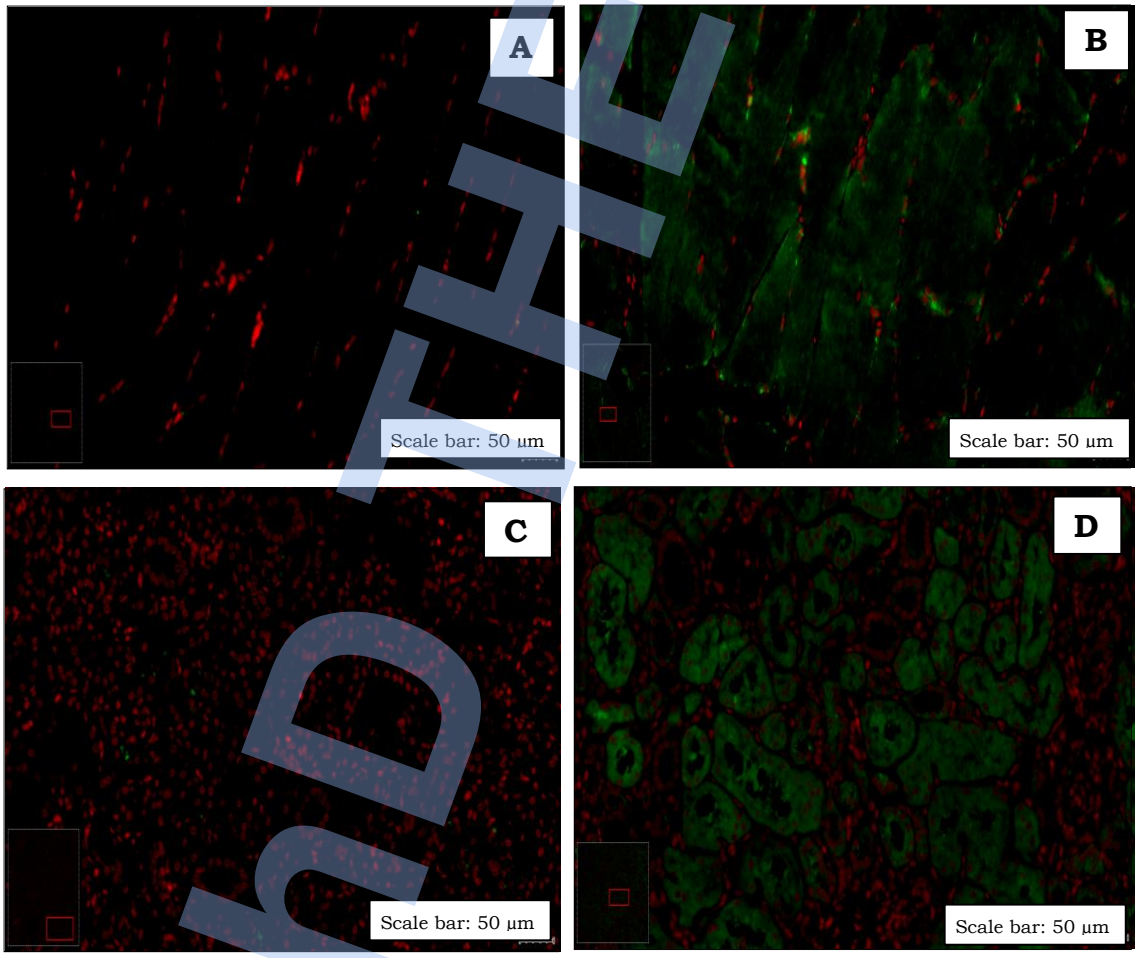


Fig. 39. Immunofluorescence staining of PTH1R by TissueFAXS*- analysis of PTH1R protein in human skeletal muscle tissues sections (A: negative control with secondary antibody only and B: with primary anti-PTH1R antibody). Analysis of CaSR protein in human Kidney tissues sections used as positive control (C: negative control with secondary antibody only and D: with primary anti-PTH1R antibody). Fluorescent microscopy with TisssueFAXS software, conventional colour: green for PTH1R protein and red for nuclei, original magnification: 10X, Scale bar: 50 µm. Experiment was carried out in triplicates of hSMts sections from three different humans.*

4.4.4 PTH1R Protein Expression Analysis in Human Satellite Cell (hSCs) Lines and during *In Vitro* Myogenic Differentiation

To evaluate the PTH1R presence in hSCs lines and to evaluate this during myogenic differentiation, we have performed a western blot analysis. The obtained data showed the presence of PTH1R in hSCs lines and an increase of PTH1R protein expression during myogenic differentiation (Fig. 40).

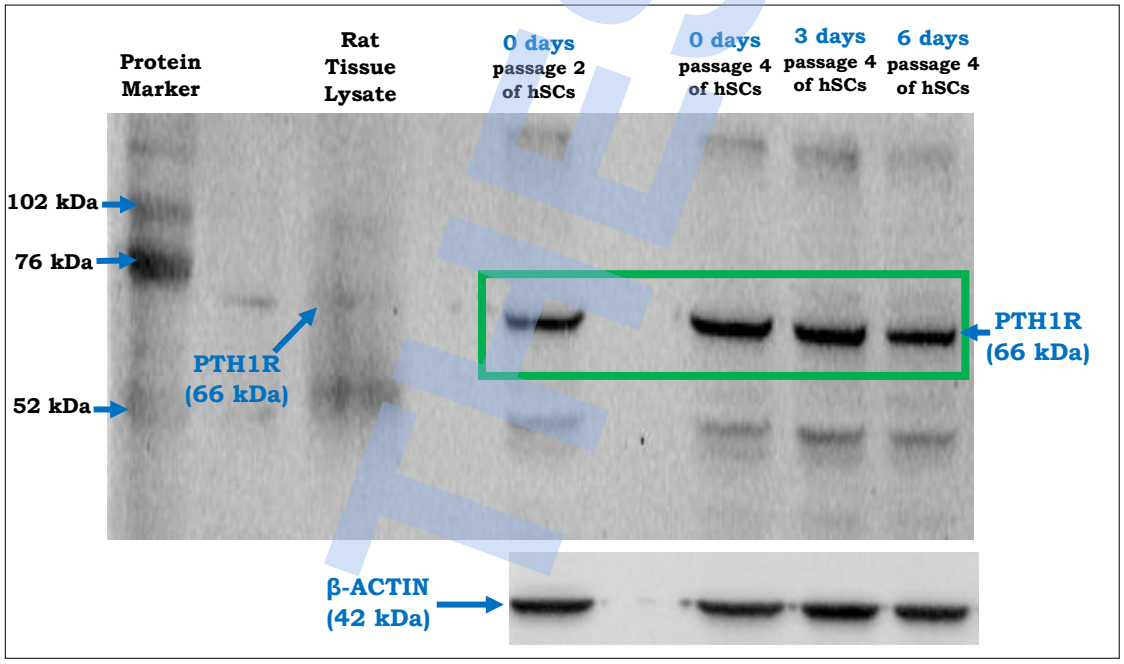


Fig. 40. Western Blot analysis of PTH1R protein in hSCs lines and during in vitro myogenic differentiation- *the analysis showed the expression of PTH1R protein in hSCs lines and an increase of the expression of PTH1R protein during myogenic differentiation. Rat kidney tissue lysate was used as positive control. Experiment was carried out in triplicates and is representative of the three established hSCs lines.*

4.4.5 PTH1R Secondary Messenger Analysis in hSCs lines: Gs- Cyclic Adenosine Monophosphate (cAMP)

To further verify the presence of PTH1R in hSCs lines, we performed the analysis of one of the secondary messengers of PTH1R, which is cAMP (Fig. 41) by ELISA assay in hSCs upon stimulation with a wide range of hPTH 1-34. The analysis was performed in un-differentiated hSCs lines at 0 days (passage 2) where we have detected the lowest expression of PTH1R protein in comparison to other days (3 days and 6 days) of myogenic differentiation (Fig. 40). The obtained data showed no cAMP response in the un-differentiated hSCs lines at 0 days (passage 2) upon stimulation with a wide range of concentrations (1 pM-1µM) of hPTH 1-34 (Fig. 42).

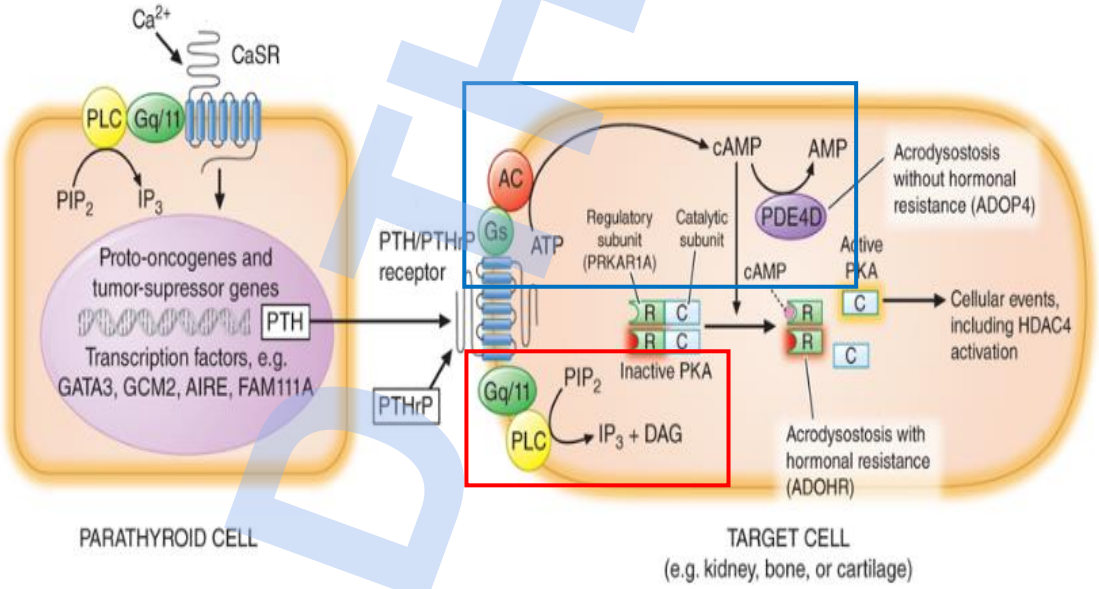


Fig. 41. PTH1R secondary messenger in signaling pathways*- PTH1R upon activation can couple to Gq/11 and Gs, which leads to an increase in secondary messenger IP_3 and cAMP, respectively. The pathways marked in blue rectangle; Gs-cAMP was analyzed in hSCs to verify the presence of PTH1R in established hSCs lines. The figure was taken from reference* (72).

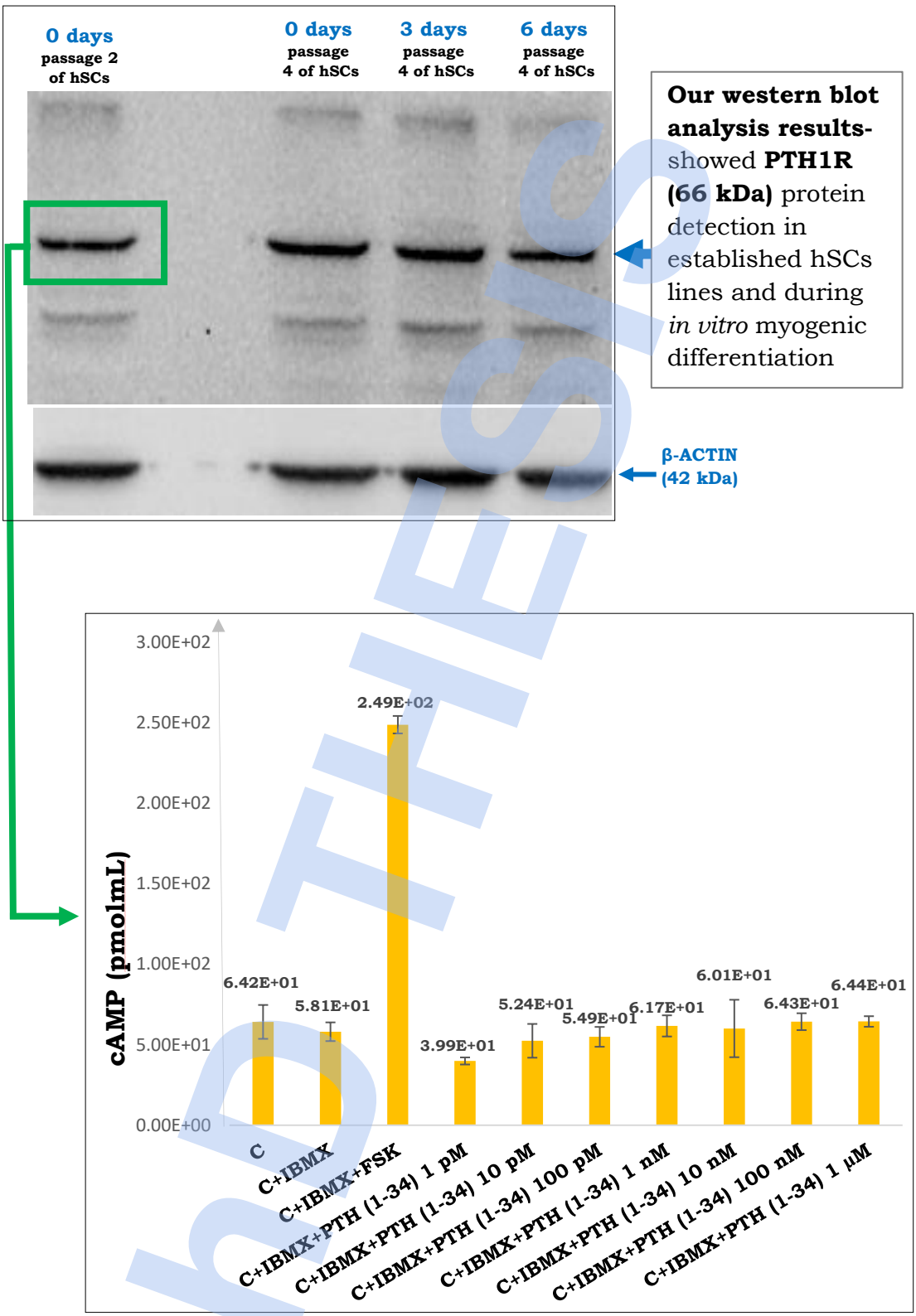


Fig. 42. Gs-cAMP ELISA assay in hSCs lines. *graph representing the effect of PTH1R stimulations in cAMP response in un-differentiated hSCs lines at 0 days (passage 2) where we have detected the lowest expression of PTH1R protein in comparison to other days (3 days and 6 days) of myogenic differentiation. Experiment was carried out in triplicates and is representative of all the three established hSCs lines.* ***C:*** *control buffer with 0.5 mM* Ca^{2+}, ***IBMX:*** *3-isobutyl-1-methylxanthine (inhibit the degradation of cAMP),* ***FSK:*** *Forskolin (positive control), Stimulation time: 15 min.*

5. DISCUSSION

The study to understand mechanisms regulating skeletal muscle physiology to find therapeutic drug targets is limited by difficulties in maintaining metabolically viable isolated muscle fibers and the inability to manipulate specific variables *in vivo* (78, 80). The development of stem cell culture has provided an invaluable tool by which cellular physiology can be studied. Satellite cells are the postnatal stem cells of skeletal muscles, which in response to injury or daily wear and tear undergo myogenesis and are so responsible for skeletal muscle regeneration (9). Probably, the failure of satellite cells to undergo myogenic differentiation program is one of the leading causes of skeletal muscle disorders such as sarcopenia and muscular dystrophies (29, 31, 33, 34). Moreover, the skeletal muscle weaknesses are also present as complications in other pathologies such as hypoparathyroidism (35-37), but the mechanism of these myopathies are just not that well understood. In the last decade, it has become possible to isolate and culture populations of human satellite cells that have been unambiguously shown the capacity to differentiate into myotubes *in vitro* (86-88). However, the methodological variations observed in primary myotubes culture, especially in regards to medium composition and the myogenic differentiation protocol, limit the reproducibility of the study.

In our work, we had the aim to establish a primary cell culture of isolated satellite cells from human skeletal muscle biopsies obtained from the discarded tissues of patients undergoing plastic surgery. In our used protocol to establish primary cell cultures of Human Satellite Cells (hSCs), we have used Matrigel-coated tissue culture dishes and a specific low serum-containing medium to enhance the growth of the hSCs and to avoid the growth of non-myogenic cells (i.e. fibroblast and neurons). In this way, we had the possibility to establish a pure hSCs cell culture. We have observed that the hSCs culture initially showed a slow growth pattern, however once maintained in culture for more

days, we observed that the growth rates were increased rapidly. The established primary hSCs lines are termed as “finite” cell lines because after 8-10 passages, they start to become senescent; so, we have cryopreserved each established hSCs lines at earlier passages (2-3) for future experimental purposes. After the establishment of primary hSCs lines, we have characterized them phenotypically by analyzing the presence of satellite cell’s specific nuclear transcriptional factor PAX7 protein by flow cytometry analysis. The flow cytometry analysis has shown that the ~ 98% of established hSCs are positive for PAX7 protein. Moreover, the RT-PCR and western blot analysis have confirmed the presence of PAX7 in hSCs lines. We have observed the presence of the PAX7 marker in all the established hSCs lines. So, we have established an *in vitro* cellular model of human satellite cell lines. Moreover, we have found and showed a method of establishment and characterization of hSCs lines, which is reliable, as we have obtained the same results in all other primary cultured hSCs lines derived from different humans.

During the myogenic differentiation, hSCs must fuse to produce long multinucleated skeletal muscle cells called myotubes. The previous studies have reported that skeletal muscle myogenic differentiation is under the synergistic regulation of a vast number of myogenic genes (main regulators are Myf5, MyoD1, Myogenin and Mrf4) and expression of terminal differentiation proteins such as myosin heavy chain (MHC), α-actin and other proteins needed for muscle contraction (9).

In our myogenic differentiation experiments, we have observed the myogenic phenotype characterized by the presence of long multi-nucleated cells (myotubes), after 7 days of myogenic induction of primary cultured hSCs lines with a specific medium without serum. We have noticed that the longer durations of differentiation (more than 8 to 10 days, depending on hSCs lines) can lead to myotube detachment and subsequent cellular death, probably due to deficiency of serum in the medium. The obtained myotubes at 7 days of myogenic induction have shown a high presence of the main terminal myogenic

differentiation marker protein myosin heavy chain (MHC) by immunofluorescence analysis using Laser Scanning Confocal Microscopy (LSCM). Moreover, we have also characterized the expression of myogenic regulatory genes *Myf5, MyoD1, Myogenin,* and of the *MHC* by RT-PCR analysis. The obtained data have shown that the genes *Myf5, MyoD1, Myo* and of *MHC* were contemporary expressed during the last period of myogenic differentiation (7 days) when myotubes were formed, while during the first phases (0 days) of the myogenic differentiation only *Myf5* and *MyoD1* were expressed, and *Myogenin* and *MHC* were utterly absent. The reported presence of *Myf5* gene and *MyoD1* expression at later stage of myogenic differentiation (7 days) in our analysis was due to that fact that not all the satellite cells can undergo myogenic differentiation (observed in the compound microscope), this observation is in line with the previous studies where it has been reported that the population of satellite cells is heterogenic due to their difference in myogenic potential (9). The earlier detection of *MyoD1* in the primary cultured hSCs is due to the fact that after collecting biopsies, the surgical procedure per se is a form of injury to initiate muscle regeneration, which leads to the activation of satellite cells and their commitment to myogenic differentiation; as a result, we have found the early detection of *MyoD1* in primary cell culture of hSCs. The suitability of our myogenic differentiation model was further shown by a significant gradual increase of MHC gene and protein analyzed at different times (0 days, 3 days, 6 day, and 9 days) during myogenic differentiation respect to the control group at 0 days, analyzed by Real-Time qRT-PCR and by western blot, respectively. We have found that all the results regarding the establishment and characterization of myogenic differentiation model were reproducible in other obtained hSCs lines. Taken together, our results have demonstrated that the isolated primary cell cultures of hSCs are capable of undergoing myogenic differentiation, showing not only the myogenic phenotypic long multinucleated cells aspect but also the expression of MHC and myogenic regulatory factors Myf5, MyoD1, Myogenin. Finally, since we have obtained the same results in all

primary cultured hSCs lines, we have demonstrated that our method of establishment and characterization of the established model of *in vitro* myogenic differentiation is reliable.

Since calcium (Ca^{2+}) plays an essential role in skeletal muscle myogenesis, we have hypothesized that the receptor CaSR (Calcium Sensing Receptor) could have a role in myogenic differentiation, becoming a possible molecular target for skeletal muscle disorder. First, we have analyzed the CaSR expression in Human Skeletal Muscle Tissues (hSMts), in their respective established hSCs lines and during *in vitro* myogenic differentiation, as there is no report about the expression of this receptor in skeletal muscle. Our results have shown the absence of CaSR mRNA in hSMts, in their respective established primary hSCs lines and during *in vitro* myogenic differentiation, analyzed using highly sensitive quantitative molecular technique TaqMan gene expression assay and 3 couples of primers specific to different exons of human CaSR. Then, we have also evaluated if the CaSR protein is present in hSMts by immunofluorescence analysis using fluorescent microscopy with TisssueFAXS software, but the data obtained have shown the absence of CaSR protein expression in hSMts. Moreover, the specificity of the used anti-CaSR antibody was verified in the same analysis by one extra internal negative control, where the antibody was incubated in the tissue sections, previously absorbed with its blocking peptide (107, 108). Regarding CaSR protein expression in primary hSCs lines, the flow cytometry analysis has shown the absence of this receptor in hSCs lines. The absence of CaSR protein was also validated by immunofluorescence staining using LSCM. Finally, the western blot analysis performed, in which we can detect the protein with immunostaining based on the size of the protein in either monomers (in reducing condition with β-mercaptoethanol or 50 mM DTT, disulfide bonds breaker) or in dimers (non-reducing condition) forms. At first, the western blot analysis was performed in reducing condition to verify the CaSR protein expression hSCs lines further and to analyze the CaSR protein expression during (0 days, 3 days, 6 days, and 9 days) myogenic differentiation of hSCs lines. The positive

detection of CaSR monomer bands 140 kDa and 160 kDa in the positive control, HEK293 cells stably transfected with human CaSR (HEK_{CaSR}) in western blot has validated our method of analysis. Moreover, we have found the faint bands of about 160 kDa and 140 kDa (size of CaSR monomers) in hSCs lines and during *in vitro* myogenic differentiation in the same western blot analysis. The observed bands were seeming like CaSR. Since we have not observed the CaSR gene expression in the established hSCs lines and during myogenic differentiation, we thought to verify the specificity of the obtained faint bands in the samples using western blot analysis for CaSR in reducing and non-reducing condition. Since CaSR is a homodimer protein, we have the advantage of verifying the detection of the CaSR protein in western blot by running side by side in the same SDS-PSGE gel in reducing and non-reducing conditions. In the results of the analysis, if the observed bands of CaSR monomer size in reducing condition disappear or shift to dimer bands in the non-reducing condition that proves that the bands that we have detected are of CaSR protein. Thus, we have performed western blot analysis in reducing and non-reducing conditions in the samples of proliferating hSCs lines where we have detected the high intensities of the bands of the size of CaSR monomer. In this particular western blot analysis, another native positive control, bovine parathyroid tissue lysate, was also included to verify the binding of the anti-CaSR antibody further. The obtained results of western blot analysis have shown that the monomers CaSR bands observed in positive control HEK_{CaSR} in reducing condition has shifted to CaSR dimers band in non-reducing condition. While the bands of CaSR monomers size observed in the hSCs lines in reducing condition, which seemed like CaSR, neither disappeared nor was shifted to CaSR dimer band size in non-reducing condition. So, the bands that we have observed in hSCs lines and during *in vitro* myogenic differentiation are due to unspecific reactivity of anti-CaSR antibodies, thus are false. Therefore, western blot analysis confirms the absence of CaSR protein expression in hSCs lines and during *in vitro* myogenic differentiation.

As CaSR is a G Protein-Coupled Receptor (GPCR) and one activated GPCR can activate many G proteins, providing a biologically important amplification step in the signaling pathway. This makes the importance of an even very low presence of CaSR receptors in physiological and pathological conditions (109). Thus, in order to verify the possibilities that might be the low presence of CaSR mRNA and protein in skeletal muscle tissues and cellular model, was not be able to be detected by CaSR probe and anti-CaSR antibodies that we have used in our analysis, we have performed CaSR secondary messengers analysis where we have induced the hSCs lines with CaSR main ligand Ca^{2+} and available CaSR drugs calcimimetic (NPS R-568) a positive CaSR allosteric modulator and calcilytic (NPS2143) a negative CaSR allosteric modulator. The obtained data of the one of the CaSR secondary messenger Inositol Trisphosphate (IP_3) in Gq-CaSR signaling pathway in hSCs lines, stimulated with 1.2 mM & 3.5 mM Ca^{2+} have shown the absence of the dose responses in IP_3 production. Moreover, we have also analyzed the response in Gq-IP_3-Intracellular Calcium Immobilization in live primary cultured hSCs lines upon stimulation with various concentrations of Ca^{2+} alone and along with CaSR drugs calcilytic (NPS2143) or calcimimetic (NPS R-568). And we have detected an absence of signal in intracellular calcium immobilization too in primary hSCs lines stimulated with Ca^{2+} and CaSR drugs. Furthermore, we have analyzed another secondary messenger of CaSR, Cyclic Adenosine Monophosphate (cAMP) in the Gi-cAMP signaling pathway in hSCs lines, because CaSR signaling pathways could vary from cell to cell depending on physiology (41). For this analysis, hSCs lines were stimulated with 3 mM Ca^{2+} alone and along with CaSR drugs 1 µM NPS2143 or 1 µM NPS R-568. We have detected a strange significant variation in cAMP response in hSCs stimulated with 3 mM Ca^{2+} alone and along with calcilytic (1µM NPS R-568) or calcimimetic (1µM NPS2143). Since, we obtained contradictory results in two main secondary messenger pathways (Gq-IP_3 & Gi-cAMP) for CaSR in hSCs stimulated with Ca^{2+} and CaSR drugs, in order to conclude the obtained results, we have analyzed the effect of Ca^{2+} and CaSR drugs

on *in vitro* myogenic differentiation by analyzing the variation in expression of one of the most important myogenic marker MHC at different time points (0 days, 3 days, and 6 days) during myogenic differentiation. The obtained data of the analysis have shown the absence of effect of Ca^{2+} alone and co-treatment of NPS2143 or NPS R-568 along with Ca^{2+} on *in vitro* myogenic differentiation. Taken together, our finding demonstrated for the first time that CaSR is not expressed in skeletal muscle tissue, in hSCs, and during myogenic differentiation.

Despite the absence of CaSR gene and protein and IP_3 (CaSR-Gq) secondary messenger in response to Ca^{2+} and CaSR drugs (NPS2143 or NPS R-568) in primary hSCs lines, we have obtained cAMP response (CaSR-Gi) in primary hSCs lines upon stimulation with Ca^{2+} alone and along with CaSR drugs (NPS2143 or NPS R-568). This led us to the thought of the presence of the highest CaSR homologous protein GPRC6A (103). The GPRC6A is the physiologically important class C family GPCR, recently discovered (103, 104). The GPRC6A reported to respond to calcium with low affinity and also with drugs NPS 2143 & NPS R-568 through secondary messenger cAMP, although the studies have been reported using mouse GPRC6A (104-106). Considering the given information, we hypothesized the presence of GPRC6A receptor in human skeletal muscle, which could be a possible drug target for skeletal muscle disorder. For that, at first, we have analyzed the presence of GPRC6A gene expression in hSMt and cultured hSCs lines. Nevertheless, our results of GPCR6A gene expression analysis have shown the absence of GPRC6A in human skeletal muscle tissue and hSCs lines.

Since one of the complications in hypoparathyroidism is the skeletal muscle weakness, we have hypothesized that the Parathyroid Hormone (PTH) might have a role in skeletal muscle disorder in that (35-37). In our studies, we have detected the presence of Parathyroid Hormone type-1 Receptor (PTH1R) mRNA in Human Skeletal Muscle Tissues (hSMts) and hSCs lines. Furthermore, we have detected the presence

of the PTH1R protein in hSMts sections by immunofluorescence staining analysis and in hSCs lines by western blot analysis. After the detection of PTH1R in hSMts and in primary hSCs lines, we have evaluated the functionality of the PTH1R protein in hSCs lines, by analyzing one of the secondary messengers of PTH1R, which is Gs-cAMP response by ELISA upon stimulation with a wide range of concentrations of hPTH 1-34. The results of this analysis have shown the no cAMP response in cultured hSCs stimulated with hPTH 1-34. At this point, it is to consider that PTH1R also acts through Gq-IP_3 signaling pathway; it could be that in muscle cells the signaling of PTH & PTH1R may occur through Gq-IP_3 pathway rather than Gs-cAMP pathway. Finally, we have also analyzed the expression of PTH1R gene and protein at different time points (0 days, 3 days, and 6 days) during myogenic differentiation of hSCs lines by Real-Time qRT-PCR and western, respectively. The obtained results have shown a significant increase in expression of the PTH1R gene and protein during *in vitro* myogenic differentiation, revealing a possible role of PTH1R in skeletal muscle myogenesis process.

6. CONCLUSION

To understand the molecular process at the base of skeletal muscle disorders, it is necessary to have good *in vitro* cellular model to understand the biological processes which can be altered in these type of disorders. In this work, we have established the human satellite cell (hSCs) lines, which are capable of growth and undergo myogenic differentiation. We have thus established a reliable *in vitro* cellular model of myogenic differentiation, which can be a useful and valid *in vitro* model for studying skeletal muscle differentiation and for finding new therapeutic molecular targets for the future treatments of skeletal muscle disorders.

In this study, for the first time, we have analyzed the expression of the Calcium Sensing Receptor (CaSR), demonstrating the total absence of this molecule in human skeletal muscle tissues, hSCs lines, and during *in vitro* myogenic differentiation. Taken together, our findings support the fact that in spite of CaSR being a very important receptor in the physiology of several organs and tissues and also in several pathologies, probably this receptor does not have any physiological importance in skeletal muscle tissue. Consequently, our study has also demonstrated that CaSR is not involved in myogenesis. Thus this receptor can't be a valid molecular target for the future development of new therapies for skeletal muscle disorders.

Quite the opposite, our results of Parathyroid Hormone type-1 Receptor (PTH1R) expression analysis have demonstrated the presence of PTH1R in human skeletal muscle tissue, in hSCs lines and during myogenic differentiation. Moreover, we have detected a significant increase of PTH1R expression during *in vitro* myogenic differentiation. In conclusion, our data for the first time suggest a possible involvement of Parathyroid Hormone (PTH) in development and in the regeneration of skeletal muscle tissue. Our last finding also indicates that probably the direct deficiency of PTH could also cause of skeletal muscle disorders in hypoparathyroidism.

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