UNIVERSITA' POLITECNICA DELLE MARCHE

Scuola di Dottorato di Ricerca della Facoltà di Medicina e Chirurgia

Curriculum "Salute dell'Uomo"

Mesoderm stem cells and inflammation: role in the pathogenesis and potential therapy of selected gynecological diseases and primary myopathies

Ph.D. Candidate

Miriam Caffarini

Tutor

Dr. Monia Orciani

XXXI ciclo

SUMMARY

Abstract

Mesenchymal stem or stromal cells (MSCs) are a specific type of adult stem cells with an extensive proliferation and differentiation potential towards specialized cells developing from the mesoderm. MSCs are also characterized by paracrine function through the release of multiple growth factors, chemokines and cytokines. MSCs play as sentinel that feel the microenvironment and act consequently, switching from a pro-inflammatory phenotype to an immunosuppressive phenotype according to the signals they receive. In the present work the existence and the role of MSCs in the pathogenesis and potential therapy of selected gynecological diseases with an inflammatory component as uterine leiomyoma, cervical intraepithelial neoplasia (CIN), and in primary myopathies, as Duchenne Muscular Dystrophy (DMD) were evaluated. In the first study, progenitor cells were identified both in leiomyomas and normal myometrium, and the correlation between these cells and inflammation in leiomyoma onset has been investigated. The data suggest that the upregulation of cytokines related to chronic inflammation in leiomyoma progenit ors could favour a microenvironment suitable for the onset of this pathology. In the second study, MSCs from cervix of young (yC-MSCs) and old patients (oC-MSCs) were isolated and results show as their immunobiology is affected by the age of donors, influencing in turn the regression rate of CIN. In addition, in the crosstalk with HeLa cells, yC-MSCs play an anti-tumoral role sustaining an acute inflammatory environment. The goal of the third study was to find a correct strategy to enhance the production of dystrophin protein in DMD through gene therapy. Therefore, myoblasts isolated from DMD donor were transduced with green fluorescent protein (GFP) and a lentiviral vector expressing the snRNA to induce exon skipping; data indicate that transduced myoblasts were able to perform myogenic differentiation expressing a functional dystrophin protein.

1. Introduction

1.1. Mesenchymal stem cells (MSCs)

Mesenchymal stem or stromal cells (MSCs) are a specific type of adult stem cells (SC) able to generate the different cell types of the mesenchyme (Caffarini M et al. 2018, Ullha I et al. 2015).

Due to their ability to differentiate into specialized cells developing from mesoderm, they were named as mesenchymal stem cells (MSCs). MSCs, also known as multipotent cells, exist in adult tissues of different sources, ranging from murine to humans.

After their discovery in bone marrow more than 40 years ago (in 1976) (Friedenstein et al. 1976), MSCs have been isolated from other many tissues, such as adipose tissue, periosteum/ synovial fluid, Wharton's jelly, umbilical cord blood, placenta, amniotic fluid, skin, dental pulp, and skeletal muscle (Toyserkani NM et al. 2015, Zuk PA et al. 2002, Nemeth K et al 2015, Troyer DL et al. 2008, Nakahara H et al. 1991, Kim KH et al. 2017, Kern S et al. 2006, Brooke G et al. 2009, Shi C et al. 2006, Sellheyer K et al. 2010). At the beginning, they were called osteogenic stem cells or bone marrow stromal cells. Because of their plastic-adherent capacity and their fibroblast-like cells morphology, they were identified as distinct from the hematopoietic stem cells (HSCs). In 1991, Caplan coined the name "mesenchymal stem cells" to define the multilineage potential of these cells and the ability to renew themselves for long periods of time without significative variations in their properties (Caplan AI. 1991). To better classify these multipotent cells as mesenchymal stem/stromal cells, Dominici et al. introduced three minimal criteria approved by the International Society for Cellular Therapy (ISCT): 1. First, cells must be plastic adherent when grown in culture under standard conditions. 2. Second, they must be positive (expression) for CD73, CD90, CD105 and negative for CD45, CD34, CD14 or CD11b, CD19 or CD79α, and HLA-DR surface molecules (tested by flow cytometry). 3. Third, MSCs must be able to differentiate into osteoblasts, adipocytes and chondrocytes *in vitro* (Dominici M et al. 2006, Soundararajan M et al. 2018).

Despite the mesodermal origin, MSCs have displayed the capacity of trans-differentiation into ectodermal lineages (Ullha I et al. 2015). In particular, MSCs isolated from different sources have shown trans-differentiation into neural cells as a result of exposure to neural induction media supplemented with cocktails of growth factors (Ullha I et al. 2015).

More in details, adding several growth factors to the neuronal medium cocktail, such as hepatocyte growth factor (HGF), FGF and EGF, it was possible to obtain specific neurons

phenotypes: oligodendrocytes, cholinergic and dopaminergic neurons (Naghdi M et al. 2009, Datta I et al. 2011, Barzilay R et al. 2009, Safford KM et al. 2002, Kang SK et al. 2003). It was also proved that MSCs were able to generate hepatocytes and pancreocytes upon induction with their corresponding conditioned media (Ullha I et al. 2015), revealing that hMSCs can also trans-differentiate into endodermal lineages. For what concern human MSCs, it is supposed that they require two passages to trans-differentiate into hepatocytes: differentiation and maturation steps, both included the addition of specific growth factors in the culture medium (Lee KD et al. 2004, Stock P et al. 2014). Therefore, differentiated cells into hepatocytes were able to express liver-specific transcription markers. So far, MSCs derived from adipose, dental, umbilical cord, amnion, Wharton jelly and placental tissues seem to have successfully differentiated into insulin producing *β*-cells (Kim et al. 2012, Criscimanna et al. 2012, Kanafi et al. 2013).

Besides their extensive proliferative and differentiation potential, it was demonstrated that MSCs lack the teratoma formation in vivo (Jiang Y et al 2007). This gives MSCs the advantage of being a good candidate for replacing embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC) in regenerative and cell therapies. MSCs are also characterized by paracrine function through the release of multiple growth factors, chemokines and cytokines (Jiang Y et al 2007).

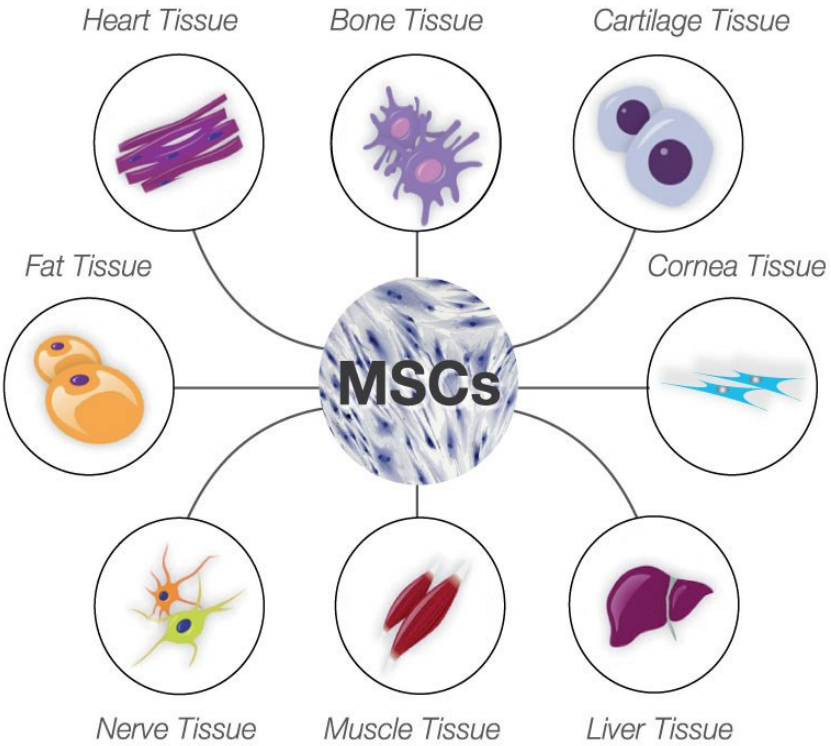

Figure 1. Representation of Mesenchymal stem cells (MSCs) and their differentiation potential into a variety of cell types.

Cytokines and chemokines are redundant secreted proteins with growth, differentiation, and activation functions that regulate and control immune cell trafficking and the cellular arrangement of immune organs (Borish LC et al. 2003).

1.2. Inflammation

Inflammation is a biological response of the organism to harmful stimuli. It happens when the immune system fights against something that could be injurious. The most common causes of inflammation are pathogens, as bacteria, viruses or fungi, external injuries and effects of chemicals and radiations. There are five signs that may indicate an inflamed process, such as redness, swelling, heat, pain and loss of function (rubor, tumor, calor, dolor et functio laesa) (Ferrero-Miliani L et al. 2007). During an inflammatory process, the innate immune system plays a pivotal role, as it mediates the first response and many cells are involved and release different factors, the inflammatory mediators. Inflammation can be classified as acute or chronic. Acute inflammation is a short-term process occurring in response to tissue injury, usually appearing within minutes or hours and it may be regards as the first line of defence against wound (Ramzi S Cotran et al. 1999). Acute inflammatory response is the result of a series of biochemical events that involves the local vascular system, the immune system and infiltration of neutrophils from the blood to the injured tissue. In some disorders the inflammatory process, which in normal conditions is self-limiting, becomes prolonged developing chronic inflammation (Ferrero-Miliani L et al. 2007). Chronic inflammation involves an infiltration of macrophages, T lymphocytes and plasma cells indicating a progressive shift in the type of cells present at the site of inflammation (Ferrero-Miliani L et al. 2007). Loss of tissue function due to fibrosis represents the final consequence of chronic inflammation.

The immune system is capable to recognize and respond to pathogenic insult (Akira S et al. 2006) through specific pathogen recognition receptors (Chen GY et al. 2010). Then, a signalling cascade promotes the release of complement components, acute phase proteins, pro inflammatory cytokines and chemokines recruiting innate immune cells (neutrophils and macrophages) (Medzhitov R. 2010). This process is named adaptive response to foreign antigens and it is the other main immune strategy (besides the innate system).

Neutrophils and monocytes/macrophages co-express similar antigens and these innate phagocytes can readily produce effector molecules such as granular proteins, oxidants, chemokines and cytokines (Daley JM et al. 2008, Nauseef WM 2007, Sunderkötter C et

al. 2004). Regardless of their similarities, emerging evidence indicates that neutrophils and monocytes/macrophages have distinct roles as innate immune cells and therefore are indispensable as key players against infection. Typically, neutrophils are the first responders to be recruited and have a higher microbicidal activity; whereas monocytes/macrophages are recruited later on. Despite this, monocytes/macrophages are able to digest and present antigens to other immune cells, thereby allowing them to interact with the adaptive immune system (Silva MT and Correia-Neves M 2012). Neutrophils and monocytes/macrophages share a complex relationship and; together, they orchestrate a more enhanced immune response by regulating other immune cells as well as each other.

Whenever T cell are activated, antigen-presenting cells, with dendritic cells (DCs), macrophages, and B cells, reduce foreign proteins to small peptides that are presented in major histocompatibility complexes (MHC) (English K. 2013). Therefore, MHC-I stimulates cluster of differentiation (CD)8^+ T cells, whereas MHC-II activates CD4+ T cells.

We now know that inflammation comes in many different forms and modalities, which are governed by different mechanisms of induction, regulation, and resolution (Auletta JJ et al. 2012, Medzhitov R. 2010). Between the variety of inflammatory phenomena, inflammation is an adaptive response to noxious conditions.

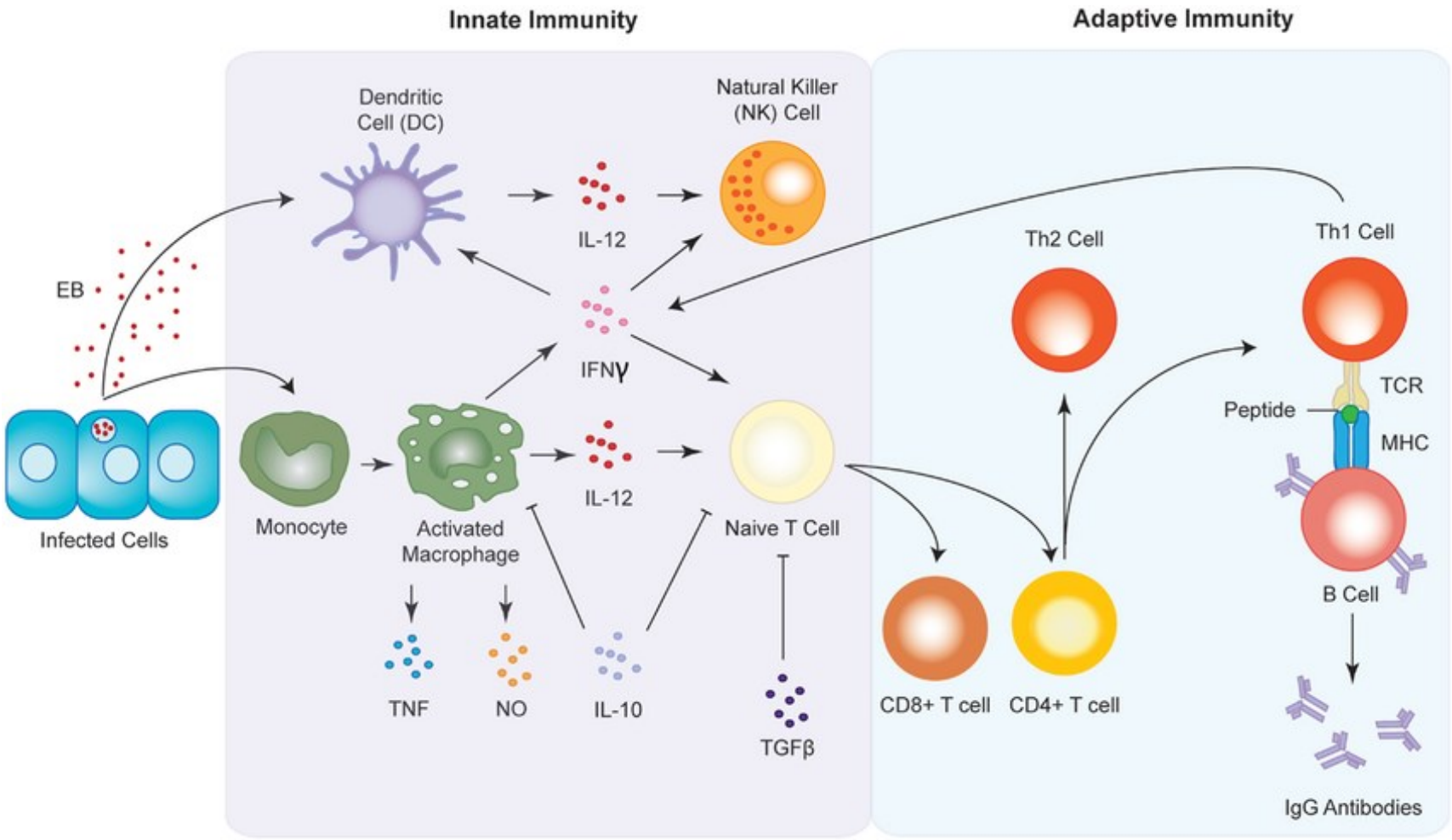

Figure 2. Upon infection, antigen presenting cells (APC) such as macrophages and dendritic cells are sequestered to the site of infection where they begin to release pro-inflammatory cytokines such as IFNγ and IL-12. The chemokines in turn activate natural killer (NK) cells and induce the maturation of T cells into either $CD8^+$ or $CD4^+$ T cells. $CD4^+$ T cells go on to form either T-helper 1 (Th1) or T-helper 2 type (Th2) T cells. Th1 cell interact with B cells via the T cell receptor (TCR) and the major histocompatibility complex (MHC) to produce antibodies against the infection. (Redgrove KA. Et al. Front. Immunol. 2014).

1.3. Role of MSCs in the inflammation

It is believed that MSCs possess many immunosuppressive mechanisms involved in the modulation of inflammation. They respond to specific signals produced by inflamed tissues both through paracrine effect and through their ability to migrate to the site of inflammation (Waterman RS et al. 2010). MSCs have sentinel functions that allow them to feel their microenvironment and act consequently (Aggarwal S et al. 2005) and they are able to switch from a pro-inflammatory phenotype to an immunosuppressive phenotype according to the signals they receive (Soundararajan M et al. 2018). Indeed, in conditions where there are large numbers of inflammatory cytokines, MSCs suppress immune responses by acting on a variety of immune cells (Jiang XX et al. 2005). Immunomodulation can be divided in some steps: 1) MSCs react to inflammation and move to the site of tissue injury, 2) receive the stimuli to be activated, 3) allow the pathogen clearance if it is required and 4) modulate inflammation (Waterman RS et al. 2010). So if on one hand, many studies confirmed that MSCs exert their immunosuppressive effects at a distance, on the other hand it has been demonstrate that MSCs require contact with immune cells to exert their effect (Waterman RS et al. 2010).They are able to directly modulate the T cell functions, inhibiting the proliferation and the activation of T-cells and also induce the generation of T-reg cells (Corcione A et al. 2006).

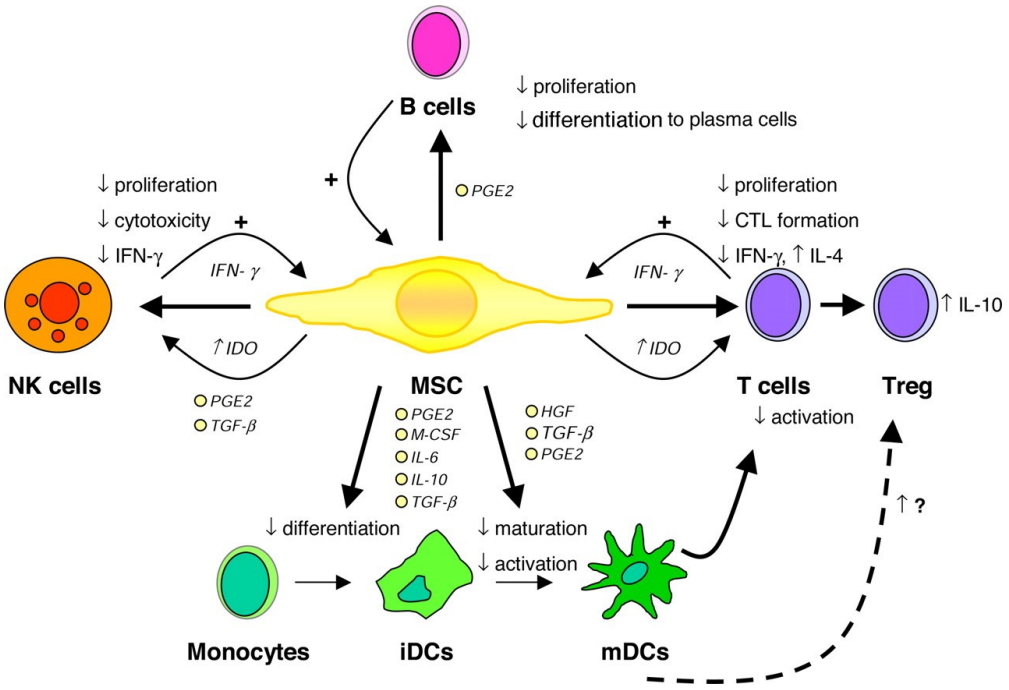

Figure 3. Immunomodulatory effects of MSCs. CTL indicates cytotoxic T cell; HGF, hepatocyte growth factor; IDO, indoleamine 2,3-dioxygenase; PGE2, prostaglandin E2; and TGF-β, transforming growth factor β. Illustration by Paulette Dennis.

It has been proven that MSCs hinder dendritic cell (DC) maturation from monocytes, which is necessary for antigen presentation and T cell response (Spaggiari GM et al. 2008). This process is considered as an important checkpoint in assembling an immune response because immature DC not only fail to trigger T cells efficiently but also support tolerance induction (Akiyama K et al. 2012).

Corcione et al. observed that MSCs dissuade the proliferation, differentiation, and chemotactic properties of B cells *in vitro*, and suppress the cytokine-induced proliferation and cytotoxicity, and cytokine production of natural killer (NK) cells (Kong QF et al. 2009, Ghannam S et al. 2010). MSCs modulation of immune responses is mediated through several mechanisms, however, most of them involve the production of immunosuppressive factors such as prostaglandin E2, indoleamine 2,3-dioxygenase (IDO), and nitric oxide (even if the exact mechanism of action is still unclear.) The majority of these soluble factors are not constitutively produced by MSCs but are induced through the licensing or activation of MSCs as described before (Waterman RS et al. 2010).

MSCs are also able to alter the helper T-cell balance (Th1/Th2/Th17). CD4+ T helper cells become activated in response to pathogen- or danger- associated signals and can differentiate into various T-cell subsets with distinct cytokine and gene expression profiles: Th1, Th2, and Th17 subsets (English K. 2013). Depending on the circumstances, MSCs can modulate T- cell proliferation and function mediating their protective effect through shifting the balance from Th1 phenotype secreting IFNγ and TNFα to a more anti-inflammatory Th2 profile secreting increased levels of IL4, IL5, IL10, Il13 and other chemokines (Bai L et al. 2009, Fiorina P et al. 2009, Batten P et al. 2009). MSCs have the capacity to modulate Th17 differentiation in favour of IL4 producing Th2 cells or the generation of Treg (Bai L et al. 2009, Ghannam S et al. 2010). MSCs allow the generation of Treg corresponding with a decrease in Th1, Th2 and Th17 lymphocytes (Akiyama K et al. 2012, Kong QF et al. 2009, Ghannam S et al. 2010, Rafei M et al. 2009). In particular, TGFβ is the major soluble factor involved in MSC promotion of Treg in vivo (Akiyama K et al. 2012, Kong QF et al. 2009, Nemeth K et al. 2010, Zhao W et al. 2008). Many studies describe the activation of MSCs to produce soluble factors, but the sequence of events that lead to the generation of Treg and subsequence induction of tolerance is quite different and influenced by the particular microenvironment (allergic/alloreactive/allotollerant) (Waterman RS et al. 2010).

1.4. STUDY 1: Uterine leiomyoma

Uterine leiomyomas (also known as uterine fibroid) is benign tumor originated from the uterine smooth-muscle tissue and are characterized by the production of excessive quantities of extracellular matrix (ECM) (Aleksandrovych V et al. 2015). Uterine leiomyoma (UL) affects up to 80% of all women in their reproductive age (Aleksandrovych V et al. 2015), but it has slight difference between women with different skin color, (nearly 70% of white women and more than 80% of black women). These benign tumors can develop into significantly sized lesions (from 10 mm to 20 cm).

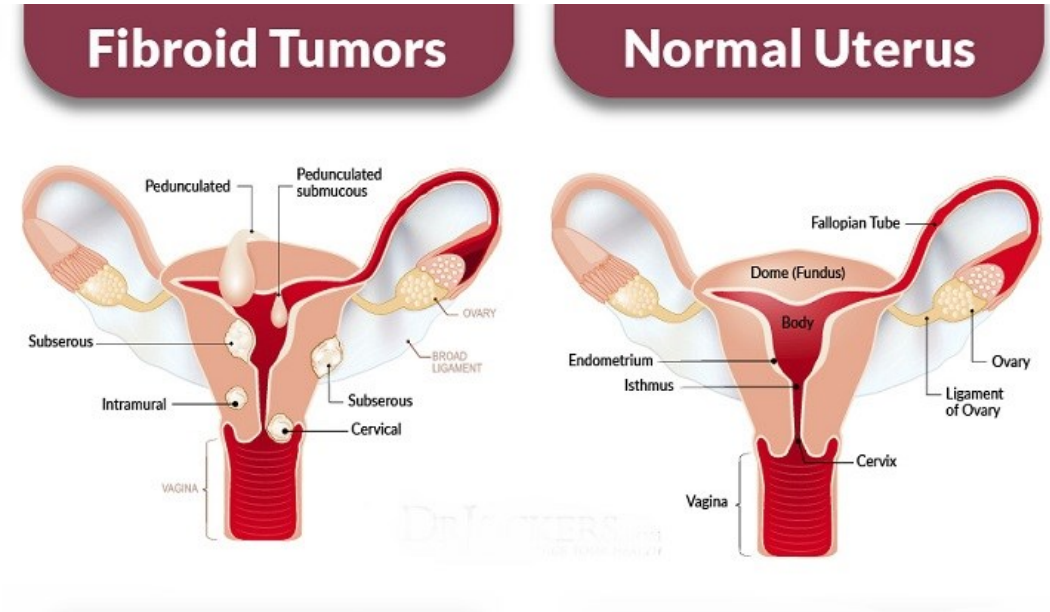

Figure 4. Schematic representation of the main type of uterine fibroids (subserous, intramural, cervical, pedunculated and submucous) and relative comparison with normal uterus. Subserosal fibroids grow mostly outside of the uterus and into the abdominal cavity. Intramural fibroids originate and grow within the wall of the uterus, and may negatively impact fertility and increase the risk of pregnancy loss. Submucosal fibroids grow and develop on the inside of the uterus and may also cause infertility and miscarriage. Subesrosal and submucosal types may also be pedunculated, meaning they have a stalk of tissue.

The main clinical symptoms are bleeding, pelvic pain, recurrent abortions and adverse obstetric outcomes often resulting in the infertility (Stewart EA. 2007, Parker WH. 2007, Ciavattini A et al. 2015). Traditionally classification of leiomyomas is based on the type of growth and location within the uterus (Brosens I. 2006). To date, there is no effective medical

drug therapy against uterine leiomyomas but some medical treatments have been shown to be effective in reducing the volume.

Hysterectomy represents the main surgery approach even if it leads to a loss of female reproductive potential (Wallach and Vlahos 2004).

Histologically, leiomyomas appear with a different phenotype from the adjacent normal myometrium. Leiomyomas are known to be fibrotic disorders displaying excessive and continuous wound healing triggered by tissue injury and characterized by disorganized distribution of collagen fibers in extracellular matrix (ECM). However, excessive deposition and remodeling of extracellular matrix (ECM) are thought to play an important role in forming the main structure of leiomyoma (Islam MS et al. 2018). Leiomyomas typically have 50% more collagen than normal myometrium and present as encapsulated collagen-rich masses of smooth muscle cells. Moreover, ECM serve as reservoir for growth factors, cytokines, angiogenic and inflammatory response mediators (Islam MS et al 2014). The autocrine/ paracrine signaling has a significative influence in events involved in myometrium cellular transformation and turnover that are implicate din leiomyoma pathophysiology (Ciarmela P et al. 2011). Fibroids growth is affected by hormones during pregnancy and postpartum periods. Estrogen/progesterone- dependent in vivo growth of human leiomyoma tissue requires the presence of these multipotent tissue-specific stem cells (Bunting KD. 2002, Sozen I et al. 2002).

Despite their high prevalence, the cellular and molecular origins of uterine leiomyomas are not well understood. Recent studies suggest the involvement of epigenetic mechanisms such as DNA methylation and micro-RNA and histone modification in leiomyoma (Izadpanah R et al. 2006, Panepucci RA et al. 2004, Orciani M et al. 2017, McWilliams MM et al. 2017, Gargett CE et al. 2012, Hubbard SA et al. 2009). At the genetic level, several mutations, such as germline mutations causing fumarate hydratase deficiency, have been associated with leiomyoma formation (Ono M et al. 2014).

Therefore, the question about the origin and pathophysiology of leiomyoma is still open and unclear. Furthermore, several factors, such as genetic aberrations (Bulun SE. 2013) and undifferentiated cell population that could give rise to them, has been investigated (Zhang P et al. 2006, Canevari RA et al. 2005). The latter theory is sustained by the uterine tissue remodeling that happens during life in physiological (Carneiro MM. 2016) and pathological conditions (Blake RE. 2007).

1.4.1. Role of MSCs in uterine leiomyomas

MSCs could be involved in the onset and development of uterine leiomyomas. Indeed, some studies (Mas A et al. 2014, Mas A et al. 2015) proposed that undifferentiated cells are involved in myometrial pathologies, and also leiomyoma onset may be the results of impaired function, proliferation, and differentiation of progenitor cells inside the myometrium that are under the effect of ovarian hormones (Kurita T et al. 2001, Flake GP et al. 2003). The dysregulation of mesenchymal stem cells activity could be a possible explanation for the development of leiomyoma (Maruyama T et al. 2013). Furthermore, this hypothesis is strongly supported by the ability of leiomyomas to originate from a single altered cell derived from myometrial smooth muscle and to maintain the replicability (Mas A et al. 2014, Chang HL et al. 2010, Ono M et al. 2012). Their growth requires the presence of mature myometrial or leiomyoma cells with higher levels of steroid receptors and their ligands. This is based on the postulate that steroid hormone action on leiomyoma stem cells is mediated by mature myometrial cells (tumor initiation) or mature leiomyoma cells (growth maintenance) in a paracrine fashion (Ono M et al. 2014).

As demonstrated for other types of tumors, also in leiomyomas (Ono M et al. 2013, Ono M et al. 2014, Yin P et al. 2015, Wegienka G. 2012) microenvironment seems to play a key role in relationship with inflammation. If acute inflammation results in chronic inflammation, tumor onset and development are promoted (Orciani M et al. 2018). In this situation, cytokines secreted by the immune system and undifferentiated cells (Protic O et al. 2016, Islam MS et al. 2014, Weiss G et al. 2009, Elinav E et al. 2013, Ma S et al. 2014), which are involved in a complex crosstalk with neoplastic cells, are able to carry on a chronic inflammation. These cytokines influence proliferation, fibrosis, and angiogenesis, which in turn sustain fibroid formation and growth (Orciani M et al. 2013, Orciani M et al. 2016, Orciani M et al. 2017).

1.5. STUDY 2: Cervical intraepithelial neoplasia (CIN)

Cervical intraepithelial neoplasia (CIN) is a pre-cancerous lesion of uterine cervix epithelium and a surgical treatment is recommended for women diagnosed with high-grade CIN (CIN2-3). It is associated with several risk factors such as persistent human papillomavirus (HPV) infection (Vasiljević N et al. 2013), immunosuppression (Lima MI et al. 2009, Lodi CT et al. 2011), genetic mutations and epigenetic events (Virmani AK et al. 2001, Widschwendter A et al. 2004, Narayan G et al. 2003), sexual behavior and smoking (Luhn P et al. 2013). In particular, HPV infection seems to be the strongest factor influencing the natural history of CIN and increasing the risk for persistent disease. CIN can either resolve spontaneously or persist or progress if not treated properly.

Depending on the severity of the lesion type, CIN may occur in grade I (CIN1), II (CIN2) and III (CIN3). CIN 2, 3 are correlated with a risk of developing cervical cancer, and are typically treated with conisation. The societal importance is highlighted by the fact that the annual incidence of CIN1, 2 and 3 among young women (under 30 years) is increasing. The loop electrosurgical excision procedure (LEEP) is an usual method for treating high-grade CIN (Bae JH et al 2007). Despite this treatment was shown to not adversely affect fertility, it was associated with an increased risk of miscarriage in the second trimester (Kyrgiou M et al. 2015) and with enhanced chance for prematurity/higher risk for preterm birth and adverse pregnancy outcomes (Kyrgiou M et al. 2015, Liverani CA et al. 2016, Ciavattini A et al. 2015). For this reason, each patient needs an individual evaluation, especially for young women in a reproductive age, with a careful selection of patients to be treated surgically and of those that can be managed conservatively, also considering the possibility of regression of the lesions (Bekos C et. al 2018). In recent studies (Himes KP et al. 2007, Ciavattini A et al. 2015), it was demonstrated the correlation between a shortened cervical length treated with LEEP procedures and an increased risk of preterm birth (Song T et al. 2016). Therefore, it is important to consider how much cone depth increase in relationship with the amount of the volume of tissue excised. About that, Founta et al evaluated the change in cervical volume with the magnetic resonance imaging reporting that the mean cervical volume at 6 months post-procedure corresponded to 97.8% of the baseline cervical volume (Founta C et al. 2010). Furthermore, several studies have suggested that younger women generally seem to have higher rates of spontaneous regression and remission (Bekos C et al. 2018, Cox JT et al. 2003, Moscicki AB et al. 2010).

In particular, Bekos and colleagues investigated about age-dependent regression and progression rates in a large number of patients (783) using histologic data. They demonstrated that the regression rates were higher in young women with CIN and, in agreement with those reported in literature, regression was more likely in patients with low- grade CIN compared to high- grade dysplasia. All of these studies provides clinically relevant findings on the influence of age on the natural history of CIN and the rates of regression are notably high in young women with CIN and with a lower risk of progression.

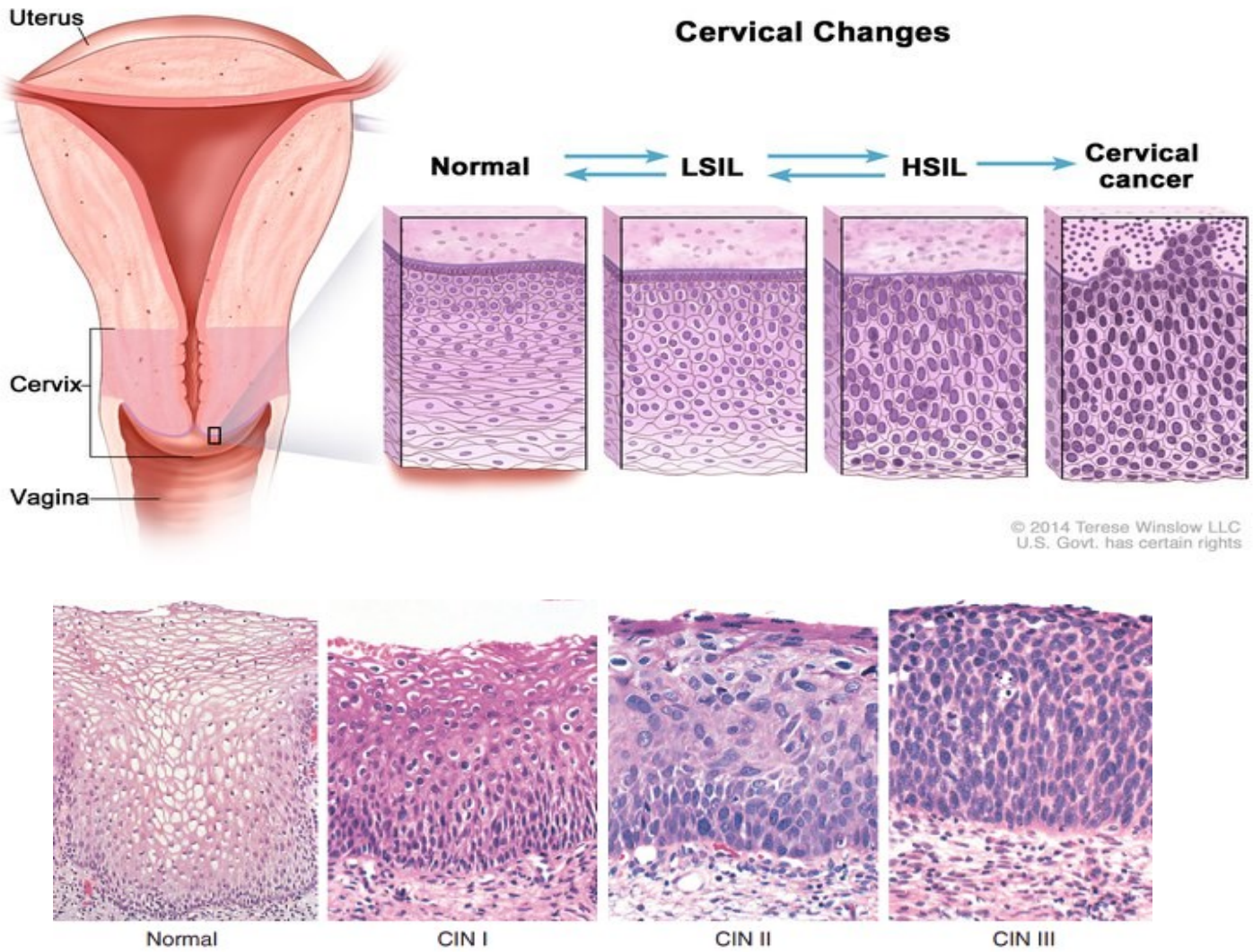

Figure 5. Representation of cervical changes in cervical intraepithelial neoplasia (CIN). Cervical dysplasia is the precursor to cervical cancer and it is characterized by transformation and abnormal growth of cervical keratinocytes. Based on histopathology, three CIN (cervical intraepithelial neoplasia) grades are defined: CIN1 (mild dysplasia), CIN2 (moderate dysplasia) and CIN3 (severe dysplasia) lesions Based on cytology, two grades are defined: LSIL (low grade squamous intra-epithelial lesions) or HSIL (high-grade squamous intraepithelial lesions). Usually LSIL and CIN-1 overlap, and HSIL and CIN-2 and CIN-3 overlap. About 10% of LSIL/CIN-1 eventually progress to cervical cancer, while about 50% of HSIL/CIN-2/CIN-3 progress to cervical cancer.

1.5.1. The role of inflammation and MSCs in age-related regression of CIN

Several researches (Bekos C et al. 2018, Cox JT et al. 2003, McCredie MR et al. 2008) have evaluated the possible correlation between patient's age and regression/progression rates of CIN, finding that younger women generally seem to have higher rates of spontaneous regression and remission. The underlying mechanisms are not yet fully known but, as for other solid tumors, inflammation may play a pivotal role in CIN fate (Hammes LS et al. 2007). The hypothesis of an involvement of inflammation in CIN progression is enforced also by studies aimed to evaluate the use of anti-inflammatory drugs in the treatment of CIN (Grabosch SM et al. 2018).

Inflammation is orchestrated by different cells of the immune system as well as by mesenchymal stem cells (Kyurkchiev D et al. 2014). The existence of MSCs in cervical tissue has been demonstrated by Eirò et al. in a study in which they isolated for the first time MSCs from the human uterine cervix (human uterine cervical stromal cells, HUCESCs) by exfoliation PAP smear (a minimally invasive procedure). HUCESCs and their secretome were able to inhibit the aggressive behavior of cancer cells (cell lines and primary tumors) *in vitro* and to reduce tumor growth *in vivo* in a mouse xenograft tumor model. They also showed an inhibitory effect on cancer associated fibroblast (CAFs) proliferation and invasion, and can inhibit and revert macrophage differentiation (Eirò N et al. 2014).

Further support has been obtained from the evidence that, within 6 months after LEEP treatment, cervix shows a regenerative process, reporting values of 71-98% of post-excision tissue deficiency (Song T et al. 2016, Papoutsis D et al. 2012, Ciavattini A et al. 2018). As inflammation is supported by undifferentiated mesenchymal stem cells (Orciani M et al. 2018), they plays a crucial role in the regression and progress of CIN. It is believed that the existence of a reservoir of MSCs inside cervical tissue represent the trigger for cervical regeneration after conization (Orciani M et al. 2018).

1.6. STUDY 3: Duchenne muscular Dystrophy (DMD)

Muscular dystrophy is a group of inherited diseases characterized by skeletal muscle deficiency and degeneration. Muscular dystrophies are progressive disorders caused by the loss of healthy muscle fibers over time that are replaced by fibrosis and fat, making muscle tissues less able to generate force for everyday activity (Gao QQ et al. 2015). Duchenne muscular dystrophy (DMD) is a severe X-linked recessive disorder and the most prevalent inherited myopathy affecting one in 3,500 live male births (Bushby K et al. 2010). DMD is usually diagnosed during early childhood, between 2 and 7 years of age, in which the first signs of muscle weakness appear evident. DMD patients present progressive muscular deficiency, in addition to orthopaedic, respiratory, and cardiac complications that lead to their death around the third or fourth decade of life (McNally EM. 2007, Bushby K et al. 2010).

At the molecular level, DMD is caused by mutations in the 2.2-Mb dystrophin gene which disrupts the protein's reading frame causing premature stop codons (Koenig et al. 1987, Kunkel et al. 1987). These mutated transcripts produce truncated proteins that are unstable and subject to degradation, leaving the cells devoid of protein products. Therefore, the final result of all these mutations is an absence of functional dystrophin in the skeletal muscle.

This gene is one of the biggest genes in the human genome with more than 2 million base pairs in Xp21.2-p21.1. The dystrophin coding sequence (11kbp) contains 79 exons encoding a 427 kDa dystrophin protein (Guiraud et al. 2015).

In skeletal and cardiac muscle, dystrophin binds to several proteins forming the Dystrophin-Associated Protein Complex (DAPC). The structure of dystrophin protein consists on an N-terminal actin-binding domain, a central rod-like domain with 24 spectrin-like triple helical coiled coils, and with a cysteine-rich C-terminus that permit aggregation of the DAPC (Ehmsen J et al. 2002). The DAPC has a pivotal role to link the actin cytoskeleton to the extracellular matrix, stabilizing the sarcolemma during contraction and relaxation, and sending force from the muscle sarcomeres to the extracellular matrix (Petrof BJ et al. 1993). Besides the fracture of the muscle fiber during the contraction, the DMD brings a modification in the intracellular signaling that contribute the progression of the disorder (Constantin B. 2014).

Most of the DMD patients come through the frame and non-sense mutations leding to reduction of the transcript level and truncation of translation (Monaco AP et al. 1988; Roberts RG et al. 1994). Dystrophin is a cytoskeletal protein crucial for the stability and function of myofibers in muscle.

It creates a mechanical link between the extracellular matrix and the cytoskeletal actin in muscle fibers through the dystrophin-associated protein complex (DAPC) (Ervasti JM et al. 2008).

Therefore, at the cellular level, the muscle of DMD patients exhibit necrosis, degeneration and regeneration, myofiber atrophy, fatty accumulation, fibrosis, and inflammation (Spencer and Tidball, 2001; Alvarez et al., 2002; Desguerre et al., 2009a,b; Serrano and Muñoz-Cá-noves, 2010; Zhou and Lu, 2010; Villalta et al., 2011).

In the last few years, different approaches (gene-based, cell-based, nano-particles, and pharmacological) have been developed to restore a functional dystrophin to DMD muscles (Negroni et al., 2016; Chamberlain and Chamberlain, 2017; Nance et al., 2017) and many of them are tested in clinical trials that are currently ongoing on DMD patients. Since 1995, among the main treatment choices for dystrophic patients in clinical trials, pharmacological approaches were found to be the primary with 57%, followed by 28% gene-based (22% antisense oligonucleotide-based exon skipping, 6% AAV gene addition), and 3% cell-base approaches (Cordova et al., 2018). The only standard therapy approved for dystrophic patients is the administration of steroids that have a number of side effects and only slow down the progression of DMD (Griggs et al. 2013). Furthermore, the clinical experimentation with novel approach reached encouraging results but none of them has achieved significant and long lasting clinical efficacy (Guirad et al. 2015). Currently steroids represent the only standard therapy for dystrophic patients but only delay the progression of the disease and come with serious side effects (Griggs et al. 2013). Many novel therapeutic approaches have entered clinical experimentation with encouraging results but none has yet reached significant and long lasting clinical efficacy (Guirad et al. 2015). These include new drugs, gene therapy, exon skipping, PTC124 (which triggers premature STOP-codon read-through), and cell therapy (Galli et al. 2018).

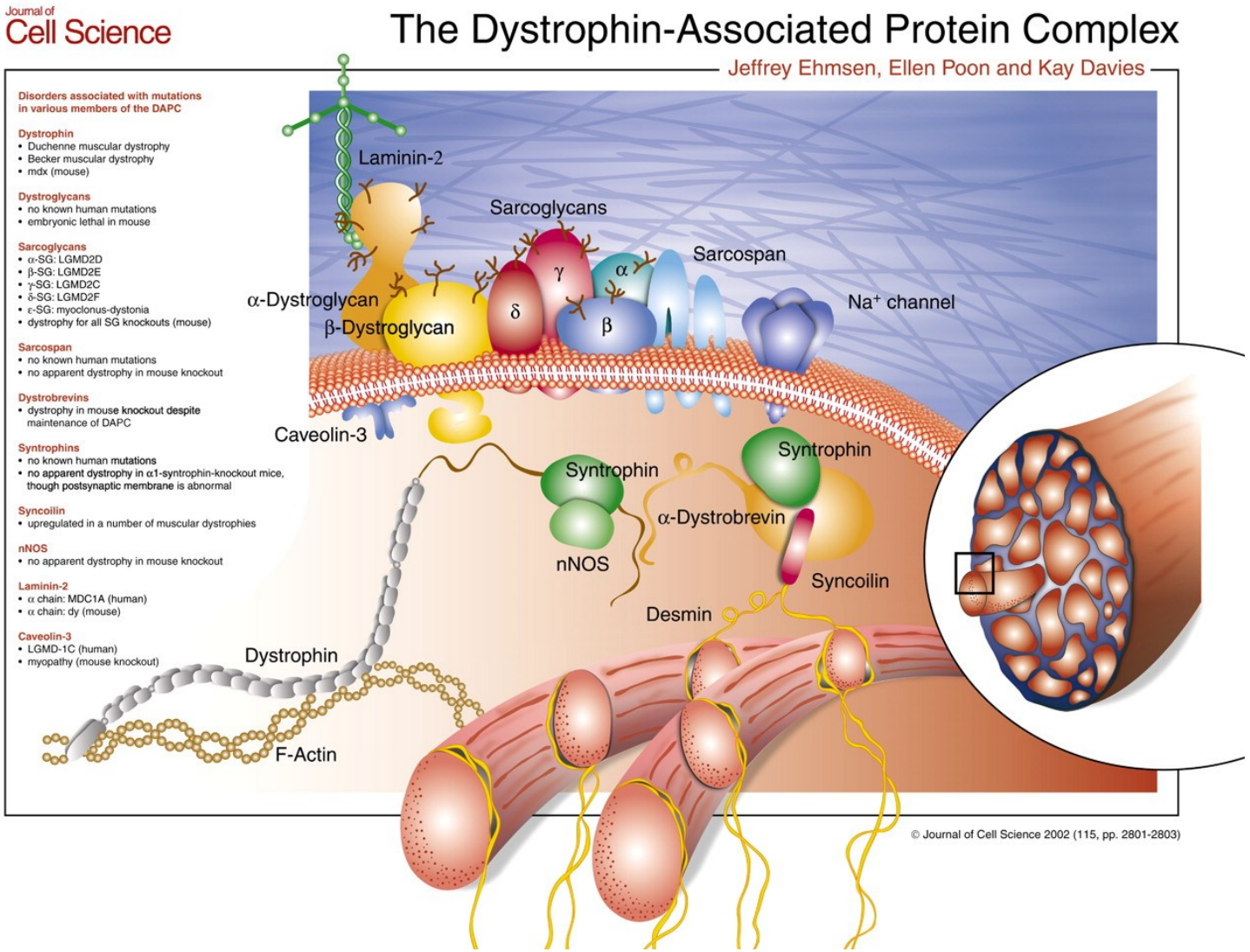

Figure 6. The dystrophin-associated protein complex.

1.6.1. Gene therapy

The basic theory of in vivo gene therapy is the therapeutic delivery of nucleic acid into the patient cells, tissues, and organs as a drug to treat disease (Kaji E et al 2001). In details, a vector carries inside the diseased cell either a wild-type (wt) copy of the mutated gene (to replace it) or molecules that repair the DNA, or the mRNA, leading in all cases to the production of a normal or semi-normal protein at a sufficient level to carry on its specific function. In DMD specific case, to obtain a structural protein like dystrophin, it has been estimated that approximately 20-30% of the normal level is the minimum level necessary to restore function (Galli F et al. 2018).

The first step for gene therapy requires the right choice of the vector. Currently, adeno-associated vectors (AAV) are center-stage in gene therapy for muscular dystrophies (Chamberlain JR et al. 2017) as for other genetic diseases. AAV are small vectors and this feature presents pros and cons: in fact, the small dimension allow them to spread easily into the tissue but at the same time they are not able to accommodate sufficiently large enough cDNAs to encode a protein such as dystrophin. A second hurdle is the immune response of the host to the AAV capsid protein and to the gene products eventually expressed by the vector (Hareendran S et al. 2013). It is estimated that about half of the human population has been exposed to one or more serotypes of AAV, therefore the patients need to be screened preliminarily to warrant that pre-existing neutralizing antibodies may avoid any effect of the vector. Moreover, it is retained that the immune system may not see a part or whole gene product, which may evoke an immune response.

Another issue is related to the nature of the AAV vector it is the inability to integrate into the host cell genome, and cells during their divisions lose the vector. In case of striated muscle the problem is solved by the fact that differentiated cells don't divide for long passages and persist during all the life of the host. Furthermore, the distribution of these vectors is considered a very important aspect, as it is related to the route of administration and on the structure of the target tissue. Skeletal muscle is the most abundant tissue of our body and the systemic way, especially the intra-venous one, is almost always the first choice of administration because represent the easiest way to distribute the vectors in this tissue. Moreover, this way permits to achieve wider vector distribution respect the intra-arterial characterization and the intra-muscular injection (Le Guiner C et al. 2014, Le Guiner C et al 2017).

Other aspects that should not be underestimated are complexity and costs that are necessary to produce large amount of AAV vectors to get significant dystrophin expression (Cohen-Haguenauer O et al. 2010).

At last, in this big scenario, gene replacement is the main method of gene correction. It was demonstrated that optimized versions of micro-dystrophin (Reza et al. 2016) and functional domains are now accessible and tests in few clinical trials with good results in patients affected by spinal muscular atrophy. However, a recent study in primates and piglets showed significant liver and neural toxicity. Another valid option is to use AAV delivered small nuclear RNA, such as U7, able to skip the mutated exon in order to restore the reading frame and produce a short but functional dystrophin (Le Guiner 2014, Vulin 2012). Currently, the strategy of using AAV as an enzyme carrier that correct the genome and repair the mutation is one of the most promising. CRISPR-Cas9 is the most innovative and current strategy that cut the damaged region restoring it with the correct sequence (Tabebordbar et al. 2016; Bengtsson et al. 2017). The fact that Cas9 is a bacterial protein is a potential cause of a strong immune reaction in vivo that could be solved using transient immune suppression or new version of the enzyme, maybe short lived to reduce exposition to the immune system.

1.6.2. Cell therapy

Nowadays, cell therapy is considered less popular and full of barriers that are more difficult to solve respect to gene therapy.

First of all, it is fundamental to choose the right type of cell that has a strong ability to differentiate into skeletal muscle cells and is also able to maintain this potential for more generations. Satellite cells are the first choice considering that they are the main stem cells of skeletal muscle responsible for both post-natal muscle growth and regeneration. In 1961, satellite cells were described for the first time and studied for their role in regeneration and senescence (Mauro et al). It has been shown that these cells, when transplanted in a mouse muscle, preserve their stemness (Rocheteau et al. 2012; Partridge et al. 1989), encouraging few clinical trials of cell transplantation for DMD. The theory at the base of this trial is that myogenic progenitors derived from satellite cell are expanded in vitro and then directly injected in some patient muscles without toxicity or efficacy. However, this route has led to two hurdles: massive death of transplanted cells and the fact that once injected, these cells remain in the same area without spreading to the whole muscle, making less favorable the possibility of intra-muscle transplant to larger muscles (Mouly et al. 2005; Peault et al. 2007). Many years were necessary to optimize the intramuscular transplantation protocol, and this approach has now become part of the clinical trial for the treatment of localized forms such as the Oculo-Pharyngeal Muscular Dystrophy (Pèrié et al. 2014). Until now, we can state that intra-muscular injection of satellite cells seems the therapy of choice for localized forms of muscular dystrophy or other muscle diseases, but not for forms affecting most of the body muscles (Galli F et al 2018). The second major issue for cell therapy is delivery. For a long time, systemic injection of myogenic cells was considered the best option to overcome this obstacle, but satellite cells are unable of extravasation and amass together in the capillaries creating micro-thrombi. However, a possible way to enhance diffusion involved the use of blood-born progenitor cells, which in bone marrow could participate to muscle regeneration in bone marrow transplantation (Ferrari et al. 1998). Nevertheless, this approach flunked to restore dystrophin expression in a considerable portion of muscle fibers in the receiving animals (Gussoni et al. 1999).

Another interesting approach consist on the use of mesangioblasts, that are mesenchymal-like cells, associated with the walls of large vessels, considered in vitro counterparts of muscle perivascular cells (Dellavalle et al. 2007).

In a clinical trial, Cossu and his group performed intra-arterially transplant mesangioblasts from human donor, usually an HLA-matched brother, in the femoral and subclavian arteries of five DMD patients in specific conditions in four consecutive injections at increasing cell dosage, resulted safety but lack of clear clinical efficacy. Indeed, expression of donor dystrophin was detected in the youngest patient, but only the 0.7% of donor DNA has been found in the biopsy of the same patient leading to the probably most difficult hurdle, the low engraftment (Cossu et al. 2015). Recently, the same authors confirmed that it is possible reach good results in disease of tissues like the blood or the epithelia, where damaged cells can be remove giving space for donor cells (Cossu et al. 2018).

Another way to act is the possibility to engineer donor cells, since muscle fibers have multinucleated cells, that may enter regenerating fibers and then also correct neighboring resident nuclei through overexpression of microdystrophin or sarcoglycan from a strong muscle promoter. It was recently shown that also MSC exosomes promote dystrophic muscle regeneration (Bier et al 2018).

An entirely innovative and different strategy consist on transducing donor myogenic cells with a lentivector expressing the U7 snRNA, engineered to skip exon 51, with the goal to exploit the diffusion of snRNA to neighboring nuclei and induce exon skipping there and thus amplify the production of dystrophin (Galli et al. 2018). Alternatively, transient expression of CRISPR Cas9 from a non integrating lentivector, may genetically correct also neighboring nuclei, thus amplifying the genetic correction. It gives the advantage of a permanent correction of the genetic defect respect to the exon skipping, but with the risk of the induction of an immune response that can be or not controlled by immune suppression.

In the last years, induced Pluripotent Stem cells (iPSc) represent another promising approach (Hotta and Yamanaka, 2015). Adult cells from the patient can be reprogrammed until a stage very close to embryonic stem cells, genetically corrected with CRISPR Cas9 for a definitive effect, and then differentiated to muscle progenitor cells. This technology provides the possibility to bypass an immune reaction, and clones with appropriate correction and genome integrity may be selected before induction of differentiation into the target cell type. Therefore, it can give good hopes for the cell therapy but the problems related to engraftment still remain.

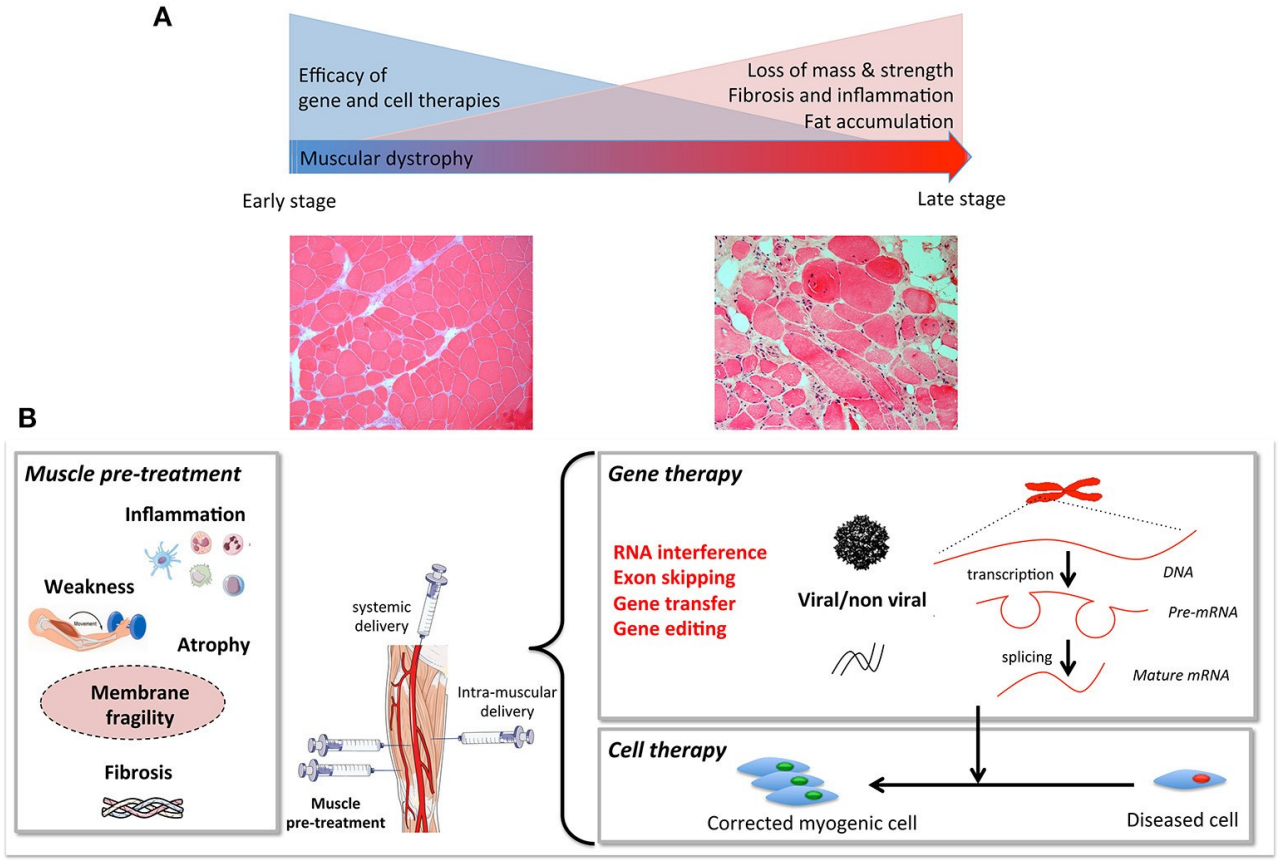

*Figure 7. (*A) Muscular dystrophies fibers, including loss of mass, weakness, fat, and extracellular matrix accumulation. Gene and cell based therapies will have to overcome the progressive degeneration of muscle fibers. When these histological changes become prominent, combined strategies are needed. (B) Muscle pre- or co-treatment may target inflammation, atrophy, membrane fragility, muscle weakness, and/or atrophy to pre-condition the tissue to increase efficiency of gene and cell therapy. (Cordova et al. 2018).

2. Aim of the study

The main aim of these projects is to evaluate the existence of mesoderm stem cells (MSCs) and understand their role in the pathogenesis and potential therapy of selected gynecological diseases with an inflammatory component as uterine leiomyoma, cervical intraepithelial neoplasia (CIN), and in primary myopathies, as Duchenne Muscular Dystrophy (DMD).

To study gynaecological diseases, we isolate MSCs from uterine leiomyomas, healthy myometrium and cervixes. Then, the relationship between MSCs and specific inflammatory pathways, such as Th1, Th17 and Th2 will be investigated. In details, in study 1 we evaluate if: 1) the existence of undifferentiated cells may correlate with leiomyoma onset, 2) inflammation may sustain leiomyomas, and 3) cytokines secreted by undifferentiated cells may create an inflammatory microenvironment.

In study 2, the intention is to understand if: 1) age may affects the behaviour of MSCs from cervixes (C-MSCs) derived from young and old patients, 2) C-MSCs in turn may modulate inflammation and regression rate of CIN, and 3) in the crosstalk with HeLa cells, C-MSCs could play an anti-tumoral role through their paracrine effect.

About study 3, the goal is to find a correct strategy to enhance the production of dystrophin protein in Duchenne Muscular Dystrophy (DMD) through gene therapy. Therefore, to address to this question, myoblasts will be isolated from DMD donor, transduced with green fluorescent protein (GFP) and a lentiviral vector expressing the snRNA to induce exon skipping, and promoted the myogenic differentiation in order to check if they express a functional dystrophin protein.

3. Materials and methods

3.1. Human tissues collection

For the first study, twelve samples of tissue respectively of leiomyoma and healthy myometrium were collected from women of childbearing age (between 30 to 35 years) by the Gynecology and Obstetrics Unit, Department of Clinical Sciences, Polytechnic University of Marche (Ancona, Italy). After a histologically confirmed diagnosis of leiomyoma, the patients underwent surgical operation such as hysterectomy or laparoscopy to remove the fibroid tissue. All tissue samples were collected in the operating room from a trained operator. One small fragment of 3-5 mm from leiomyoma and one from normal myometrium has been obtained by a cold-blade scalpel. The samples were placed in MSCGM medium (Mesenchymal Stem Cell Growth Medium, Lonza, Basel, Switzerland) and sent to our laboratory for processing. We reported the size (in cm), topographic site (anterior, posterior, left lateral, right lateral, and fundal), and location (subserosal, intramural or submucosal) of fibroids from where the samples were obtained. The removal of the sample did not alter the histopathological analysis in any case. All patients displayed good general condition; none of them had a history of myomectomy or uterine surgery, had received medical therapy or oral contraceptives in the previous three months, or had evidence of genital tract infection, endometriosis, or ovarian disease. All had a negative cervical vaginal swab collected prior to surgery, which was performed in the proliferative phase of the cycle. Adenomyosis or other uterine disorders demonstrated on histopathological examination were exclusion criteria.

For the second study, fourteen women undergoing cervical excision for high-grade cervical intraepithelial neoplasia (CIN 2-3) were recruited. The diagnosis was confirmed after colposcopy-directed cervical punch biopsy following an abnormal cervical cytology by the Gynecology and Obstetrics Unit, Department of Clinical Sciences, Polytechnic University of Marche (Ancona, Italy). The patients were divided into two groups according to age: 7 of them defined “young” (mean age 28±2) and the other 7 defined “old” (mean age 45±3).

The cervical excisional procedures were performed with the loop electrosurgical excision procedure (LEEP) technique, in an outpatient setting under local anesthesia and strict colposcopic guidance, with 1.5 – 2.0 cm rounded loops, chosen according to the type of the transformation zone and the area of cervical tissue to remove.

All patients provided their written informed consent to participate in the studies, which was approved by the institutional ethics committees and was conducted in accordance with the Declaration of Helsinki.

3.2. Cell culture

Tissue fragments (2-3 mm^3) from leiomyomas and myometrium were firstly subjected to mechanical digestion then to enzymatic digestion with 0.2% type II collagenase (Sigma-Aldrich, Milan, Italy) at 37°C for 4 hours; subsequently, partially digested solution was placed into 6-well plates containing MSCGM medium which enhances the growth of undifferentiated cells and maintained in culture using same media at 37°C in 95% air and 5% CO_2. Tissue fragments, obtained from cone specimen (cervixes), were directly placed into 6-well plates containing MSCGM medium. The growth medium was changed after 24 hours to remove unattached cells/fragments or debris and then twice a week. Cell morphology was evaluated by phase-contrast microscopy (Leica DM IL; Leica Microsystems GmbH, Wetzlar, Germany) and viability by an automated cell counter (Invitrogen, Milano, Italy). All further analyses involved separate assays of the specimens from each participant up to the first five passages.

3.3. Characterization of MSCs from different tissues

After samples collection, cells were characterized by testing the minimal criteria identified by Dominici for mesenchymal definition; in detail, the plastic adherence, the immunophenotype and the multipotency were evaluated. In addition, the expression of genes (OCT4, SOX2, NANOG, KFL4) related to stemness was analyzed by Real Time PCR.

For immunophenotyping, 2.5×10^5 cells were stained for 45' with fluorescein isothiocyanate (FITC)-conjugated antibodies (Becton-Dickinson) against: HLA-DR, CD14, CD19, CD34, CD45, CD73, CD90 and CD105 and analyzed by flow cytometry. For differentiation assay, cells were induced to differentiate into osteocytes, chondrocytes and adipocytes using STEMPRO® Osteogenesis, Chondrogenesis and Adipogenesis Kits (GIBCO, Invitrogen,) respectively. For osteogenic differentiation, after 21 days cells were assessed by Von Kossa staining and after 7 days by Alkaline phosphatase (ALP) reaction; for adipogenic differentiation, after 21 days, cells were tested by Oil Red O staining; for chondrogenesis, cells were cultured in pellet culture system for 14 days and fixed in 4% neutral buffered formalin for 24 hours at 4°C. The pellet was then processed with different grades of alcohol and xylene and paraffin embedded at temperatures

not exceeding 60°C. The sections were exposed to a solution of Safranin-O. Cells cultured in MSCGM alone were used as negative controls. For the analysis of the expression of genes related to stemness, total RNA was isolated from cells using Master Script RT-PCR System (5 PRIME, Hamburg, Germany) and quantified at Nanodrop instrument (Thermo Scientific™ NanoDrop™ instruments 2000/2000c UV-Vis). 1µg of RNA was retrotranscribed in cDNA following the manifacturer's instructions (GoScript™ Reverse Transcription System, Promega, Italy). The amplification program and the primer sequences are reported respectively in Table 1 and Table 2. All samples were tested in triplicate with the housekeeping genes RPLP0 and GAPDH. GAPDH was the most stable and used for subsequent data normalization. After amplification, melting curves were acquired.

Table 1. Amplification program for RT-PCR

	cDNA		
Cycling step	**Temperature**	**Time**	**# Cycles**
Enzyme activation	95°C	30 sec	1
Denaturation	95°C	5 sec	30-40
Annealing/ Extension	60°C	20 sec	
Melt Curve	50-95°C	20 min	1

Direct detection of PCR products was monitored by measuring the fluorescence produced by SYBR Green I dye (EVA Green PCR Master Mix, Bio-Rad) binding to double strand DNA after every cycle. The parameter threshold cycle (Ct) was defined as the cycle number at which the first detectable increase above the threshold in fluorescence was observed.

The amount of mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The values of the relative expression of genes of interest are referred as mean ± DS, over three independent experiments.

Table 2. Primer sequences related to stemness genes.

Gene Symbol	Forward	Reverse
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
RPLP0	CCATTCTATCATCAACGGGTACAA	TCAGCAAGTGGGAAGGTGTAATC
NANOG	TGAACCTCAGCTACAAACAG	CTGGATGTTCTGGGTCTGGT
SOX2	ACACCAATCCCATCCACACT	GCAAACTTCCTGCAAAGCTC
OCT4	AGCGAACCAGTATCGAGAAC	TTACAGAACCACACTCGGAC
KLF4	CCCACACAGGTGAGAAACCT	ATGTGTAAGGCGAGGTGGTC

3.4. Doubling time

To assess doubling-time, 8 x 10^4 cells per well were plated using an algorithm available online (http://www.doubling-time.com): DT = t x lg2 / (lgN t – lgN 0)

where N0 is the number of plated cells (always the same), Nt is the number of harvested cells, and t is culture time in hours. The values are reported as mean ± SD of three independent experiments.

After the characterization, cells from normal myometrium and leiomyomas (study 1) and cell from young and old cervixes (study 2) were subjected to different analyses aimed to confirm the two working hypotheses.

3.5. STUDY 1

3.5.1. Immunocytochemistry

For ICC, 1.5 x 10^4 cells from leiomyoma and myometrium were plated in each well of a 4- well culture chamber slides (Lab Tek, Nunc, USA) and cultured overnight. Cells were fixed in 4% formalin for 30' at room temperature, washed in Phosphate Buffer Saline (PBS) 1X and in hydrogen peroxide diluted in distillate water. Then each sample were permeabilized with 0.1% Triton X-100 in PBS 1X for 20' at room temperature and incubated overnight at 4°C with the following monoclonal antibodies: anti-collagen type I (1:1000 dilution, Sigma-Aldrich), anti-cellular fibronectin (1:400 dilution, Biorbyt) and anti-αSMA (1:400 dilution, Sigma-Aldrich). After washing in PBS 1X, cells were subsequently immunostained using the streptoavidin– biotin–peroxidase technique (LSAB universal peroxidase kit, Dako Cytomation, Milan, Italy). After incubation with 3,3-diaminobenzidine (0.05 diaminobenzidine in 0.05 M Tris buffer, pH 7.6 and 0.01% hydrogen peroxide), the slides were counterstained with Mayer's hematoxylin, dehydrated and cover slipped with Dako Cytomation Glycergel mounting medium (Dako North America, Inc., Carpinteria, CA, USA). Negative control was performed omitting primary antibodies.

3.5.2. Immunofluorescence

For IIF, 1.5 x 10^4 cells from leiomyoma and myometrium were plated in each well of a 4- well culture chamber slides (Lab Tek, Nunc, USA) and cultured overnight. Cells were fixed in 4% formalin for 30' at room temperature, washed three times with Phosphate Buffer Saline (PBS) 1X and treated with 0.1% of Triton X-100 in PBS 1X for 30' at room temperature. After other three washes in PBS 1X, cells were blocked with PBS containing 2% bovine serum albumin (BSA) for 15' at room temperature and incubated with mouse anti-human primary antibody diluted in PBS with 2% BSA anti-collagen type I (1:1000 dilution, Sigma-Aldrich), anti-cellular fibronectin (1:400 dilution, Biorbyt) and anti-αSMA (1:400 dilution, Sigma-Aldrich) overnight at 4°C.

Samples were washed three times and incubated with goat anti-mouse FITC-conjugate antibody (1:1000 dilution, Sigma-Aldrich) for 30' at room temperature.

Then cells were washed three times and incubated for 5' at room temperature with Hoechst 33342 (1:1000 dilution, Molecular Probe) in PBS to visualize nuclei and mounted onto a glass slide using Vectashield Vector mounting medium for fluorescence H-1000 (Vector Laboratories,

Inc. Burlingame, CA). Fluorescence was detected with Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany) AxioVision. Negative control was performed omitting primary antibodies.

3.5.3. Western blot

For protein extraction from cells derived from myometrium and leiomyoma, pellets were collected and incubated in ice with RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1 % SDS, 1.0 % Triton X-100, 5 mM EDTA, pH 8.0) containing protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) for 45 minutes and then centrifuge at 14000 g for 15 minutes at 4°C. Supernatants were aliquoted and stored at -20°C before the use. To determine protein concentration, the Bradford protein assay with Bradford reagent was performed (Bradford reagent, Sigma-Aldrich, Milan, Italy). Different concentrations of BSA (Albumin Bovine Serum) in distilled water were used to draw the standard curve. Total protein extracts (35 μg of protein for each sample) were reduced in DTT (0.5 M) for 10 minutes at 70°C before the electrophoresis. Precast polyacrylamide gels at 4-12% gradient (Invitrogen NuPAGE Bis-Tris protein gels) were chosen for electrophoresis separation. Electroblotting was performed using iBlot® Dry Blotting System (Invitrogen). Membranes were incubated with 5% milk in PBS 1X with 0.05% Tween 20 (T-PBS) to block nonspecific sites for 1 hour. Then, membranes were hybridized with monoclonal mouse antibodies anti-MDR1 (Santa Cruz, 1:400) diluted in 5% milk T-PBS at 4°C overnight. After washing with T-PBS, membranes were incubated with secondary antibody anti-mouse conjugated with horseradish peroxidase (Santa Cruz Biotechnology) at 1:10000 dilution for 1 hour. Detection of antibody binding was performed with the chemiluminescence substrate reagent (ImmunoCruz™ Western blotting Luminol reagent, Santa Cruz Biotechnology) and images were acquired with UVITEC Alliance Q9 Advanced (UVITEC Cambridge). GAPDH and β-Actin were used as housekeeping to normalize. As negative control, Normal Human Lung Fibroblasts (NHLF) were used.

3.5.4. Expression of inflammation-related cytokines by RT-PCR and ELISA

Selected cytokines related to acute and chronic inflammation, IL6, IL12, IFN-γ, TNF-α, IL2, IL4, IL5, IL13, IL10, TGF-β1, IL17A, and GM-CSF, were investigated by RT-PCR (as above reported) and by ELISA (Multi-Analyte ELISArray kit, Qiagen, Milan, Italy).

Briefly, medium conditioned for 72 hours by each sample of MSCs derived from myometrium and leiomyoma was collected, concentrated and dispensed into a 96-well microtiter plate from Row B to G. The appropriate Sample Dilution Buffer was added in each well of Row A of the ELISArray plate to set up the negative control, and the final Antigen Standard Cocktail diluted following the protocol was added in the Row H to set up the positive control. The ELISArray plate was incubated for 2 hours at room temperature. After three washes with a Wash Buffer 1X, the diluted Detection Antibodies were transfer to the appropriate rows of the ELISArray plate and incubated for 1 hour. Then, the plate was washed for three times and the Avidin-HRP conjugate, diluted in Assay Buffer, was added into all wells and incubated for 30' at room temperature in the dark. After four washes, the plate was incubated with a Development Solution for 15' at room temperature in the dark. Finally, the Stop Solution was added in each well and the absorbance was read at 450 nm and 570 nm using a microtiter plate reader (Multiskangomicroplate reader, Thermo Scientific).

The levels of the cytokines secreted by leiomyoma cells are reported as a percentage of the levels measured in the corresponding myometrial sample. After, mean ± SD from three independent experiments in triplicates was calculated and displayed.

For mRNA analysis, RT-PCR was performed as above mentioned and the primer sequences are summarized in Table 3; the amount of mRNA detected in MSCs from leiomyomas was calculated as X-fold respect to MSCs from myometrium (expressed as 1) by the $2^{-\Delta\Delta Ct}$ method, where ΔCt=Ct (gene of interest)—Ct (control gene) and Δ (ΔCt) = ΔCt (leiomyomas)—ΔCt (myometrium). X-fold was calculated for the selected genes in all the twelve samples of MSCs. Subsequently, mean ± SD from three independent experiments in triplicates was calculated and displayed.

Since both mRNA levels and ELISA revealed a strong downregulation of Th1/Th17 pathway cytokines, the expression of other Th1/Th17-related soluble factors (IL22, NFKB1, IL23A, STAT3, CCR5, IL17A, IL17RA, CX3CL1, CXCL12, CXCL5) was also assessed by RT-PCR and the amount of mRNA calculated as above described.

Table 3. Primer sequences related to inflammation pathways: Th1, Th2, Th17.

Gene Symbol	Forward	Reverse
IL2	CCTCAACTCCTGCCACAATG	TGTGAGCATCCTGGTGAGTT
IL4	TTTGCTGCCTCCAAGAACAC	GTCGAGCCGTTTCAGGAATC
IL5	GCCATCCCCACAGAAATTCC	CCCCTTGCACAGTTTGACTC
IL6	ATTCTGCGCAGCTTTAAGGA	AACAACAATCTGAGGTGCCC
IL10	GCCAAGCCTTGTCTGAGATG	AAGAAATCGATGACAGCGCC
IL12	AATGTTCCCATGCCTTCACC	CCAATGGTAAACAGGCCTCC
IL13	CATCCGCTCCTCAATCCTCT	CACAGTACATGCCAGCTGTC
IL17A	AGGCAGGAATCACAATCCCA	ACTTTGCCTCCCAGATCACA
IFNγ	GCTCTGCATCGTTTTGGGTT	CACTCTTTTGGATGCTCTGGT
TNFα	GGTGCTTGTTCCTCAGCCTC	AGATGATCTGACTGCCTGGG
GM-CSF	GGACATGGTTTGACTCCCGA	CTTCCTTTCACACACAGGCC
TGβ1	CAAGTGGACATCAACGGGTTC	TGCGGAAGTCAATGTACAGC
IL22	TTGAGGTGTCCAACTTCCAGCA	AGCCGGACGTCTGTGTTGTTA
NFKB1	CACTGCTCAGGTCCACTGTC	CTGTCACTATCCCGGAGTTCA
IL23A	CGTCTCCTTCTCCGCTTCAA	ACCCGGGCGGCTACAG
STAT3	GAGGACTGAGCATCGAGCA	CATGTGATCTGACACCCTGAA
CCR5	CAAAAAGAAGGTCTTCATTACACC	CCTGTGCCTCTTCTTCTCATTTCG
IL17RA	CCCAGTAATCTCAAATACCACAGTTC	CGATGAGTGTGATGAGGCCATA
CX3CL1	GGATGCAGCCTCACAGTCCTTAC	GGCCTCAGGGTCCAAAGACA
CXCL12	TCAGCCTGAGCTACAGATGC	CTTTAGCTTCGGGTCAATGC
CXCL5	TGGACGGTGGAAACAAGG	CTTCCCTGGGTTCAGAGAC

3.6 STUDY 2

3.6.1 Proliferation assay

To assess the proliferation rate, XTT Cell Proliferation Assay (Trevigen, Gaithesbrug MD, USA) was performed on cells after 72 hours of culture for the first 6 passages. The data about yC-MSCs are reported as percentages of the values measured in parallel in oC-MSCs (referred as 100%), over three independent experiments.

3.6.2 Senescence associated β-galactosidase assay

Senescence associated β-gal activity was detected with a senescent cell staining kit (Sigma-Aldrich, Milan, Italy) according to the manufacturer's instructions. 7×10^4 MSCs from old and young cervixes (o-CMSCs and Y-CMSCs) at passage 6^{th} were seeded in a 6-well plate and incubated with the staining solution overnight at 37°C without CO_2. β- Gal was microscopically revealed by the presence of a blue, insoluble precipitate within the cell, the percentage of SA- β-gal positive cells was determined by counting at least 500 cells in each sample and calculating the mean.

3.6.3. PCR array for the senescence

The expression of 86 genes related to senescence was analysed by PCR array (Qiagen, Milan) in MSCs isolated from cervixes derived from young and old patients.

Total RNA was isolated by using Master Script RT-PCR System (5 PRIME, Hamburg, Germany). cDNA synthesis was performed using SABiosciences RT² First Strand Kits, following the manufacturer's instruction. mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method, where ΔCt = Ct (gene of interest)—Ct (control gene) and Δ (ΔCt) = ΔCt (yC-MSCs)—ΔCt (oC-MSCs).

3.6.4. Indirect co-culture with HeLa cells

Human cervix epithelioid carcinoma cell line (HeLa, Sigma Aldrich) was cultured in EMEM (EBSS) + 2mM Glutamine + 1% Non-Essential Amino Acids (NEAA) + 10% Foetal Bovine Serum (Sigma Aldrich). HeLa were co-cultured with MSCs isolated from young and old cervixes.

Table 4. PCR-array plate layout for the senescence pathway.

	1	2	3	4	5	6	7	8	9	10	11	12
A	ABL1	AKT1	ALDH1A3	ATM	BMI1	CALR	CCNA2	CCNB1	CCND1	CCNE1	CD44	CDC25C
B	CDK2	CDK4	CDK6	CDKN1A	CDKN1B	CDKN1B	CDKN2A	CDKN2B	CDKN2C	CDKN2D	CHEK1	CHEK2
C	CITED2	COL1A1	COL3A1	CREG1	E2F1	E2F3	EGR1	ETS1	ETS2	FN1	GADD45A	GLB1
D	GSK3B	HRAS	ID1	IFNG	IGF1	IGF1R	IGFBP3	IGFBP5	IGFBP27	ING1	IRF3	IRF5
E	IRF7	MAP2K1	MAP2K3	MAP2K6	MAP2K14	MDM2	MORC3	MYC	NBN	NFKB1	NOX4	PCNA
F	PIK3CA	PLAU	PRKCD	PTEN	RB1	RBL1	RBL2	SERPINB2	SERPINE1	SIRT1	SOD1	SOD2
G	SPARC	TBX2	TBX3	TERF2	TERT	TGFB1	TGFB111	THBS1	TP53	TP53BP1	TWIST1	VIM
H	ACTB	B2M	GAPDH	HPRT1	RPL0	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

In a 6-well plate, 5 x 10^4 HeLa were seeded at the lower surface of each well and the day after, 5 x 10^4 MSCs derived from young and old cervixes were individually added in the upper surface of a polycarbonate transmembrane filter in a transwell filter system (pore size 0.4 µm, BD Falcon). Cells were co-cultured for 72 hours. After co-cultures, the medium was collected and store at -80°C for ELISA assay and tumor cells were recovered, counted by an automated cell counter and the pellets were freeze down for further analysis. HeLa cells cultured individually served as controls (mock). Data are reported as mean ± SD from three independent experiments.

3.6.5. Expression of selected genes in co-cultured HeLa cells by RT-PCR

RT-PCR, performed as above mentioned, was performed to evaluate the expression of genes associated with specific cellular mechanisms, such as oncogenesis (cMET, cFOS, cJUN), proliferation (mKi67), invasion and migration (MMP11) and angiogenesis (VEGF, CXCL12). Quantification of mRNA expression was calculated with the $2^{-\Delta\Delta Ct}$ method, where ΔCt = Ct (gene of interest) − Ct (control gene) and Δ(ΔCt) = ΔCt (HeLa cells co-cultured with cervix-MSCs) − ΔCt

(HeLa cells cultured alone). The values of the relative expression of genes of interest are referred as mean ± DS, over three independent experiments.

The primer sequences are reported in Table 5.

Table 5. Primer sequences of genes related to oncogenesis, proliferation, invasion and migration and angiogenesis.

Gene Symbol	Forward	Reverse
cMET	TACCACTCCTTCCCTGCAAC	TCATTGCCCATTGAGATCAT
cFOS	AGAATCCGAAGGGAAAGGAA	CTTCTCCTTCAGCAGGTTGG
cJUN	TAACAGTGGGTGCCAACTCA	CCAAGTCCTTCCCACTCGT
mKi67	ACAGAAAAATCGAACTGGGAAA	GTTTATGAAGCCGATTCAGACC
MMP11	TCTCGTGGGTCCTGACTTCT	GTTGTCATGGTGGTTGTACCC
VEGF	CCTCCGAAACCATGAACTTT	ATGATTCTGCCCTCCTCCTTCT
CXCL12	TGAGAGCTCGCTTTGAGTGA	CACCAGGACCTTCTGTGGAT

3.6.6. Analysis of the effects of age and co-culture in secretion of inflammatory cytokines.

Potential effects related to the age of donors on secretion of cytokines involved in inflammation were tested by ELISA test (Multi-Analyte ELISArray kit, Qiagen, Milan, Italy) as above described. Mean ± SD has been calculated for MSCs derived from cervixes of young and old patients over three independent tests and expressed as pg/ml. Subsequently, MSCs were co-cultured with HeLa cells and the amount of the differentially secreted cytokines was re-evaluated. Levels detected in co-cultured HeLa have been reported as a percentage of the levels measured in HeLa cells cultured alone, and data are presented as mean ± SD over three independent tests.

3.6.7. Statistical Analysis

Statistical analysis was performed by using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). All data are presented as mean ± SD. Statistical significance was calculated for two-sample comparisons by Student's t test using SPSS 17.0 software. Statistical significance was analysed for data from at least three independent experiments. P values ≤ 0.05 were defined as statistically significant.

3.7 STUDY 3

The study has been conducted in the laboratory of Division of Cell Matrix Biology and Regenerative Medicine at the University of Manchester. Human biopsies were collected from 3 dystrophic patients at the Wytenshaw Hospital, South Manchester (Manchester).

3.7.1. Sample collection and cell culture

Before these experiments, human Mesoangiobalst from patients were isolated from a donor skeletal muscle sample, using the protocol described by Tonlorenzi et al (Tonlorenzi R et al. 2007). All cells used were from a single DMD donor and had been previously transduced with a lentiviral vector encoding the green fluorescent protein (GFP). These myoblasts were cultured in SKM medium (1 vol 199 medium, 4 vol DMEM, 20% FBS, Gentamycin 50 µg/ml), with Supplement mix (Fetuin 25 µg/ml, hEGF 5 mg/ml, hFGF 0.5 mg/ml, Insuline 5 µg/ml, DEX 0.2 µg/ml). They were grown in 25 cm^2 flasks at 37°C in 5% CO_2. Cells from wild type myoblasts isolated from dystrophic patient (KM) with a mutation in exon 51 and cells with only GFP and GFP plus #51 were also mixed at different ratio: 1/10 and 1/30. Myoblasts with only lentiviral vector and GFP, and myoblasts with lentiviral vector expressing the U7 snRNA, to skip exon 51, and GFP.

3.7.2. Myogenic differentiation

For myogenic differentiation of dystrophic myoblasts, U7#T2AGFP expressing only GFP and corrected myoblasts, U7#51T2AGFP expressing the snRNA, to induce exon skipping, were cultured in Differentiation media + supplements (DMEM 50µg/ml gentamicin, 10 µg/ml insulin e 5µM forskolin to increase the fusion) for 10/14 days in CO_2 5% at 37°.

3.7.3. Immunofluorescence assay

Immunofluorescence assay was performed on 2 x 10^4 wild type Mesoangioblast, Dystrophic myoblast transduced with U7#T2AGFP and corrected myoblasts transduced with, U7#51T2AGFP. Cells plated in a 24 multi-well plate. After 24h in culture, differentiation media was added to induce differentiation (14 days). At the end of 14 days of differentiation, cells were fixed with 4% of paraformaldehyde (PFA) for 20 minutes at 4°C, permeabilize with 200 µl of 1% bovine serum albumin (BSA) + 0,2% Triton X-100 in PBS, for 30 minutes at room temperature (RT). Cells were incubated in the blocking solution (10% Foetal Bovine Serum, FBS + 0,2 % Triton X-100 in PBS) for 30 minutes at RT. Primary antibodies were diluted in 1% BSA + 0.2% Triton X-100 in PBS, to the

following concentrations: 1:2 for Myosin Heavy Chain (MyHc) Antibody Monoclonal Mouse IgG2B Clone # MF20 (Hybridoma bank) and 1:100 for MyOD (Abcam) and were incubated overnight at 4°C in the dark. After washing in 1% BSA + 0.2% Triton, the secondary antibody (Alexa Fluor IgG anti-mouse 568, donkey, Life Technology) was added at the specific dilution of 1:300 in 1% BSA, 0.2% Triton in PBS with Hoechst 33258 (1 µg/ml; Thermo Fisher Scientific) for 1h at RT/4°C in the dark. Samples were visualized with a ZEISS Axio Vert.A1 microscope.

3.7.4. Protein extraction and quantification

After 14 days of differentiation, cells were detached with scrapers, collected in PBS 1X and centrifuged for 15 minutes at 14000 rpm at RT (or at 4°C). PBS was removed and cells were lysed in Dystrophin Lysis buffer (10% SDS, 150 mM NaCl and 50 mM Tris HCl pH 7.5) plus 0.5 M EDTA. To avoid protein degradation Protease Inhibitor Cocktail (1:100) was added at the buffer (Halt™ Protease and Phosphatase Inhibitor Cocktail (100X), Thermo Scientific™). The samples were centrifuged for 15' at RT at 5000 rpm, in order to separate the lipid component from proteins. The supernatant with the protein fraction, was transferred in a new eppendorf tube and put at 98°C for 10 minutes to denaturate the protein.

Protein samples were quantified by Bradford protein assay. The assay was performed in 96-well. A mix containing protein sample diluted 1:10 in H_2O and Coomassie Blue dye (Bio-Rad) diluted 1:5 in H_2O were prepared. Different concentrations of BSA (Albumin Bovine Serum) were used to draw the standard curve. Absorbance was read at 595 nm. A comparison between the sample's absorbance and the standard curve's absorbance allow to determinate the samples' concentration.

3.7.5. Western blot

The loading samples are made mixing: protein lysates (35/50 ng) were mixed with Reducing Agent 10X (1:10), Loading Buffer 4X (1: 10) and Nuclease-free H_2O to make volume. Samples are denaturated at 98°C for 10 minutes. Samples were loaded for fractionating on 4-12% NuPAGE novex precast gel Bis- Tris Gel (Invitrogen). The gel was run in NuPAGE MOPS SDS Running Buffer 1X in H_2O for 2h 30' at 100 V, at RT. Proteins were blotted on a nitrocellulose blotting using NuPAGE Transfer Buffer 20X (1:20 in Milliq water Ultrapure) in methanol 10% for 1h 30' at constant 400 mA/30 V.

After transfer, membranes were incubated in Ponceau S Solution (Sigma) for 1' at RT (to visualize that the proteins have been properly transferred into the membrane). Membranes were washed in fresh TBS 1X + Tween 20 (TBST) and incubated in blocking solution (5% milk) in TBST for 1h in agitation at RT. Primary antibody MF20 (Hybridoma bank) 1:4 and Dystrophin (Mandra 17) 1:250 was added in fresh, overnight at 4°C in agitation. After the incubation membranes were washed 3 times with TBS-Tween. Secondary antibody, Polyclonal Goat Anti-Mouse HRP coniugated (Dako) 1:1000 (in 5% milk in TBST) were added to the membranes and incubated for 1h in agitation at RT. They were washed 3 times with TBS-Tween after incubation. GAPDH, (Abcam 1: 500) was used as housekeeping to normalize. As negative control, protein lysates from healthy patient were used. Immunoreactive signal was detected with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermoscientific) in a developmental room by using automatic X-ray film processor JP-33. Dedicated Uvitech software was used to analyzed the data and quantify the signal.

4. Results

4.1 STUDY 1

4.1.1 Cell isolation and characterization

Leiomyoma and normal myometrium samples from the same 12 patients were used to establish cell culture. After few passages, cells appeared homogeneous, with a mesenchymal-like morphology, (Fig. 8a) and also the cytofluorometric analysis revealed the presence of just one cell population.

All subsequent experiments were performed separately on each cell sample. Since no differences were detected among the samples from the two tissue groups, no pairwise analysis was necessary and values are the average of 12 samples. Doubling time was stable up to the 3rd passage and almost identical in the two cell groups; it then increased, the increment being greater in myometrium cells (Fig. 8b).

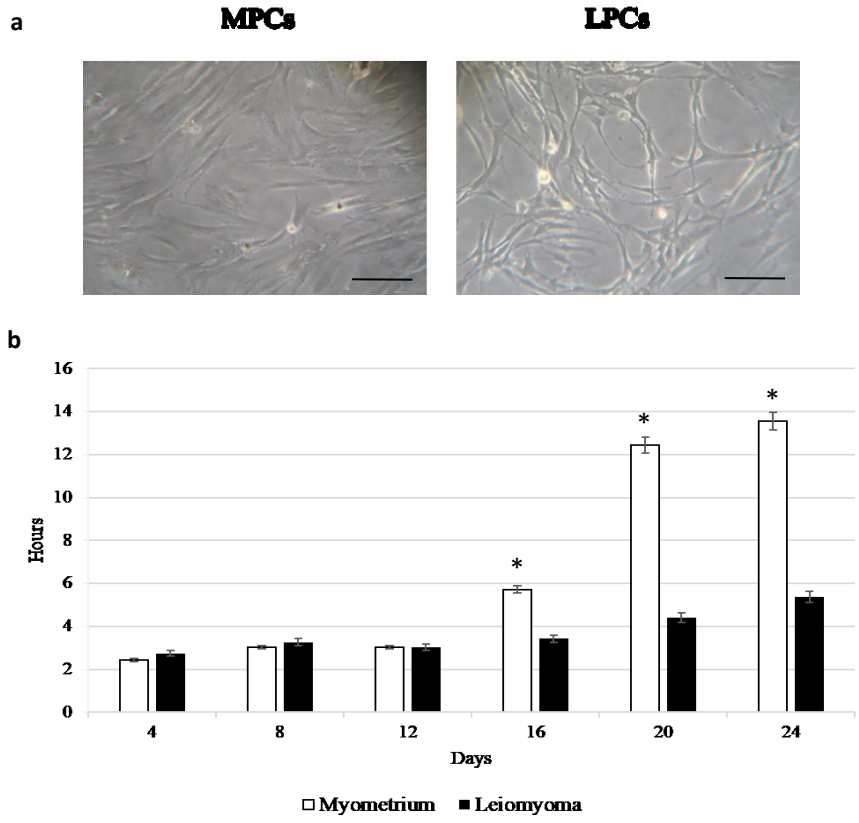

Figure 8. Cell morphology and doubling time. (a) Phase-contrast images of myometrium progenitor cells (MPCs, left) and leiomyoma progenitor cells (LPCs, right) at 2nd passage of culture. Scale bar = 100 µm. (b) Doubling time was calculated over 21 days (6th passage). Data are mean ± SD of experiments performed on 12 samples. ∗p < 0 05 LPCs versus MPCs.

Evaluation of the stemness criteria identified by Dominici et al. demonstrated that cells adhered to plastic and that they were strongly positive for CD73, CD90, and CD105 and negative for HLA-DR, CD14, CD19, CD34, CD45, and for the key marker CD9. Cells were also capable of differentiating to osteogenic, chondrogenic, and adipogenic lineages (Fig. 9). Both cell types expressed NANOG, OCT4, SOX2, and KLF4, tested by RT-PCR, with a higher expression in leiomyoma cells (Fig. 10). Since all experiments confirmed their undifferentiated status, the two cell types were designated, respectively, as myometrium progenitor cells (MPCs) and leiomyoma progenitor cells (LPCs).

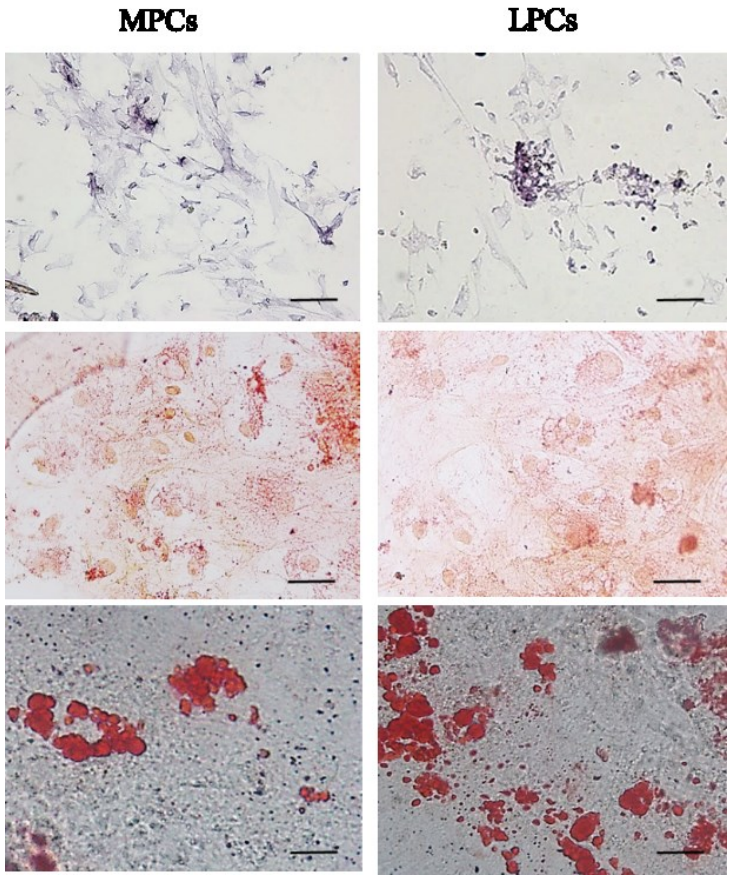

Figure 9. Multilineage differentiation of MPCs and LPCs. Representative images of differentiation experiments. Osteogenic differentiation: ALP staining (top); chondrogenic differentiation: acid mucopolysaccharide coloration with Safranin-O (center); adipocyte differentiation: Oil Red staining (bottom). No differences were noted among different leiomyoma and myometrium samples. Scale bar = 100 µm. MPCs: myometrium progenitor cells; LPCs: leiomyoma progenitor cells.

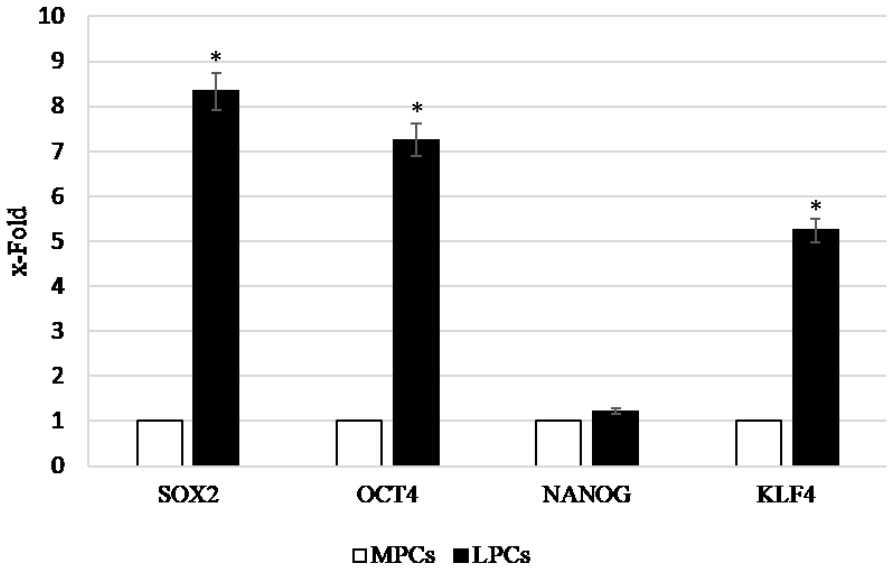

Figure 10. OCT4, SOX2, NANOG, and KLF4 expression. Selected markers of self-renewal and differentiation potential (OCT4, SOX2, NANOG, and KLF4) were evaluated by RT-PCR. The expression levels measured in LPCs are considered as X-fold with respect to MPCs (considered as 1). Data are mean ± SD of analyses performed in 12 different MPC and LPC cultures, upon three independent experiments in triplicates. ∗p < 0 05 LPCs versus MPCs.

4.1.2. MDR1, α-SMA, Collagen Type 1, and Fibronectin Expression

Western blotting demonstrated a reactive band (molecular weight 170 kDa, corresponding to MDR1) in the MPC and LPC lanes. Densitometric analysis revealed that MDR1 expression was higher in LPCs (Fig. 11), whereas no signal was detected in NHLFs (negative control).

MPCs and LPCs were positive for *α*-SMA, collagen type 1, and fibronectin on IIF (Fig. 12) and IIC (Fig. 13), without significant differences between the two cell types. Although more than 90% of MPCs and LPCs were strongly positive for all three proteins, the staining for collagen type 1 was fainter than the other two.

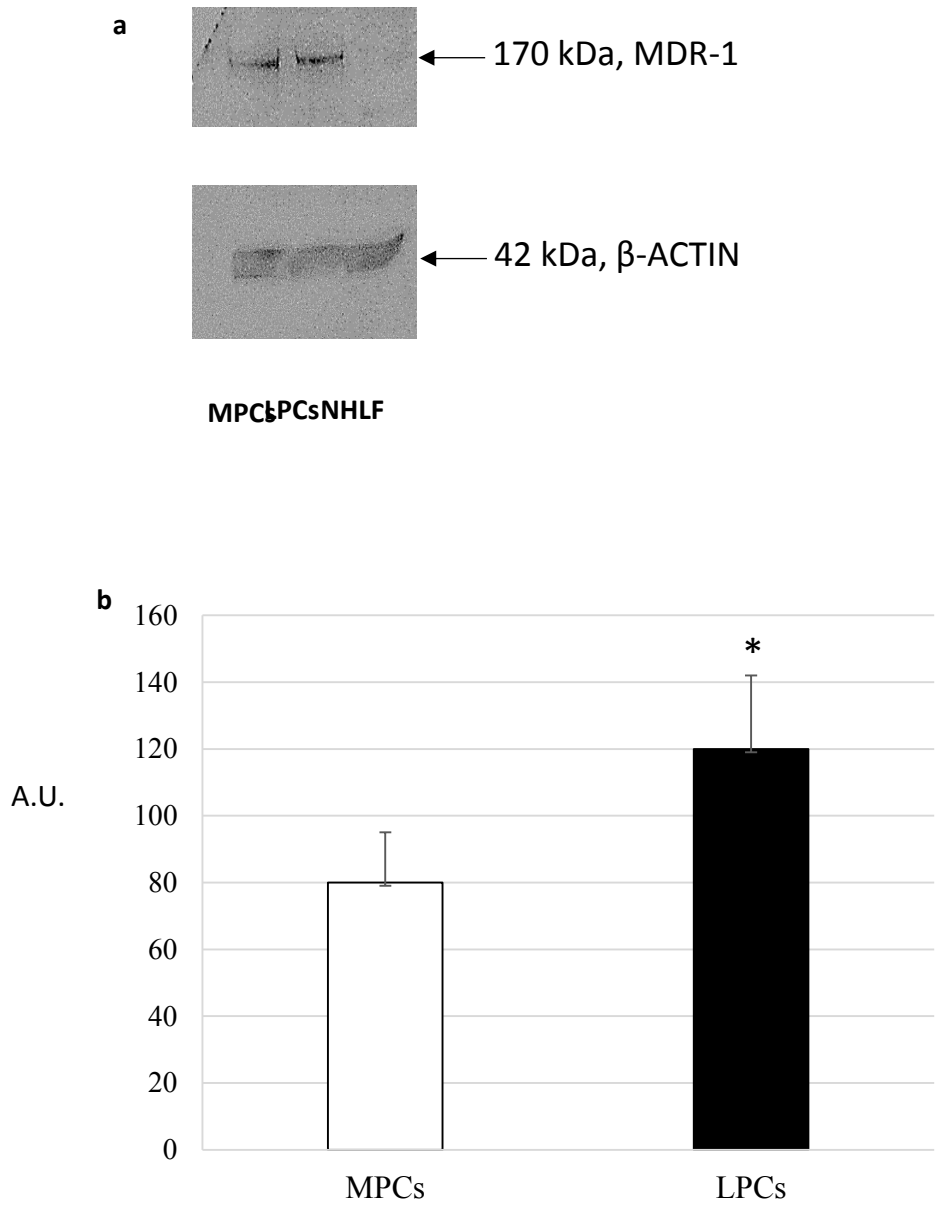

Figure 11. Western blots and densitometric analyses of MDR1 expression. (a) representative Western blot gels showing the bands of MDR1 and of the endogenous control β-actin. (b) densitometric analyses of the immunoreactive bands (quantified as MDR1/β-actin bands in corresponding samples and expressed as arbitrary units, A.U.). Data are mean ± SD of analyses performed in MPCs and LPCs from the 12 patients. ∗p < 0 05 LPCs versus MPCs. MPCs: myometrium progenitor cells; LPCs: leiomyoma progenitor cells.

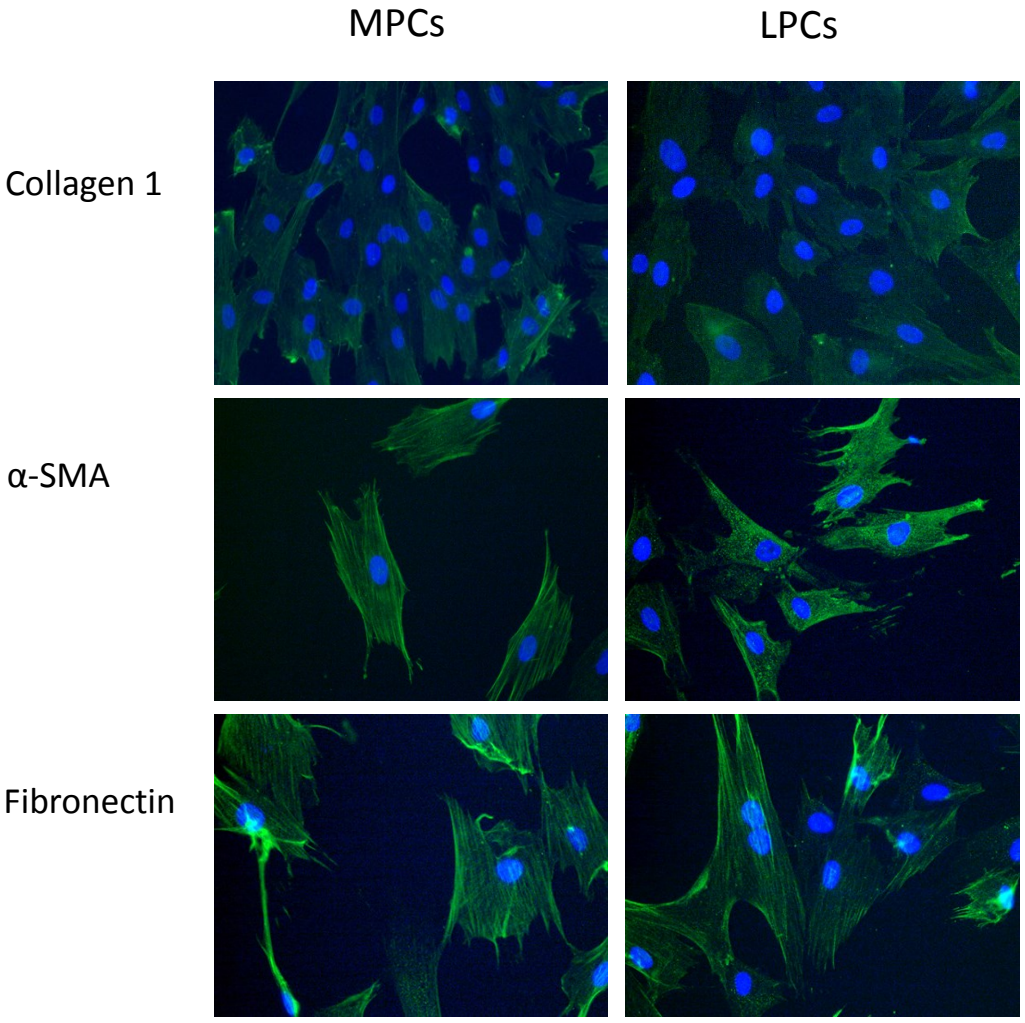

Figure 12. Indirect immunofluorescence analysis of α-SMA, collagen type 1, and fibronectin. A secondary FITC-conjugated antibody was used after incubation with the primary antibodies. Nuclei were counterstained with Hoechst 33342. Myometrium progenitor cells (MPCs) and leiomyoma progenitor cells (LPCs) showed a similarly strong positivity for α-SMA and fibronectin, whereas collagen type 1 expression was fainter. Differences between MPCs and LPCs were not significant (×200 original magnification).

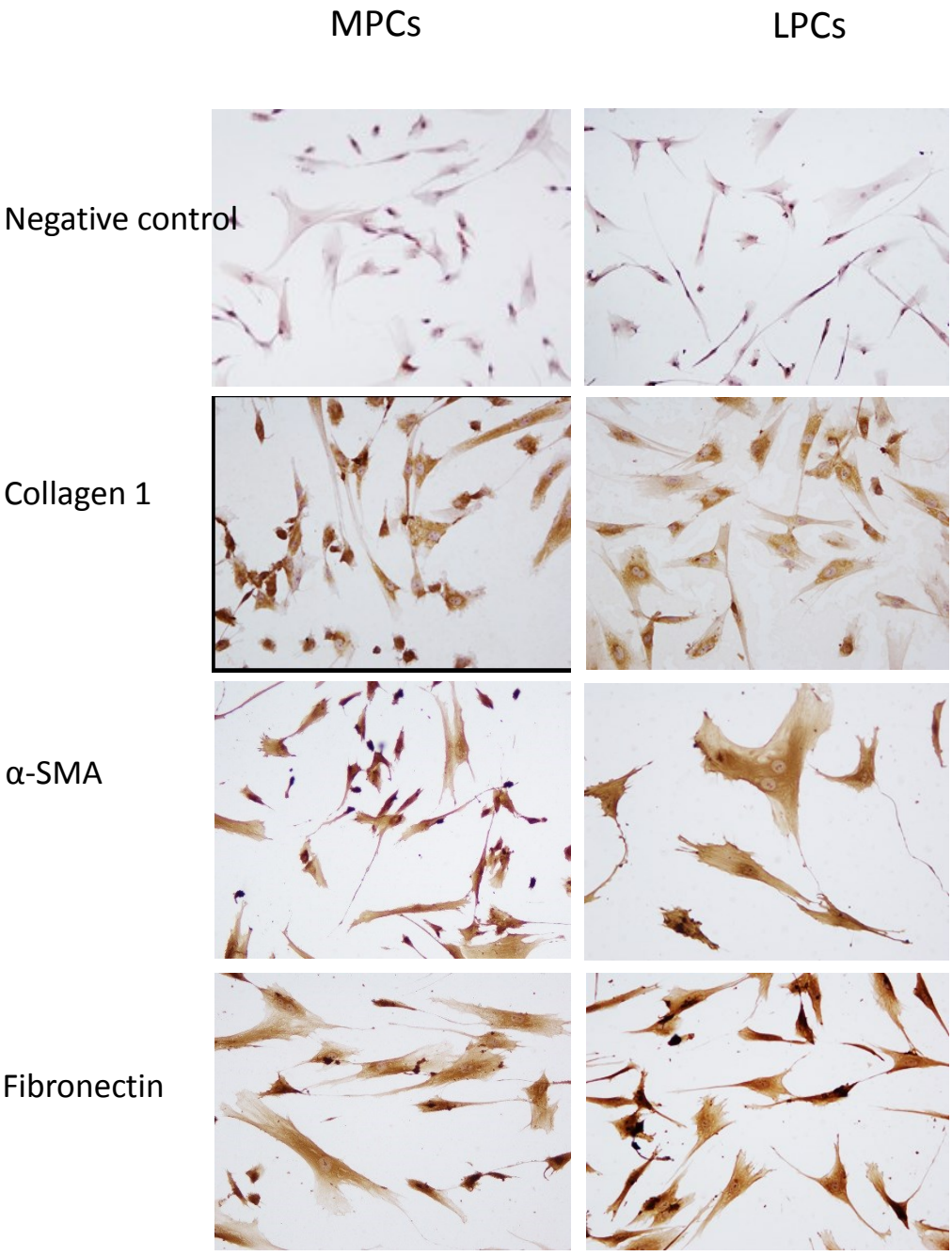

Figure 13. Immunocytochemical analysis of α-SMA, collagen type 1, and fibronectin. Compared to the negative control (secondary antibody alone), the primary antibodies induce brownish staining in myometrium progenitor cells (MPCs) and leiomyoma progenitor cells (LPCs). The reaction was weaker for collagen type 1 than for α-SMA and fibronectin. Differences between MPCs and LPCs were not significant (immunoperoxidase, ×400 original magnification).

4.1.3. Expression Profile of Inflammatory Cytokines

The expression and secretion of inflammation-related cytokines were evaluated by ELISA (Fig. 14). Compared to MPCs, LPCs exhibited significantly ($p < 0\ 05$) higher levels of Th2 pathway cytokines (IL4, IL5, IL10, and IL13), with IL10 showing a 40% increase, and significantly ($p < 0\ 05$) lower levels of Th1/Th17 cytokines (IL6, IL12, IL17A, IFN-γ, G-CSF, and TGF-β1). Finally, IL2 and TNF-α expression was not significantly different between MPCs and LPCs. Since both mRNA levels and ELISA revealed a strong downregulation of Th1/Th17 pathway cytokines in leiomyoma, the expression of other soluble factors belonging to these pathways was assessed by RT-PCR and found to be lower in LPCs (Fig. 15).

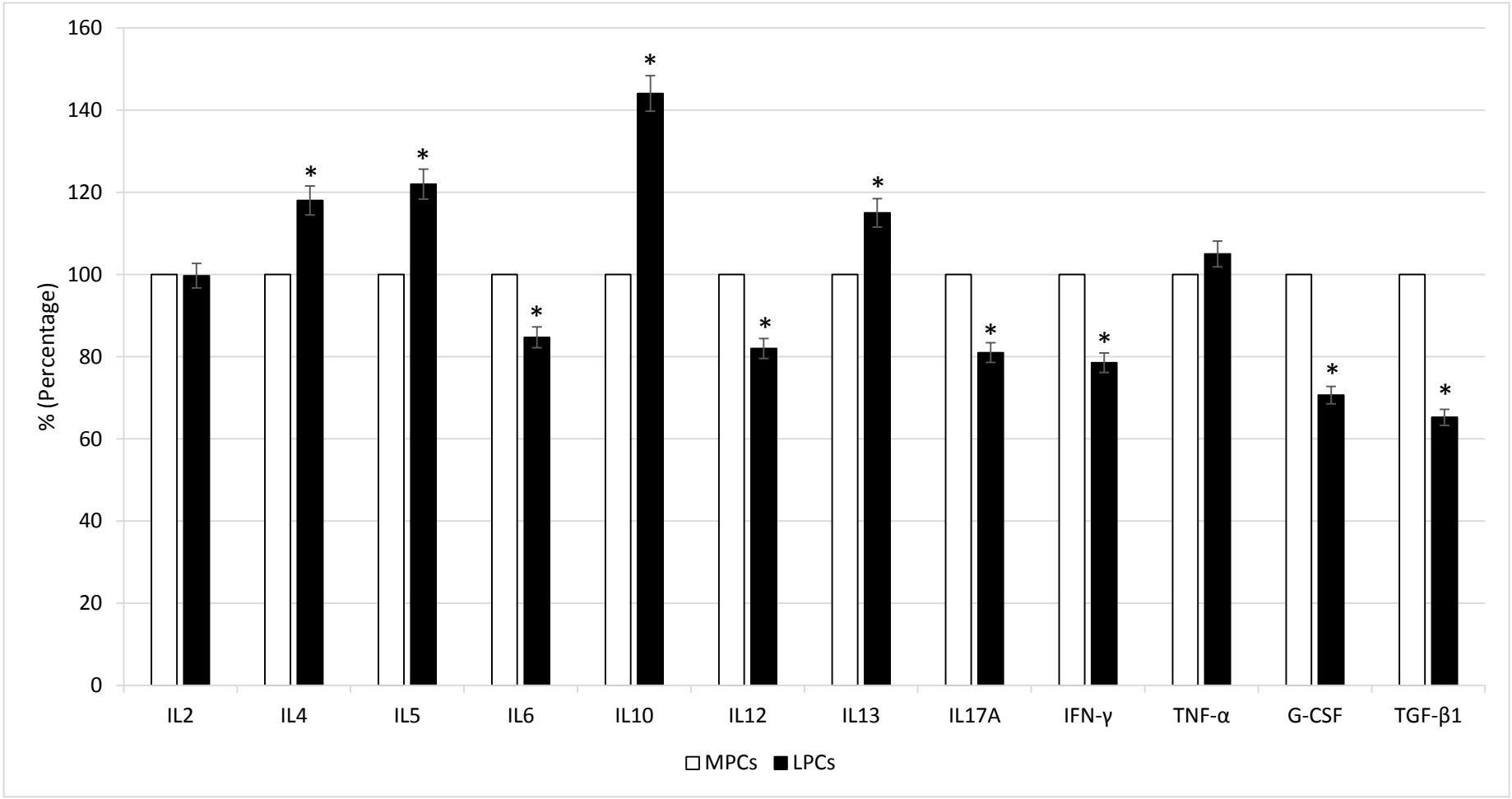

Figure 14. Expression of selected cytokines in myometrium progenitor cells (MPCs) and leiomyoma progenitor cells (LPCs). ELISA test; the levels measured in MPCs were considered as 100% and those detected in LPCs accordingly calculated; $*p < 0.05$ LPCs versus MPCs.

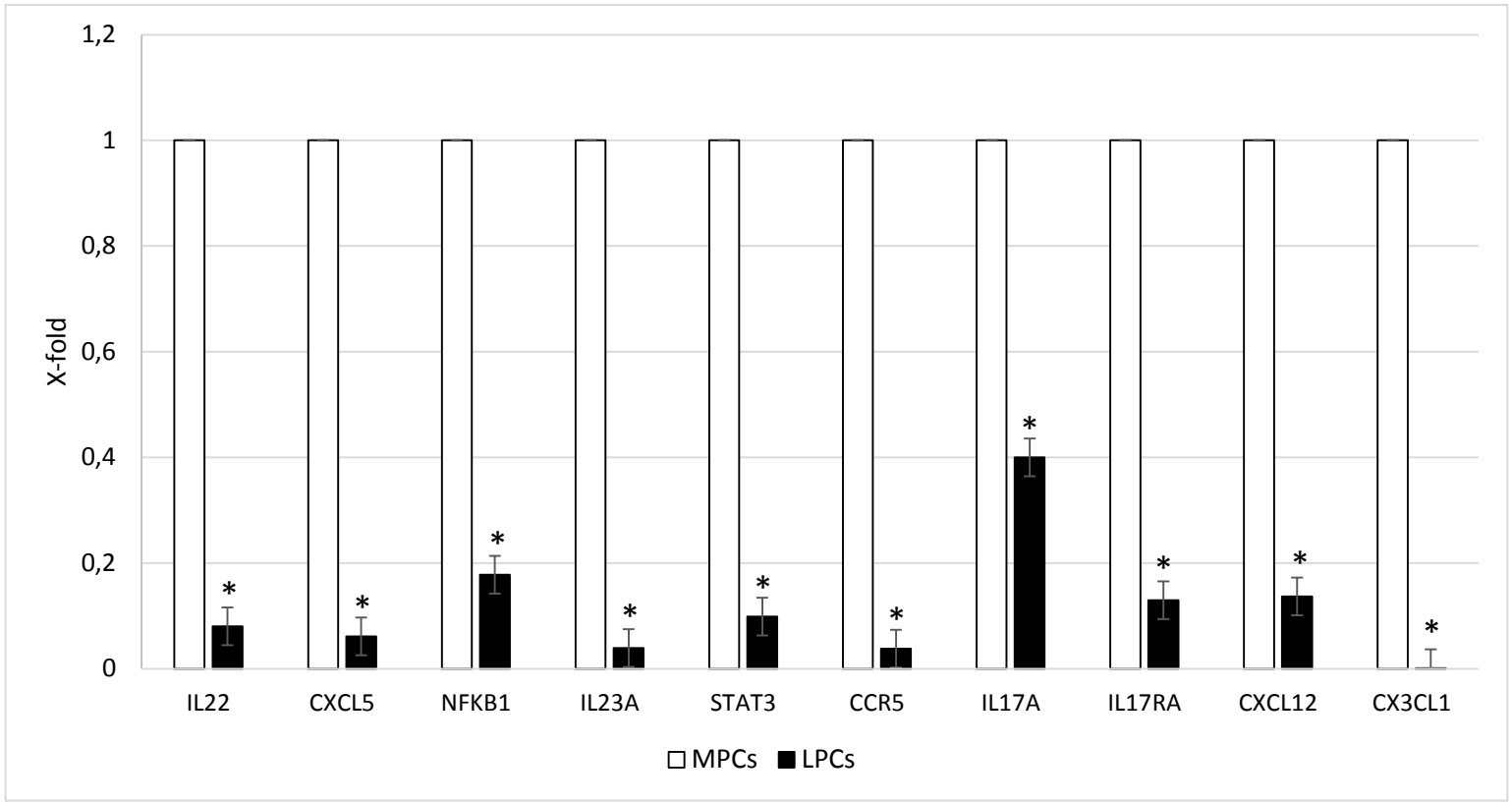

Figure 15. Expression of selected cytokines in myometrium progenitor cells (MPCs) and leiomyoma progenitor cells (LPCs). Quantification of mRNA expression in MPCs and LPCs was calculated with the $2^{-\Delta Ct}$ method, where $\Delta Ct = Ct$ (gene of interest) $- Ct$ (housekeeping gene). ΔCt was calculated for the selected genes on 12 different cultures. Subsequently, mean ± SD from three independent experiments in triplicates was calculated for LPCs and displayed. $*p < 0.05$ LPCs versus MPCs.

4.2. STUDY 2

4.2.1. Cell isolation and characterization

Cell cultures (from cervix of young and old patients) showed a fibroblastoid morphology (Fig. 16a).

Both cell types expressed all the stemness genes tested by RT-PCR, with a higher expression in cells derived from cervix of young patients. Considering as 1 the expression detected in MSCs from old cervix, the same genes are from 1.74±0.21 (OCT4) up to 12.05±0.53 (KLF4) fold higher in MSCs from young patients (Fig. 16b). This trend was confirmed at protein level, as revealed by the cytofluorimetric analysis (Fig. 16c).

Cells were also capable of differentiating towards osteogenic, chondrogenic, and adipogenic lineages (Fig. 17). Since these cells satisfied all the Dominici criteria, we considered them as young cervix MSCs (yC-MSCs) and old cervix MSCs (oC-MSCs). Evaluation of the stemness criteria identified by Dominici et al. demonstrated that cells were strongly positive for CD73, CD90, and CD105 and negative for HLA-DR, CD14, CD19, CD34, CD45, and for the key marker CD9 (Fig. 18).

XTT assay was performed after 72 hours of cultures of y- and oC-MSCs for the first six passages. (Fig. 19a). yC-MSCs displayed a higher proliferation rate than oC-MScs (referred as 100%).

Looking to the data from proliferation assay and with the aim to assess potential differences between MSCs derived from cervixes of young and old patients, cellular senescence was tested. Senescence was evaluated in yC-MSCs and oC-MSCs at passages 6 and interestingly the number of blue cells (indicating senescence) was notably different between yC-MSCs and oC-MSCs (11% and 23% respectively) (Fig. 19b).

Doubling time was stable up to the 10th passage (42 days) and almost identical in the two cell groups (yC-MSCs: 2.84 ±0.78; oC-MSC: 2.86±0.78). It then gradually increased from the 11th to the 13th passage (yC-MSCs: 5.62 ±1.35; oC-MSC: 7.02±2.06); at the 14th passage (62 days) it reached the highest values (yC-MSCs: 19.41 ±2.74; oC-MSC: 26.28±2.75) and cells became enable to proliferate (Fig. 20). Looking to the data from doubling time and with the aim to assess potential differences between MSCs derived from cervixes of young and old patients, cellular senescence was tested.

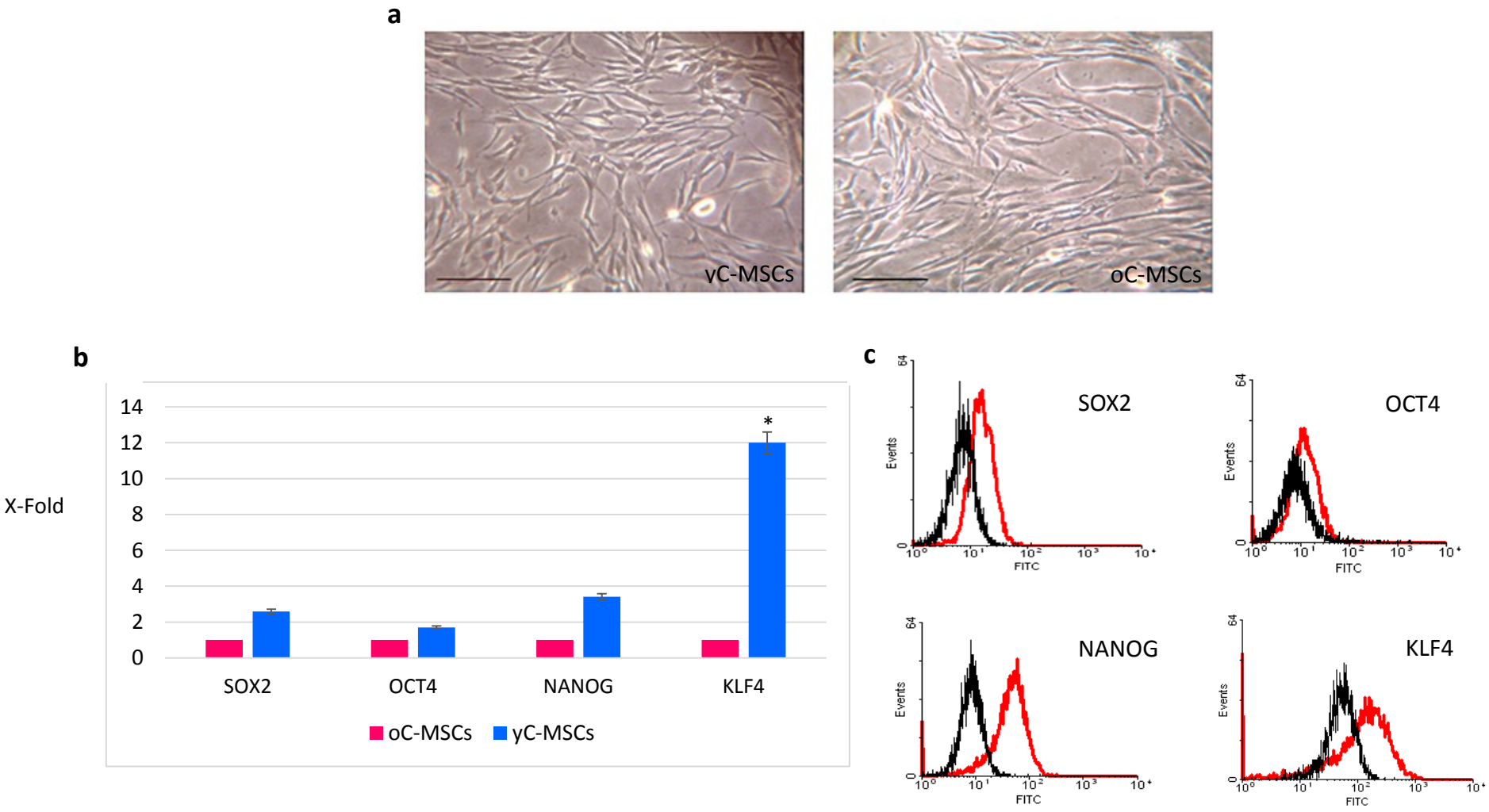

Figure 16. Morphology and expression of genes related to stemness. (a) Phase-contrast images of MSCs derived from cervix of the younger (yC-MSCs, left) and older patient (oC-MSCs, right) after 14 days of culture. Scale bar, 100 µm. The expression of selected markers related to self-renewal and differentiation potential (OCT4, SOX2, NANOG, KLF4) was evaluated by RT-PCR (b) and cytofluorimetry (c). (b) Levels of expression detected in yC-MSCs are referred as X-fold respect to oC-MSCs (expressed equal to 1). Data are means ± SD from analyses performed on three separate experiments in triplicates. * $p < 0.05$ yC-MSCs versus oC-MSCs. (c) Representative FACScan analyses of cell-surface antigen expression, as indicated. Black histograms refer to oC-MSCs, red histograms to yC-MSCs.

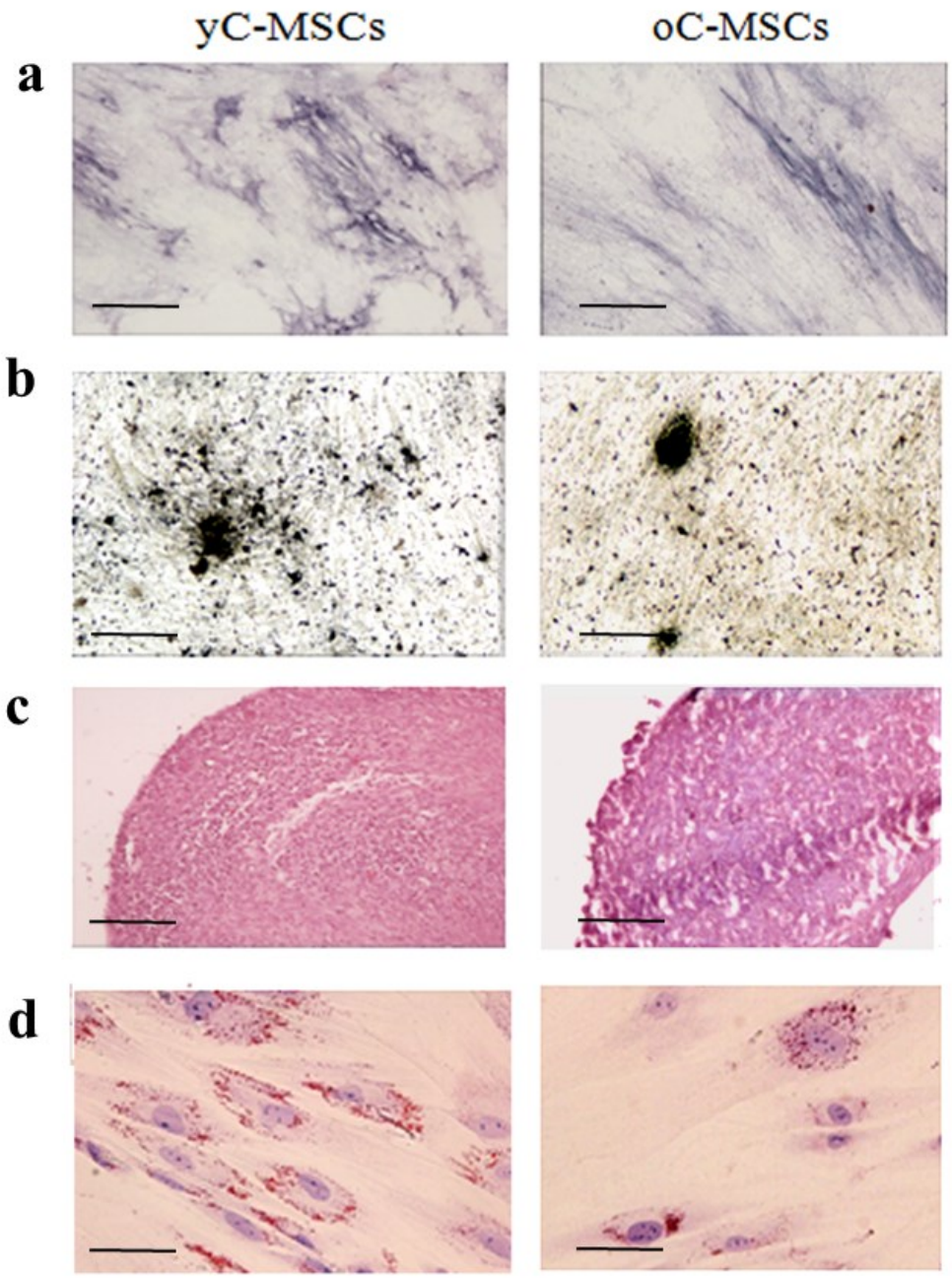

Figure 17. Multilineage differentiation of MSCs derived from young (yC-MSCs) and old (oC-MSCs) cervix. Representative images of osteogenic differentiation assessment by ALP reaction (a, Scale bar 100µm) and von Kossa staining (b, Scale bar 100µm); chondrogenic differentiation by Safranin-O coloration (c, Scale bar 200µm); adipocyte differentiation by Oil red staining (d, Scale bar 30µm).

.

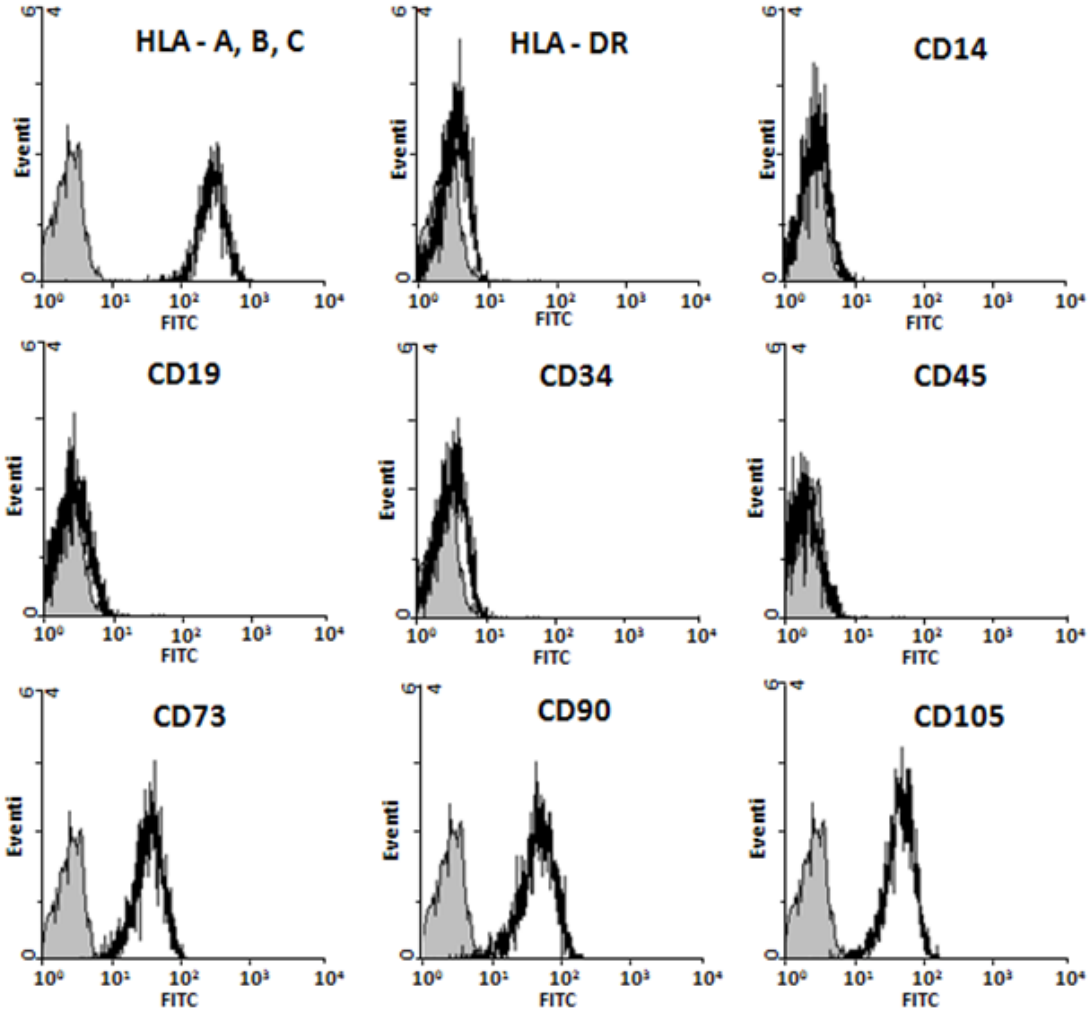

Figure 18. Representative FACScan analyses of cell-surface antigen expression, as indicated. No significant differences were noted between yC-MSCs and oC-MSCs. Solid gray fluorescence histograms, negative control (FITC-labeled IgG1 isotype control).

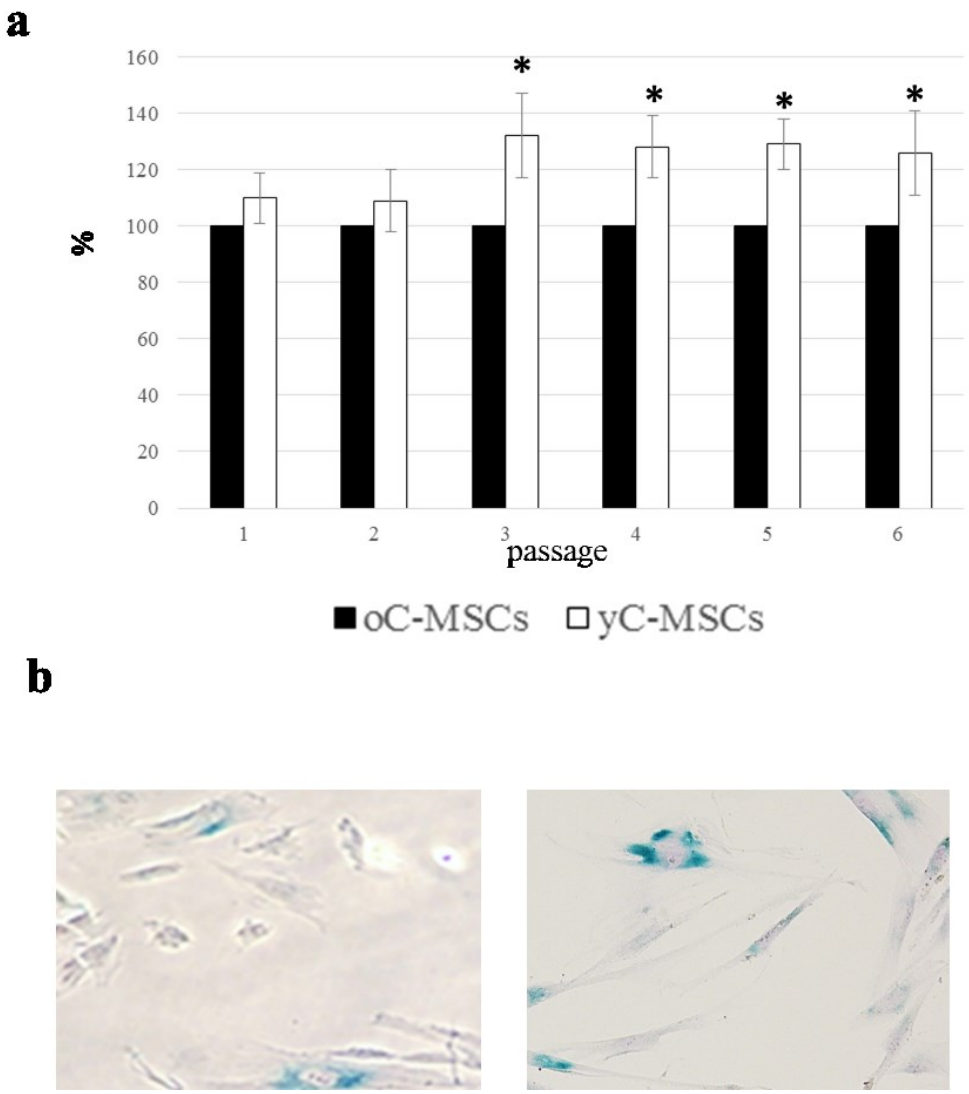

Figure 19. XTT assay and Senescence-associated beta-galactosidase assays of MSCs derived from young (yC-MSCs) and old (oC-MSCs) cervix MSCs. (a) XTT assay performed after 72 hours of culture for the first 6 passages. Data from oC-MSCs are expressed as 100% and those for yC-MSCs accordingly calculated. Data are reported as mean±SD SD of three independent experiments. * $p < 0.05$ yC-MSCs versus oC-MSCs. (b) Representative micrographs obtained with phase contrast microscopy showing SA-β-Gal activity in cells at passage 6th.

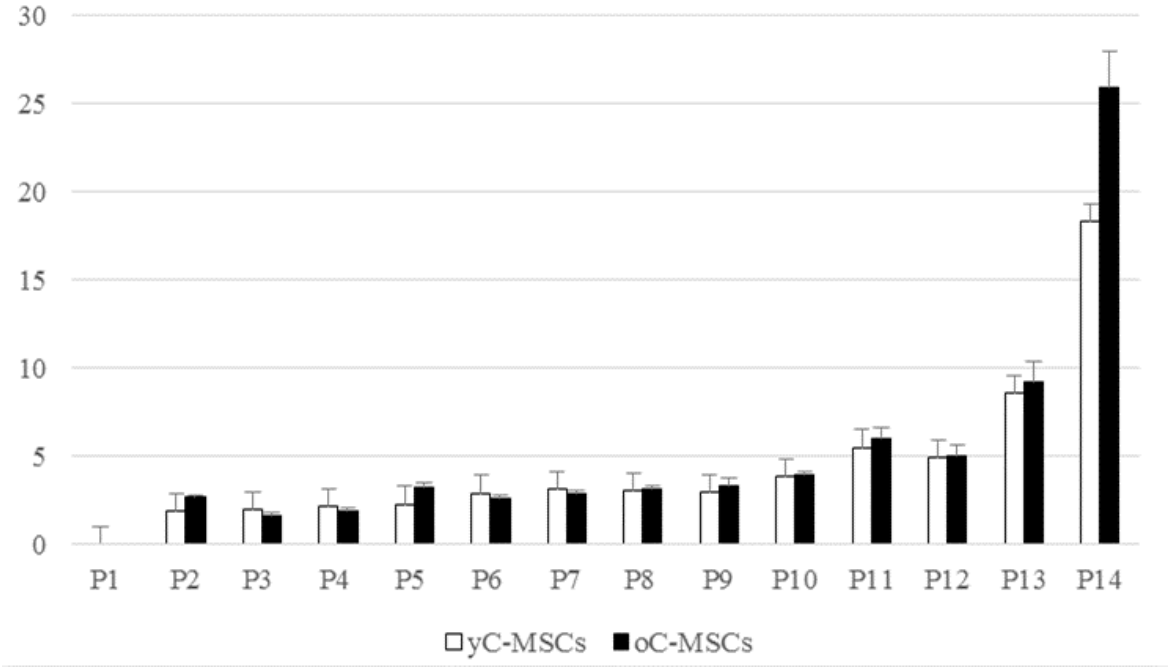

Figure 20. Doubling time was calculated over 62 days (14th passage) between yC-MSCs and oC-MSCs.

4.2.2. PCR Array for senescence in oC-MSCs and yC-MSCs

The expression of more than 80 genes related to senescence was analysed by PCR and expressed as 1 in oC-MSCs and accordingly as x-fold in yC-MSCs. The expression of all genes revealed a dysregulation in yC-MSCs compared to oC-MSCs. A cut-off of 3-fold was chosen for the significance; the expression of CCNA2, CCNB1, CDC25, CHEK1, E2F1, TERT, PCNA was significantly higher in yC-MSCs than in oC-MSCs, while CDKN2A, CCNE1, NOX4 and SOD2 were downrelugated in yC-MSCs compared to oC-MSCs (Fig. 21).

4.2.3. Proliferation of HeLa cells after co-cultures with MSCs

Tumour cells were recovered from co-cultures and cell number assessed by an automated cell counter. Compared with the mock sample, only HeLa cells co-cultured with MSCs from cervixes of old women had a significantly ($p<0.05$) higher proliferation rate (Fig. 22).

4.2.4. Gene expression

The expression of selected genes was analyzed by RT-PCR in HeLa cells before (mock) and after co-cultures with yC-MSCs and oC-MSCs. Compared to mock HeLa (calculated as 1), co-cultured HeLa exhibited significantly ($p<0.05$) higher levels of gene expression related to oncogenesis (cFOS, cJUN, cMET), invasion and migration (MMP11), proliferation (mKI67) and angiogenesis (VEGF, CXCL12). These variations were significantly ($p<0.05$) more evident in HeLa co-cultured with oC-MSCs than with yC-MSCs (Fig. 23).

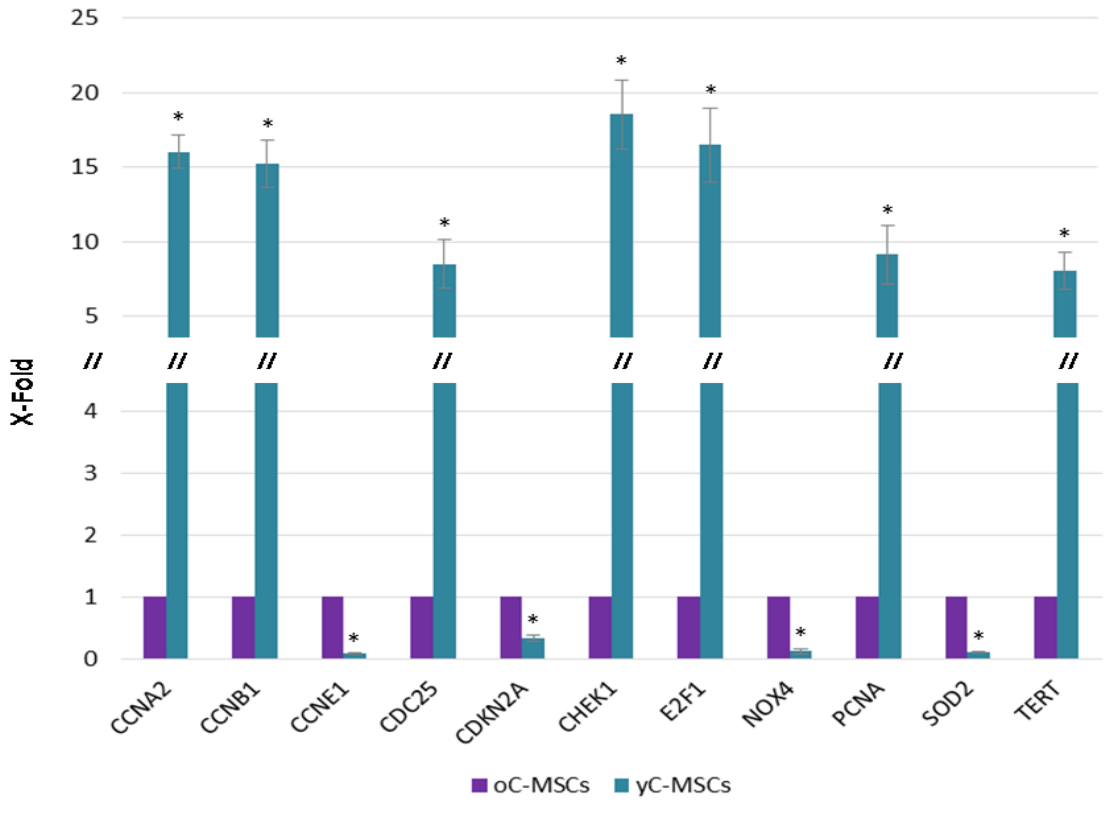

*Figure 21. Expression of genes related to senescence. The expression of 86 genes referred to senescence was evaluated by PCR-Array. Choosing a cut-off of 3-fold, CCNA2, CCNB1, CDC25, CHEK1, E2F1, TERT, PCNA were upregulated in yC-MSCs compared to oC-MSCs, while CDKN2A, CCNE1, NOX4 and SOD2 were less expressed. Data are means ± SD from analyses performed on three separate experiments. * p < 0.05 yC-MSCs versus oC-MSCs.*

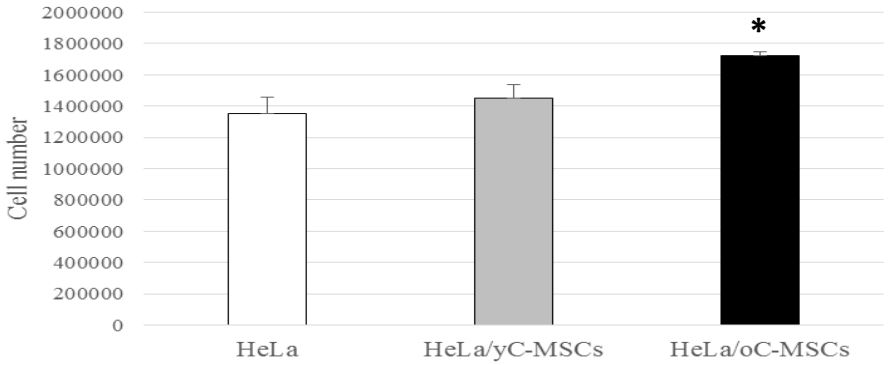

Figure 22. Proliferation and expression of genes related to tumorigenesis in HeLa cells after indirect-co-culture with MSCs. HeLa cells were indirect co-cultured for 72 hours with yC-MSCs (HeLa/yC-MSCs) or oC-MSCs (HeLa/oC-MSCs). HeLa cultured alone were used as control. Proliferation was assessed by an automated cell counter * p < 0.05 HeLa/yC-MSCs or HeLa/oC-MSCs versus HeLa; § p < 0.05 HeLa/yC-MSCs versus HeLa/oC-MSCs.

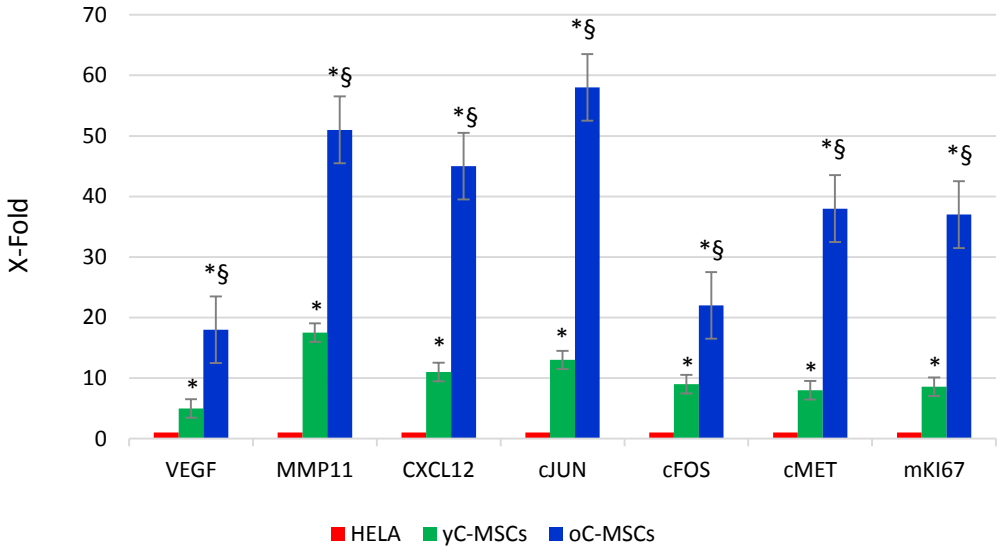

Figure 23. Expression of selected genes related to oncogenesis, invasion and migration, proliferation and angiogenesis in HeLa cells after co-cultures with the yC-MSCs and oC-MSCs. Levels of expression are referred as X-fold respect to individually cultured HeLa (express as 1). *p<0.05 HeLa/yC-MSCs or HeLa/oC-MSCs versus HeLa; § p < 0.05 HeLa/yC-MSCs versus HeLa/oC-MSCs.

4.2.5. Expression profile of inflammatory cytokines

The secretion of inflamed-related cytokines was evaluated by ELISA. Firstly, the detection was performed in yC-MSCs and oC-MSCs (Fig. 24) to identify potential age-related differences. Independently from age, the most secreted cytokines were IL6 and TGF-β, followed by IL2, IL10 and TNF-α. Compared to oC-MSCs, MSCs from cervixes of young patients exhibited significantly ($p<0.05$) higher levels of IL2, IL6, IL10, IL12, IFN-γ and TGF-β. The expression of IL4, IL5, IL13, IL17A, TNF-α and G-CSF was not significantly different between oC-MSCs and yC-MSCs. Subsequently, C-MSCs were co-cultured with HeLa cells and the secretion of the differently expressed cytokines was measured in HeLa mock (expressed as 100%) and in co-cultured HeLa (accordingly calculated). Compared to mock sample, HeLa co-cultured with yC-MSCs showed a significantly ($p<0.05$) increased secretion of IL6, IL10 and TGF-β, where the production of IL2, IL12 and IFN-γ did not change. Co-cultures with oC-MSCs produce a significant increase only in the secretion of TGF-β (Fig. 25).

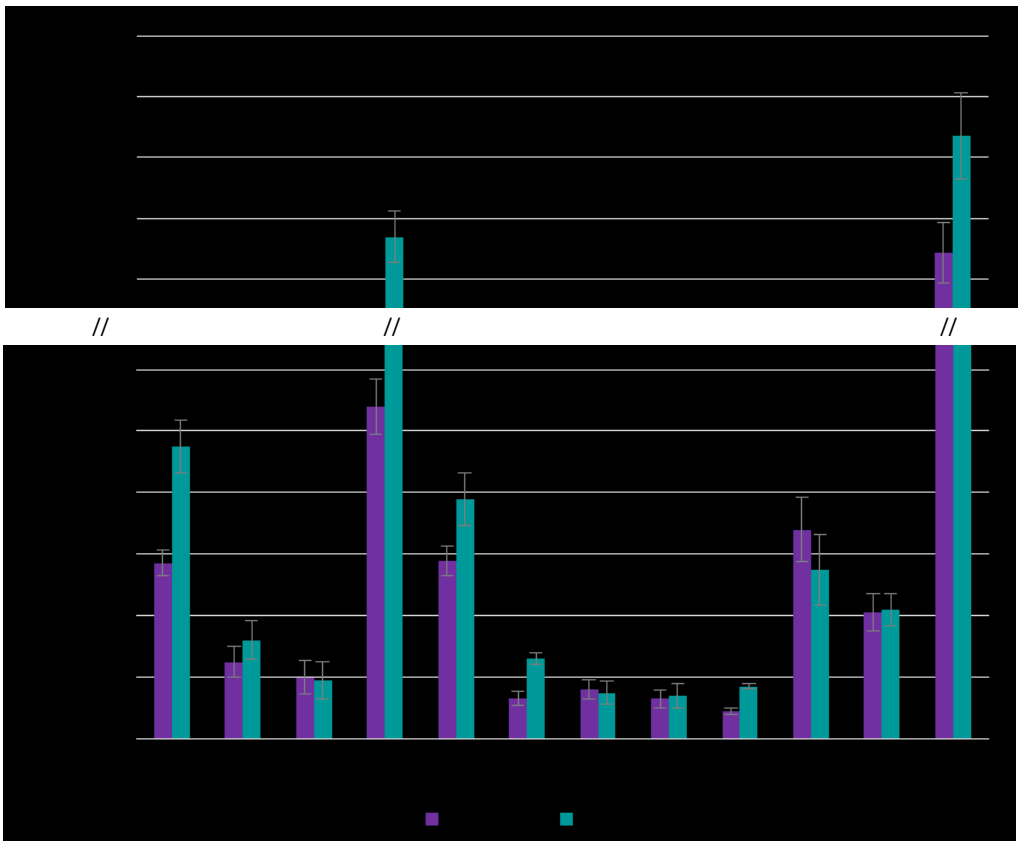

Figure 24. Detection of secreted cytokines in yC-MSCs and oC-MSCs by ELISA before co-cultures with HeLa cells. Histograms displaying the levels of the same cytokines detected after indirect co-cultures with HeLa cells for 72 hours. The level of each cytokine is shown as % (mean ± SD from three independent experiments) of the level detected in control HeLa cells. * $p < 0.05$ HeLa/yC-MSCs or HeLa/oC-MSCs versus HeLa; § $p < 0.05$ HeLa/yC-MSCs versus HeLa/oC-MSCs.

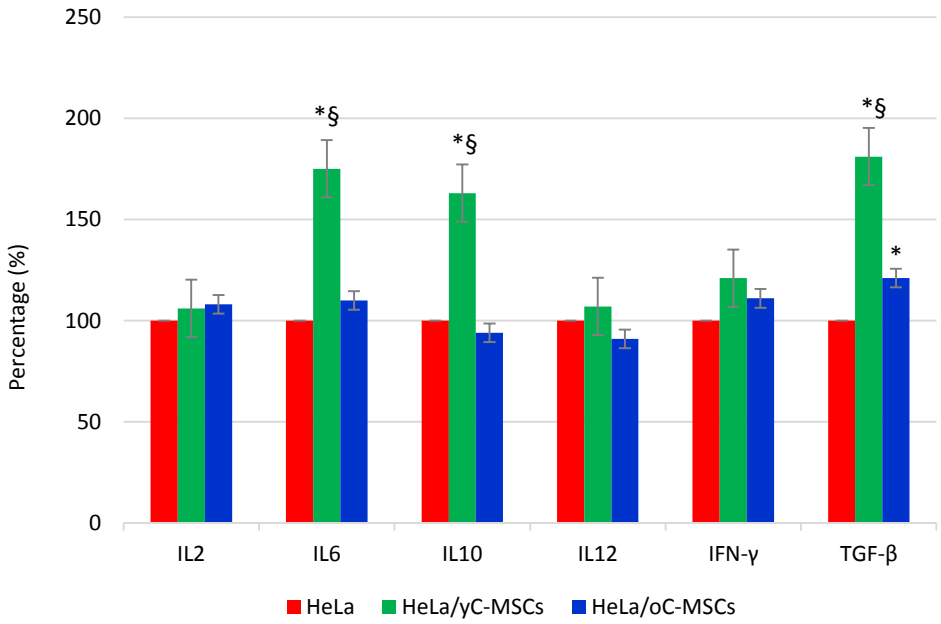

Figure 25. Detection of secreted cytokines in yC-MSCs and oC-MSCs by ELISA after co-cultures with HeLa cells. The level of each cytokine is shown as % (mean ± SD) of the level detected in control HeLa cells (referred as 100%). * $p < 0.05$ HeLa/yC-MSCs or HeLa/oC-MSCs versus HeLa; § $p < 0.05$ HeLa/yC-MSCs versus HeLa/oC-MSCs.

4.3. STUDY 3

4.3.1. Cell culture and myogenic differentiation

Myoblasts were successfully grown in culture and they start to differentiate 48h after adding the supplemented medium for differentiation (as shown in fig. 26). In figure 17 it is possible to observe in the bottom lane that at 72h the myoblasts start to fuse forming multinucleated fibers, the myotubes. At 7 days myoblasts are fused and the size of myotubes continue to increase until day 14.

4.3.2. Determination of expression of MyHC and DyS on differentiated cells

After 14 days of differentiation, cells are blocked and immunofluorescence assay confirmed the expression of MyHC (myogenic marker). In figure 27 it is showed in red the expression of Myosin Heavy Chain (MyHC) in all three samples. The presence of multinucleate fibers highlighted a high level of fusion, important for the cross correction induced by snRNA.
Dystrophic Myoblasts not transduced (No virus) and dystrophic myoblasts transduced with scramble (T2AGFP) did not show expression of Dystrophin protein while myoblasts transduced with lentiviral vector expressing snRNA (U7#51T2AGFP) displayed expression of Dystrophin protein.

4.3.3. Western blot analysis

Western blot analysis was performed to quantify the amount of dystrophin expressed. The results of WB on U7#51T2AGFP and wild type (WT) biopsy samples are shown in Figure 28. MyHC was expressed in all samples indicating that all cells were differentiated, and GAPDH highlighted that the same amount of protein was loaded in all samples. Dystrophin protein was only detected in the U7#51T2AGFP sample and WT Biopsy sample. A comparison between the different concentrations (1:10, 1:30) of U7#51T2AGFP and WT protein was performed in order to quantify the amount of dystrophin produced after the correction. In details, the 1 means the positive control, and in this case it is a biopsy from an healthy patient (no DMD), 1:10 and 1:30 indicate the two different ratios between the mixed cells corrected and cells un-transduced and the column in the middle, which divide the gel into two parts, is the negative control (cells derived

from the dystrophic patient) where no signal was detected. Dystrophin was strongly expressed in the WT biopsy (column 1), mildly expressed in the 1:10 ratio of U7#51T2AGFP and WT protein (column 2) and partially in 1:30 ratio (column 3) (figure 28). In row 2 are highlighted the positive bands related to MyHC antibody (molecular weight 220kDa) indicating that all samples have differentiated, and GAPDH in row 3 is used as housekeeping.

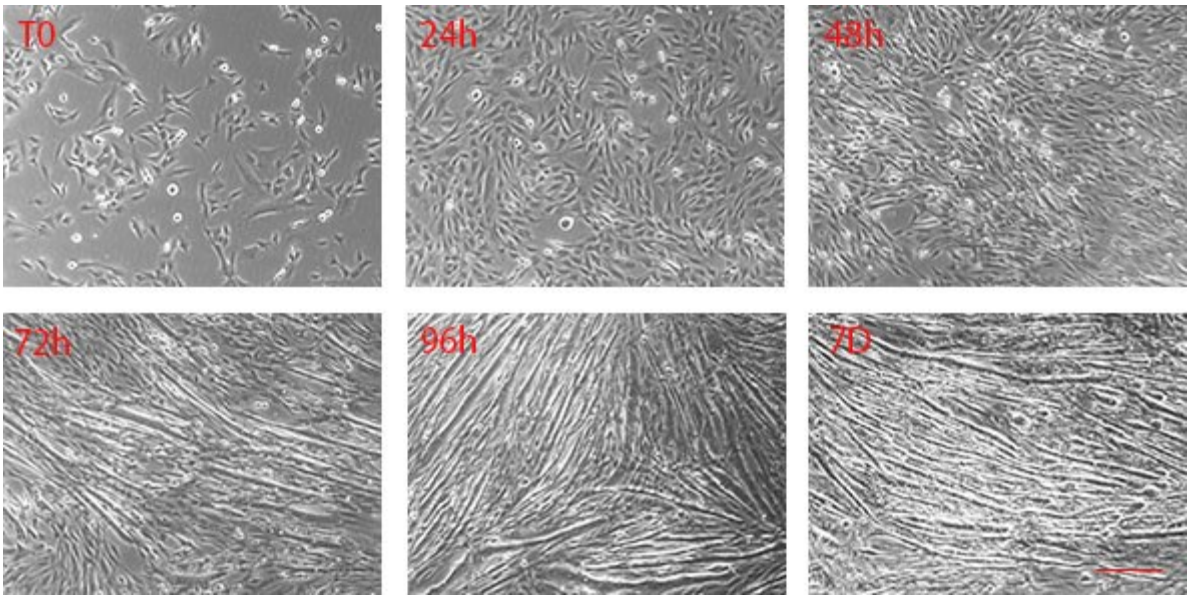

Figure 26. Cells morphology and differentiation at different time point.

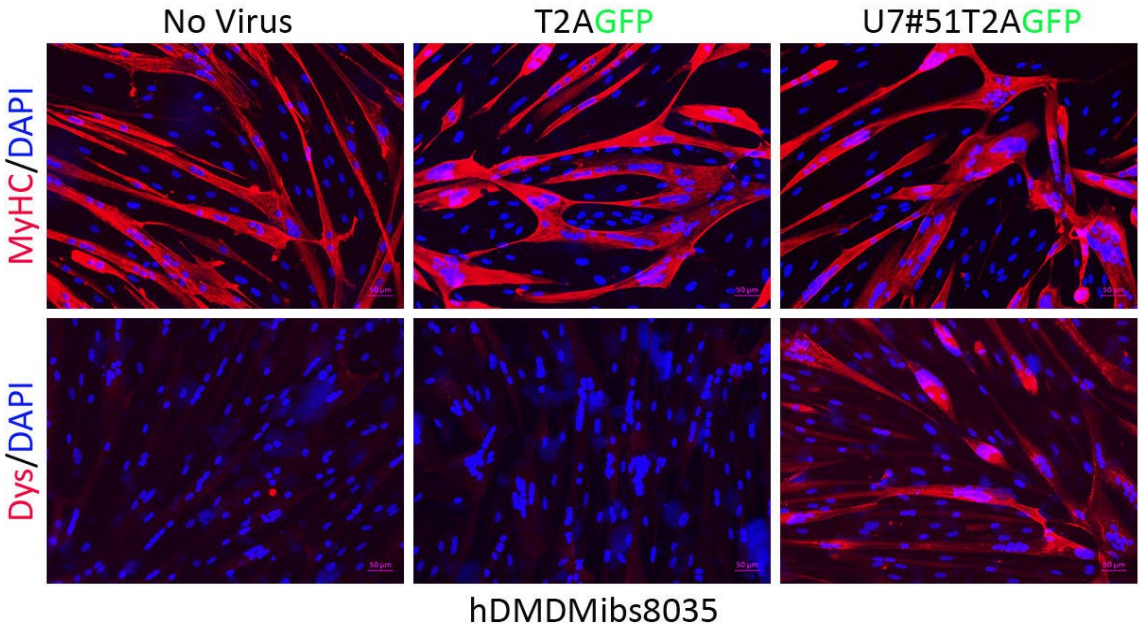

Figure 27. Dys and MyHC expression by Immunofluorescence assay.

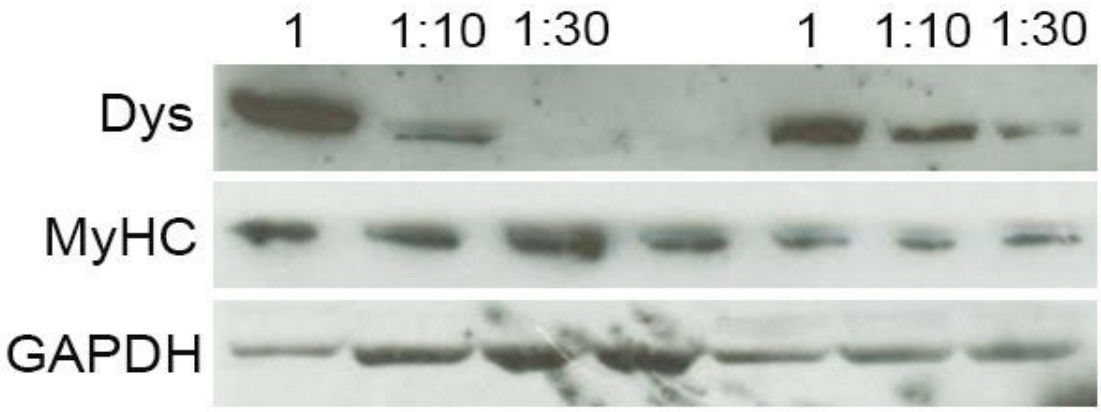

Figure 28. Dys, MyHC and GAPDH expression by Western blot analysis. Western blot detection of Dys, MyHC in differentiated, both corrected and uncorrected cells (undergoing myogenic differentiation) and in positive control (1). 1:10 ratio between uncorrected and U7#51T2AGFP cells is positive for Dys and MyHC; 1:30 ratio between uncorrected and U7#51T2AGFP cells is weakly positive for Dys and positive for MyHC. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) is the loading control.

5. Discussion

5.1 STUDY 1

Uterine leiomyomas are highly common lesions of unclear etiology. Several hypotheses have been formulated and predisposing factors have been described (McWilliams MM et al. 2017). Investigation of the factors responsible for the significant plasticity of the uterus has led to the identification of a undifferentiated cell population, promoting the hypothesis that its dysregulation may be implicated in the development of uterine pathologies (Canevari RA et al. 2005, Gargett CE et al. 2012, Hubbard SA et al. 2009).

Since inflammation is a recognized mechanism underlying the onset of several tumors, the role of an inflammatory microenvironment has also been explored in leiomyoma development. The overall hypothesis is that leiomyomas are caused in part by an immune milieu that is chronically inflammatory (Orciani M et al. 2016). In addition, the chronic inflammatory state increases estrogen which in turn may enhance leiomyoma growth (Modugno F et al. 2005). Chronic inflammation is sustained by specific cytokines secreted by immune, undifferentiated, and tumor cells (Elinav E et al. 2013, Ma S et al. 2014) and seems to be exploited by tumor cells to escape the host immune system (Elinav E et al. 2013). Undifferentiated cells play a central role in the microenvironment and modulate the cellular functions of a variety of immune cells including B and T lymphocytes, natural killer cells, monocytes, and dendritic cells (Corcione A et al. 2006, Plumas J et al. 2005, Sotiropoulou PA et al. 2006, Spaggiari GM et al. 2006, Beyth S et al. 2005, Jiang XX et al. 2005). Presumably, this role is operated by a complex interplay of short- and long-range signaling that may entail a wide spectrum of molecular mediators, including soluble cytokines and growth factors (Park CW et al. 2009).

However, a correlation between undifferentiated cells and inflammation in leiomyoma onset has never been explored. In the present study, this issue was investigated through the isolation and extensive characterization of undifferentiated progenitor cells from normal myometrium and leiomyoma. Demonstration of a stem-like immunophenotype and of the ability to differentiate into osteoblasts, chondrocytes, and adipocytes allowed their designation as MPCs (myometrium progenitor cells) and LPCs (leiomyoma progenitor cells). About the rate of growth, the DT showed an increment from passage 6 higher in MPCs than in LPCs (75.36 ± 4.19 versus 61.55 ± 1.32 hours, resp.). This increase corresponded with a reduction in proliferation, which in cultured cells is a sign of differentiated cells, and it is more evident in MPCs.

This result is consistent with the higher expression by LPCs of stemness genes *(SOX2*, *OCT4*, and *KLF4)* and of MDR1 (as demonstrated by Western blot and densitometric analysis). MDR1 is a member of the ABC transporter family, which is believed to protect stem cells from genetic damage by naturally occurring xenobiotics (de Grouw EP et al. 2006, Shervington A et al. 2008). ABC family members are considered as stem cell markers and may be used for stem cell purification. Different roles have been attributed to MDR1, such as drug efflux and protection of cells against apoptotic cell death induced by a variety of causes, and to modulate signal transduction pathways enhancing cell survival (Bunting KD. 2002).

Further analysis were conducted to better characterize the two cell populations by IIF and ICC through the expression of α-SMA, collagen type 1, and fibronectin. Their expression was strong and similar in MPCs and LPCs, although staining for collagen type 1 was weaker than the other two. It is now well accepted that mesenchymal cells are a very heterogeneous reservoir of cells; even if cells satisfied all the three essential criteria identified by Dominici, progenitor displays biologic properties that may differ according to the tissue of derivation. Specific molecules, receptors that characterized a particular tissue, may be expressed by undifferentiated cells derived from it. In this case, myometrium is characterized by abundant amounts of α-SMA, collagen type 1, and fibronectin. We found a detectable expression of these three molecules also at mRNA level; interestingly, progenitors from leiomyoma do not over-express these factors compared to cells from myometrium even if it is known their involvement in fibroid development. This apparent contradiction may reside in the fact that accumulation of extracellular matrix (ECM) in leiomyoma may be the result of a dysregulated proliferation of cells; in fibroids, ECM is more abundant because of a more elevated number of producing cells. In vitro experiments were performed using the same amount of cells derived from myometrium and leiomyoma (Iwahashi M et al. 2011). The expression of collagen type 1 was weaker than the other two molecules; it may depend by the low secretion of TGF-β1 observed by ELISA test. TGF-β1 is in fact known as a great promoter of collagen type 1 production (Tang XM et al. 1997, Sozen I et al. 2002).

As regards the role of inflammation, it is known that leiomyoma onset may correlate with active inflammation (Wegienka G. 2012) and that undifferentiated cells participate in microenvironment formation. We tested 12 cytokines related to acute and chronic inflammation in order to evaluate the difference in expression and secretion between both cell groups.

Both cell types expressed detectable level of all cytokines and interleukins, but the most notable finding was the significantly different expression of Th2 and Th1/Th17 pathways in LPCs and

MPCs. Indeed, LPCs exhibited a significantly greater expression of IL4, IL5, IL10, and IL13 (Th2 pathway) and a significantly lower expression of Th1/Th17 pathway cytokines. In particular, they secreted less TGF- β1 which, alone or combined with IL6, is involved in the differentiation of naive T-cells to Treg T-cells and Th17 T-cells. Treg T-cells are actively involved in inhibiting tissue inflammation, and their suppression may enhance the maintenance of the inflammatory microenvironment that favors leiomyoma development. IL12 and IFN-γ, which allow differentiation of naive cells to Th1 T-cells, were lower in LPCs, whereas secretion of IL4, IL5, and IL13, which drive the differentiation to Th2 T-cells, was lower in MPCs.

To lend support to the downregulation of Th1/Th17 pathway cytokines in LPCs, other soluble factors of the same subgroups (IL22, NFKB, IL23A, STAT3, CCR5, IL17A, IL17RA, CXCL12, CX3CL1, and CXCL5) were evaluated at mRNA level. This panel of molecules with different functions (chemokines, cytokines, transcription factors, and signaling pathway molecules) provided a general picture of the involvement of Th1/Th17 pathways. All molecules were downregulated in LPCs, confirming the upregulation of the Th2 profile. Th2 cells and cytokines are associated with chronic inflammation, whereas the Th1/Th17 pathways are related to acute inflammation. The upregulation of the Th2 pathway in LPCs may reflect a protracted inflammatory status that is maintained by paracrine effect exerted also by undifferentiated cells, which create a stroma favoring leiomyoma development.

These observations suggest a relationship between chronic myometrial inflammation and uterine leiomyomatosis, infertility, and adverse obstetric outcomes (Practice Committee of the American Society for Reproductive Medicine). Indeed, a chronic inflammatory reaction induced by fibroids and altered myometrium contractility may hinder embryo implantation, affecting fertility (Rackow BW et al. 2010, Kido A et al. 2014, Yoshino O et al. 2012). Among the mechanisms invoked to explain the increased myometrial contractility are an excess of cytokines, growth factors, neurotensin, neuropeptides, enkephalin, oxytocin modulators, and chronic inflammation of the fibroid capsule (Malvasi A et al. 2013, Ben-Nagi J et al. 2010, Cakmak H et al. 2011).

Alterations in the endometrial-myometrial junction (EMJ) seem to play a key role in implantation failure and recurrent miscarriage. The EMJ, the inner third of the myometrium adjacent to the endometrium, provides macrophages and uterine natural killer cells, which are essential for endometrial decidualization in the midluteal window of implantation (Sinclair DC et al. 2011).

It is conceivable that intramural/submucosal fibroids not only physically disrupt the EMJ (Kitaya K et al. 2010, Tocci A et al. 2008, Purohit P et al. 2016) but also cause chronic inflammation, steroid receptor alterations, and ultimately implantation failure. A chronic proinflammatory

effect exerted by leiomyoma progenitor cells may explain why even small myomas or early-stage diffuse leiomyomatosis hamper embryo implantation.

In conclusion, the present data suggest that (i) progenitor cells are found both in leiomyomas and normal myometrium, (ii) these progenitors show a differential expression of cytokines related to acute and chronic inflammation, and (iii) the upregulation of cytokines related to chronic inflammation in leiomyoma progenitors may favor the formation of a microenvironment suitable for leiomyoma onset and development.

5.2 STUDY 2

Cervical intraepithelial neoplasia of grade 2 (CIN-2) is a pre-cancerous lesion of uterine cervix epithelium that can evolve into cervical cancer if not treated immediately. The annual regression rate of CIN-2, in old women is estimated to range from 15 to 23% while in young women the regression rate is of 65% (Moscicki AB et al. 2010); a lower regression rate (6-38%) is instead reported for CIN-3 (Motamedi M et al. 2015, Munk AC et al. 2007).Inflammation, sustained also by undifferentiated mesenchymal stem cells (MSCs) (Kyurkchiev D et al. 2014), plays a pivotal role in the progression or regression of the CIN. The existence of a pool of MSCs inside the cervix (C-MSCs) is well accepted and sustained and they are considered also the promoters of cervical regeneration after LEEP treatment.

We wondered if MSCs may display age-related features that help to clarify the different regression rate in young and old women, and if, in case of progression towards cervical tumor, there is a correlation between MSCs role and age.

Firstly, fourteen patients were enrolled in this study and divided by the age in “young” (mean age 28±2), and “old” (mean age 45±3). From all tissue biopsies it was possible to isolate an undifferentiated cellular population, and after the characterization following the minimal criteria proposed by Dominici et al., cells from young cervixes were named yC-MSCs and cells from old cervixes oC-MSCs. The subsequent analysis of stemness genes, OCT4, SOX2, NANOG, KLF4 revealed the first discrepancy between the two groups of patients. yC-MSCs expressed higher value of KLF4 than oC-MSCs.

KLF4 directly binds to the promoter of NANOG to help OCT4 and SOX2 in regulating the expression of NANOG (Zhang P et al. 2010). This observation confirmed the critical role of KLF4 in stem cell self-renewal as well as pluripotency. Another evident difference was related to the proliferation rate that was faster in yC-MSCs than in oC-MSCs.

The analysis through β-galactosidase assay clarified the possible involvement of senescence. At passage 6 oC-MSCs displayed higher percentage of senescent cells than yC-MSCs. Subsequently, the expression of genes related to senescence by PCR array showed a dysregulation of 13 genes between MSCs derived from young and old cervixes, choosing a cut-off of 3-fold; in detail, CCNA2, CCNB1, CDC25, CHEK1, E2F1, TERT, PCNA were upregulated in yC-MSCs compared to oC-MSCs, while CDKN2A, CCNE1, NOX4 and SOD2 were less expressed. The observed differences in gene expression reflected the role of these genes in senescence (Kundrotas G et al. 2016). CCNA2 is fundamental for cell proliferation (Gopinathan L et al. 2014), TERT plays a pivotal role in telomerase length and its expression is higher in dividing cells, and gradually decreases in senescent cells (Flores I et al. 2006, Flores I et al. 2010, Sahin E et al. 2010). E2F1 and CHEK1 regulate respectively the expression of genes referred to cell cycle progression and the G2 DNA damage checkpoint (Hong S et al. 2008, Koniaras K et al. 2001) and are both less expressed in senescent MSC (Noh H et al. 20010. A downregulation of CDC25C is associated with cell cycle arrest (Welford SM et al. 2011), more precisely in the phase G2/M (Magimaidas A et al. 2014). Finally, the expression of CCNB1 and PCNA are lower in senescent MSCs.

Among the downregulated genes in yC-MSCs, CCNE1 (Dulić V et al. 1993) and CDKN2A are known to up-regulated in senescent cells compared to early-passage cells and play a role in achieving the senescent status (Kamijo T et al. 1997, Baker DJ et al. 2008); NOX4, a member of the NADPH oxidase family, increased ROS production and is related to senescence (Ryan A et al. 2016); the overexpression of SOD2 in oC-MSCs is in agreement with Estrada (Estrada JC et al. 2013) and confirms as senescence significantly alters MSCs metabolism and oxidative status.

To test if the involvement of inflammation in CIN progression could be age-related, the secretion of soluble factors related to acute and chronic inflammation was analyzed by ELISA in y- and oC-MSCs. The first evidence is the increased secretion of cytokines related to the acute inflammation (IL2, IL6, IL10, IL12, IFN-γ, TGF-β1) in yC-MSCs. Previous studies have suggested that decreased Th1 responses (strictly connected to acute inflammation) are associated with cervical carcinogenesis (Clerici M et al. 1997). This different ability of MSCs to secrete cytokines may correlate with the altered age-related regression rate.

Acute inflammation allows to the organism to counteract infection, such as the infection with oncogenic human papillomaviruses (HPV) that plays a central etiologic role in the development of squamous carcinomas of the cervix and their precursor lesions like CIN.

The profile of cytokines secreted by MSCs derived from old cervixes seems to indicate that the cervical microenvironment is not able to respond with an acute inflammation, with subsequent inability to counteract HPV and CIN as demonstrated by the lower percentage of regression.
If CIN does not regress, it may develop towards cervical carcinogenesis.
As for other tumors, inflammation appears to exert a major effect. If the condition causing acute inflammation is not resolved, it may become chronic, favoring tumor onset and development. Following this hypothesis, MSCs from cervixes of old and young patients were indirectly co-cultured with a cervix epithelioid carcinoma cell line (HeLa). We assessed if factors secreted by MSCs may alter the proliferation of tumor cells, finding that co-cultures with oC-MSCs produce an increase in HeLa proliferation rate. Afterward, the PCR analysis of genes related to different mechanisms leading to tumor development showed upregulation of cJUN, cFOS and cMET in Hela cells co-cultured with oC-MSCs. This data correlates with other works that underlined as these genes were implicated in cervical carcinogenesis (van Riggelen J et al. 2005, Comunoğlu C et al. 2012). VEGF and CXCL12, considered as master regulators of neoangiogenesis that drives tumor development, were more expressed in oC-MSCs, as well as MMP11 that is related to invasion and migration. Moreover, mKI67, a marker of proliferation, was upregulated in co-cultures with oC-MSCs compared with yC-MSCs and this correlates with the increased proliferation rate observed in co-cultures with MSCs derived from cervixes of old patients.
The detection of secreted cytokines in HeLa cells after co-culture with MSCs from the two groups of cervixes revealed that the level of IL6, IL10 and TGF-β were increase. Previous study demonstrated that the expression of TGF-β decreased as tumor cells progressed from CIN to cervical carcinoma, indicating that the disruption of this signalling pathway might contribute to the malignant progression of cervical dysplasia (Sanjabi S et al. 2009). In addition, under inflammatory conditions, TGF-β in the presence of IL6 drives the differentiation of T helper 17 (Th17) cells, which can promote further inflammation and augment autoimmune conditions (Korn T et al. 2009). The exact role of IL6 in CIN progression is still unclear; besides its increased expression reported by someone during cervical tumor development (Wei LH et al. 2003), there is the important role exerted as master switch of acute inflammation.
Finally, IL10 plays a dual, controversial role in cervical carcinogenesis (Wang Y et al. 2013); even if IL10 mRNA and/or protein are enhanced in several types of human cancer (including cervix), other works report that higher levels of IL10 may prevent cervical neoplasia by assisting in the elimination of HPV (Brower V. 2005, Farzaneh F et al. 2006). IL10 enhances the proliferation and

expression of immunologically important surface molecules and increases Th1 cytokine production.

In conclusion, our results show that a mesenchymal stem cell (MSCs) population persist inside cervix and displays age-related properties. Age may affect the regression rate of CIN by influencing the paracrine effect exerted by MSCs; in the crosstalk with HeLa cells, MSCs from young cervixes drive an anti-tumoral effect by sustaining an acute inflammatory environment. These data reinforce the evidence of the possibility of a conservative management of high-grade lesions (especially CIN2) in young women with desire for future pregnancy, avoiding unnecessary treatments with preservation of reproductive potential.

5.3 STUDY 3

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive disorder and the most prevalent inherited myopathy affecting one in 3,500 live male births (Bushby K et al. 2010). At the molecular level, DMD is caused by mutations in the dystrophin gene leading to the absence of the protein (Cordova G et al. 2018). Dystrophin is a cytoskeletal protein critical for the stability and function of myofibers in muscle because it forms a complex that connects the cytoskeleton and the basal lamina (Galli F et al. 2018). The deficiency of this protein leads to the break of the muscle fiber membrane during contraction (Allen and Whitehead K et al. 2011), causes compromised intracellular signalling (Constantin B. 2014) and increases fragility of the sarcolemma. The various forms of muscular dystrophy differ in severity, which correlates with the amount of fibers are lost and muscle tissue is progressively replaced by connective and adipose tissue (Galli F et al. 2018). It is believed that at this late step all therapies will be unsuccessful. Currently, steroids represent the only standard therapy for dystrophic patients, but they only delay the progression of the disease and have serious side effects.

Many novel therapeutic approaches with clinical experimentation have brought hopeful results, but no one has yet achieved significant and lasting clinical efficacy (Guiraud et al., 2015).

These include new drugs, gene therapy, exon skipping, PTC124 (which triggers premature STOP-codon read-through), and cell therapy. Exon skipping is one of the most promising approach: it consists in the use of an antisense oligonucleotide or small nuclear RNA (snRNA) that restore the correct reading frame in patients with eligible out-of-frame deletions by an exon skipping. The results of this strategy are similar to what happened in Becker muscular dystrophy, where naturally deletions are induced to produce in-frame proteins (Cossu G et al. 2017).

The most important exon is the 51, which is the most targeted by deletions in patients with DMD (about 13%). Also other exons are involved, at least nine, and therapies on them brings to about 70% of correction rate of boys carrying deletions (Cossu G et al. 2017). Indeed, phase 1 studies targeting exons 45 and 53 are currently on-going. Study on exon 51 are the most encouraging: since 2009 several studies demonstrated promising results using two compounds, the 2'OMethyl (2'OMe) backbone and the morpholino backbone (Sheridan C. 2016, van Deutekom JC et al. 2007, Cirak S et al. 2011). One of them, the morpholino antisense oligonucleotide namely eteplirsen and developed by Sarepta, received the approval of the FDA (Cossu G et al. 2017). Other *in vivo* therapies rely on adenovirus associated vectors that delivered snRNA, such as U7, that can skip the mutated exon to restore the reading frame and produce a shorter but functional dystrophin. This strategy is the same that as that achieved through systemic administration of oligonucleotides, which has been widely tested in several trials with different oligo backbones (Le Guiner C et al. 2014, Vulin A et al. 2012).

In this study, a new strategy is currently pursuing: by transducing donor myogenic cells with a lentivector expressing the U7 snRNA, engineered to skip exon 51, the aim is to exploit the diffusion of snRNA to neighboring nuclei and induce exon skipping and thus amplify the production of dystrophin. As the lentiviral vectors have a lower risk of insertional mutagenesis respect to the retroviral vectors, they represent the vectors of choice for next trials. This lower risk is due to the fact that lentiviruses integrate randomly in the genome, while retroviruses select for transcriptionally active regions, thus increasing the possibility of activating ''dangerous'' genes (Galli F et al. 2018, Biasco L. 2017).

In this study, myoblasts were previously isolated from DMD donor and transduced with GFP and a lentiviral vector expressing the snRNA to induce exon skipping. Then, they were stimulated to differentiate in myogenic lineage. At 7 days myoblasts showed a good level of fusion and the size of myotubes continue to increase until day 14 as demonstrated in figure xx. (as primary myoblasts are already committed to the myoblasts fate).

The immunofluorescence assay confirmed the expression of a skeletal muscle differentiation marker, Myosin Heavy Chain (MyHC). In according with Gibsong and collegues, MyHC is considered a late marker for myogenic differentiation, for contractile fiber type determination (Gibsong TM et al. 2015). As recently expected by Galli et al., the presence of multinucleate fibers validates a high level of fusion, important for the cross correction induced by snRNA (Galli F et al. 208). To examine how the diffusion of snRNA influenced the neighbor nuclei between the myotubes in order to allow exon skipping and obtain major amount of protein, Dystrophin

expression was analyzed by immunofluorescence. Dystrophic Myoblasts not transduced (No virus) and dystrophic myoblasts transduced with scramble (T2AGFP) do not show expression of Dystrophin protein while myoblasts transduced with lentiviral vector expressing snRNA (U7#51T2AGFP) show expression of Dystrophin protein.

To determine if there was a direct correlation between the number of transduced cells and the level of detectable functional Dystrophin, experiments with two different ratios (1:10, 1:30) of corrected and un-corrected cells of the same patients were performed. Cells from healthy patients were used as positive control, while cells derived from dystrophic patients were the negative control.

Firstly, all mixed samples expressed MyHC confirming the differentiated status but only cells receiving the U7#51T2AGFP displayed a detectable expression of Dystrophin; the level of expression was linear to the amount of transduced cells. This result is of particular interest since it means that *in vivo* it will be possible to reach a good outcome with a relatively low amount of corrected cells.

In conclusion, these preliminary data confirm the initial hypotheses: to induce the skipping of exon 51 through a lentivector expressing U7snRNA and to expand this snRNA to neighboring nuclei will enhance the cross correction to neighboring nuclei in order to obtain a functional Dystrophin. These results are essential for the next and most important progresses of the project: the *in vivo* experimentation and the clinical trial.

6. References

- Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005 Feb 15;105(4):1815-22.
- Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006; 124: 783–801.
- Akiyama K, Chen C, Wang D, et al. Mesenchymal-stem-cellinduced immunoregulation involves FAS-ligand-/FAS-mediated T cell apoptosis. Cell Stem Cell. 2012 May 4;10(5):544-55.
- Aleksandrovych V, Bereza T, Sajewicz M, et al. Uterine fibroid: common features of widespread tumor (Review article). Folia Med Cracov. 2015;55(1):61-75.
- Allen and Whitehead K, Finkel R, Birnkrant DJ, et al. DMD Care Considerations Working Group. Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. Lancet Neurol. 2010 Jan;9(1):77-93.
- Alvarez K, Fadic R, Brandan E. Augmented synthesis and differential localization of heparan sulfate proteoglycans in Duchenne muscular dystrophy. J Cell Biochem. 2002;85(4):703-13.
- Auletta JJ, Deans RJ, Bartholomew AM. Emerging roles for multipotent, bone marrow derived stromal cells in host defense. Blood 2012; 119: 1801–1809.
- Bae JH, Kim CJ, Park TC, et al. Persistence of human papillomavirus as a predictor for treatment failure after loop electrosurgical excision procedure. Int J Gynecol Cancer 2007;17(6):1271–1277.
- Bai L, Lennon DP, Eaton V, et al. Human bone marrow-derived mesenchymal stem cells induce Th2-polarized immune response and promote endogenous repair in animal models of multiple sclerosis. Glia. 2009 Aug 15;57(11):1192-203.
- Baker DJ, Jin F, van Deursen JM. The yin and yang of the Cdkn2a locus in senescence and aging. Cell Cycle. 2008;7:2795-802.
- Barzilay R, Ben-Zur T, Bulvik S, et al. Lentiviral delivery of LMX1a enhances dopaminergic phenotype in differentiated human bone marrow mesenchymal stem cells. Stem Cells Dev. 2009 May;18(4):591-601.
- Batten P, Sarathchandra P, Antoniw JW, et al. Human mesenchymal stem cells induce T cell anergy and downregulate T cell allo-responses via the TH2 pathway: relevance to tissue engineering human heart valves. Tissue Eng. 2006 Aug;12(8):2263-73.
- Bekos C, Schwameis R, Heinze G et al. Influence of age on histologic outcome of cervical intraepithelial neoplasia during observational management: results from large cohort, systematic review, meta-analysis Sci Rep. 2018; 8: 6383.
- Bengtsson NE, Hall JK, Odom GL, et al. Musclespecific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. Nat Commun 2017;8:14454.
- Ben-Nagi J, Miell J, Mavrelos D, et al . Endometrial implantation factors in women with submucous uterine fibroids. Reprod Biomed Online. 2010 Nov;21(5):610-5.

- Beyth S, Borovsky Z, Mevorach D, et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. Blood. 2005 Mar 1;105(5):2214-9.
- Biasco L. Integration site analysis in gene therapy patients: expectations and reality. Hum Gene Ther 2017;28(12):1122–1129.
- Bier A, Berenstein P, Kronfeld N, et al. Placentaderived mesenchymal stromal cells and their exosomes exert therapeutic effects in Duchenne muscular dystrophy. Biomaterials 2018;174:67–78.
- Blake RE. Leiomyomata uteri: hormonal and molecular determinants of growth. J Natl Med Assoc. 2007 Oct;99(10):1170-84.
- Borish LC, Steinke JW. 2. Cytokines and chemokines. J Allergy Clin Immunol. 2003 Feb;111(2 Suppl):S460-75.
- Brooke G, Rossetti T, Pelekanos R, et al. Manufacturing of human placenta-derived mesenchymal stem cells for clinical trials. Br J Haematol. 2009;144(4):571–579.
- Brosens I. Uterine Leiomyomata: Pathogenesis and Management. The New England Journal of Medicine. Abingdon, England, Informa Healthcare/Taylor & Francis, 2006.
- Brower V. Researchers attempting to define role of cytokines in cancer risk. J Natl Cancer Inst. 2005;97:1175–7
- Bulun SE. Uterine fibroids. The New England Journal of Medicine, vol. 369, no. 14, pp. 1344–1355, 2013.
- Bunting KD. ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells. 2002;20(1):11-20. Review. Erratum in: Stem Cells 2002;20(3):274.
- Caffarini M, OrcianiM, Trementino L, et al. Pituitary adenomas, stem cells, and cancer stem cells: what's new? J Endocrinol Invest. 2018 Jul;41(7):745-753.
- Cakmak H, Taylor HS. Implantation failure: molecular mechanisms and clinical treatment. Hum Reprod Update. 2011 Mar-Apr;17(2):242-53.
- Canevari RA, Pontes A, Rosa FE, et al. Independent clonal origin of multiple uterine leiomyomas that was determined by X chromosome inactivation and microsatellite analysis. Am J Obstet Gynecol. 2005 Oct;193(4):1395-403..
- Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991 Sep;9(5):641-50.
- Carneiro MM. Stem cells and uterine leiomyomas: what is the evidence? JBRA Assist Reprod. 2016 Mar 1;20(1):33-7.
- Chamberlain JR, Chamberlain JS. Progress toward Gene Therapy for Duchenne Muscular Dystrophy. Mol Ther. 2017 May 3;25(5):1125-1131.
- Chang HL, Senaratne TN, Zhang L et al. Uterine leiomyomas exhibit fewer stem/progenitor cell characteristics when compared with corresponding normal myometrium. Reprod Sci. 2010 Feb;17(2):158-67.
- Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol 2010; 10: 826–837.

- Ciarmela P, Islam MS, Reis FM, et al. Growth factors and myometrium: biological effects in uterine fibroid and possible clinical implications. Hum Reprod Update. 2011 Nov-Dec;17(6):772-90.
- Ciavattini A, ClementeN, Delli Carpini G, et al. Number and size of uterine fibroids and obstetric outcomes. J Matern Fetal Neonatal Med. 2015 Mar;28(4):484-8.
- Ciavattini A, Clemente N, Delli Carpini G, et al. Loop electrosurgical excision procedure and risk of miscarriage. Fertil Steril. 2015;103:1043-8.Ciavattini A, Delli Carpini G, Moriconi L, et al. Effect of age and cone dimensions on cervical regeneration: an Italian multicentric prospective observational study. BMJ Open. 2018;8:e020675.
- Cirak S, Arechavala-Gomeza V, Guglieri M, et al. Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. Lancet 2011; 378: 595–605.
- Clerici M, Merola M, Ferrario E, et al. Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection. J. Natl. Cancer Inst. 1997;89:245-50.
- Cohen-Haguenauer O, Creff N, Cruz P, et al. Relevance of an academic GMP Pan-European vector infra-structure (PEVI). Curr Gene Ther 2010;10(6): 414–422.
- Comunoğlu C, Boynukalin K, Uğur MG, et al. Overexpression of c-Met in cervical intraepithelial neoplasia. Eur J Gynaecol Oncol. 2012;33:625-8.
- Constantin B. Dystrophin complex functions as a scaffold for signalling proteins. Biochim. Biophys. Acta 2014.1838, 635–642.
- Corcione A, Benvenuto F, Ferretti E, et al. Human mesenchymal stem cells modulate B-cell functions. Blood. 2006 Jan 1;107(1):367-72.
- Cordova G, Negroni E, Cabello-Verrugio C, et al. Combined Therapies for Duchenne Muscular Dystrophy to Optimize Treatment Efficacy. Front Genet. 2018 Apr 10;9:114.
- Cosenza S, Toupet K, Maumus M, et al. Mesenchymal stem cells-derived exosomes are more immunosuppressive than microparticles in inflammatory arthritis. Theranostics 2018;8(5): 1399- 1410.
- Cossu G, Birchall M, Brown T, et al. Lancet Commission: Stem cells and regenerative medicine. Lancet. 2018;391(10123):883–910.
- Cossu G, Previtali SC, Napolitano S, et al. Intraarterial transplantation of HLA-matched donor mesoangioblasts in Duchenne muscular dystrophy. EMBO Mol Med 2015; 7(12):1513–1528.
- Cox JT, Schiffman M, Solomon D, Group A-LTS. Prospective follow-up suggests similar risk of subsequent cervical intraepithelial neoplasia grade 2 or 3 among women with cervical intraepithelial neoplasia grade 1 or negative colposcopy and directed biopsy. American journal of obstetrics and gynecology. 2003;188:1406–12.
- Criscimanna A, Zito G, Taddeo A, et al. In vitro generation of pancreatic endocrine cells from human adult fibroblast-like limbal stem cells. Cell Transplant. 2012;21(1):73-90.
- Daley JM, Thomay AA, Connolly MD et al. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. J Leukoc Biol. 2008 Jan;83(1):64-70.

- Datta I, Mishra S, Mohanty L., et al. Neuronal plasticity of human Wharton's jelly mesenchymal stromal cells to the dopaminergic cell type compared with human bone marrow mesenchymal stromal cells. Cytotherapy. 2011 Sep;13(8):918-32.
- de Grouw EP, Raaijmakers MH, Boezeman JB, et al. Preferential expression of a high number of ATP binding cassette transporters in both normal and leukemic CD34+CD38- cells. Leukemia. 2006 Apr;20(4):750-4.
- Dellavalle A, Sampaolesi M, Tonlorenzi R, et al. Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. Nat Cell Biol 2007;9(3):255–267.
- Desguerre I, Christov C, Mayer M, et al. Clinical heterogeneity of duchenne muscular dystrophy (DMD): definition of sub-phenotypes and predictive criteria by long-term follow-up. PLoS One. 2009;4(2):e4347.
- Desguerre I, Mayer M, Leturcq F, et al. Endomysial fibrosis in Duchenne muscular dystrophy: a marker of poor outcome associated with macrophage alternative activation. J Neuropathol Exp Neurol. 2009 Jul;68(7):762-73.
- Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-7.
- Dulić V, Drullinger LF, Lees E, et al. Altered regulation of G1 cyclins in senescent human diploid fibroblasts:accumulation of inactive cyclin E-Cdk2 and cyclin D1-Cdk2 complexes. Proc Natl Acad Sci U S A. 1993;90:11034-8.
- Ehmsen J, Poon E, Davies K. The dystrophin-associated protein complex. J Cell Sci. 2002 Jul 15;115(Pt 14):2801-3.
- Eiró N, Sendon-Lago J, Seoane S et al. Potential therapeutic effect of the secretome from human uterine cervical stem cells against both cancer and stromal cells compared with adipose tissue stem cells. Oncotarget. 2014;5:10692-708.
- Elinav E, Nowarski R, Thaiss CA, et al. Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. Nat Rev Cancer. 2013 Nov;13(11):759-71. English K. Mechanisms of mesenchymal stromal cell immunomodulation. Immunology and Cell Biology 2013; 91: 19-26.
- Ervasti JM, Sonnemann KJ. Biology of the striated muscle dystrophin-glycoprotein complex. Int Rev Cytol. 2008;265:191-225.
- Estrada JC, Torres Y, Benguría A, et al. Human mesenchymal stem cell-replicative senescence and oxidative stress are closely linked to aneuploidy. Cell Death Dis. 2013;4:e691.
- Farzaneh F, Roberts S, Mandal D, et al. The IL-10 -1082G polymorphism is associated with clearance of HPV infection. BJOG. 2006;113:961–4.
- Ferrari G, Cusella-De Angelis G, Coletta M, et al. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 1998;279(5356):1528–1530.
- Ferrero-Miliani L, Nielsen OH, Andersen PS, et al. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. Clin Exp Immunol 2007 Feb;147(2):227-35.

- Fiorina P, Jurewicz M, Augello A, et al. Immunomodulatory function of bone marrow-derived mesenchymal stem cells in experimental autoimmune type 1 diabetes. J Immunol. 2009 Jul 15;183(2):993-1004.
- Flake GP, Andersen J, Dixon D. Etiology and pathogenesis of uterine leiomyomas: a review. Environ Health Perspect. 2003 Jun;111(8):1037-54..
- Flores I, Benetti R, Blasco MA. Telomerase regulation and stem cell behaviour. Current Opinion in Cell Biology 2006;18: 254–60.
- Flores I, Blasco MA. The role of telomeres and telomerase in stem cell aging. FEBS Lett. 2010; 584: 3826–30.
- Founta C, Arbyn M, Valasoulis G, et al. Proportion of excision and cervical healing after large loop excision of the transformation zone for cervical intraepithelial neoplasia. BJOG. 2010 Nov;117(12):1468-74.
- Friedenstein AJ, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Experimental hematology. 1976. 4 (5): 267–74.
- Galli F, Bragg L, Meggiolaro L, et al. Gene and Cell Therapy for Muscular Dystrophies: Are We Getting There? Hum Gene Ther. 2018 Oct;29(10):1098-1105.
- Gao QQ, McNally EM. The Dystrophin Complex: Structure, Function, and Implications for Therapy. Compr Physiol. 2015 Jul 1;5(3):1223-39.
- Gargett CE, Nguyen HP, Ye L. Endometrial regeneration and endometrial stem/progenitor cells. Rev Endocr Metab Disord. 2012 Dec;13(4):235-51.
- Ghannam S, Pène J, Moquet-Torcy G, et al. Mesenchymal stem cells inhibit human Th17 cell differentiation and function and induce a T regulatory cell phenotype. J Immunol. 2010 Jul 1;185(1):302-12.
- Gibson TM, Gersbach CA. Single-molecule analysis of myocyte differentiation reveals bimodal lineage commitment. Integr Biol (Camb). 2015 Jun;7(6):663-71.
- Gopinathan L, Tan SL, Padmakumar VC, et al. Loss of Cdk2 and cyclin A2 impairs cell proliferation and tumorigenesis. Cancer Res. 2014;74:3870-9.
- Grabosch SM, Shariff OM, Helm CW. Non-steroidal anti-inflammatory agents to induce regression and prevent the progression of cervical intraepithelial neoplasia. Cochrane Database Syst Rev. 2018 Feb 12;2:CD004121.
- Griggs RC, Herr BE, Reha A, et al. Corticosteroids in Duchenne muscular dystrophy: major variations in practice. Muscle Nerve. 2013 Jul;48(1):27-31.
- Guiraud S, Aartsma-Rus A, Vieira NM, et al. The Pathogenesis and Therapy of Muscular Dystrophies. Annu Rev Genomics Hum Genet. 2015;16:281-308.
- Gussoni E, Soneoka Y, Strickland CD, et al. Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 1999;401(6751):390–394.
- Hammes LS, Tekmal RR, Naud P, et al. Macrophages, inflammation and risk of cervical intraepithelial neoplasia (CIN) progression--clinicopathological correlation. Gynecol Oncol. 2007;105:157-65.

- Hareendran S, Balakrishnan B, Sen D, et al. Adeno-associated virus (AAV) vectors in gene therapy: immune challenges and strategies to circumvent them. Rev Med Virol. 2013 Nov;23(6):399-413.
- Himes KP, Simhan HN. Time from cervical conization to pregnancy and preterm birth. Obstet Gynecol. 2007 Feb;109(2 Pt 1):314-9.
- Hong S, Paulson QX, Johnson DG. E2F1 and E2F3 activate ATM through distinct mechanisms to promote E1A-induced apoptosis. Cell Cycle. 2008;7:391–400.
- Hotta A, Yamanaka S. From genomics to gene therapy: induced pluripotent stem cells meet genome editing. Annu Rev Genet 2015;49:47–70.
- Hubbard SA, Friel AM, Kumar B, et al. Evidence for cancer stem cells in human endometrial carcinoma. Cancer Res. 2009 Nov 1;69(21):8241-8.
- Islam MS, Akhtar MM, Ciavattini A, et al. Use of dietary phytochemicals to target inflammation, fibrosis, proliferation, and angiogenesis in uterine tissues: promising options for prevention and treatment of uterine fibroids? Mol Nutr Food Res. 2014 Aug;58(8):1667-84.
- Islam MS, Ciavattini A, Petraglia F, et al. Extracellular matrix in uterine leiomyoma pathogenesis: a potential target for future therapeutics. Hum Reprod Update. 2018 Jan 1;24(1):59-85.
- Izadpanah R, Trygg C, Patel B, et al. Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. J Cell Biochem. 2006 Dec 1;99(5):1285-97.
- Jiang XX, Zhang Y, Liu B, et al. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. Blood. 2005 May 15;105(10):4120-6.
- Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2007 Jun 14;447(7146):879-80.
- Kaji EH, Leiden JM. Gene and stem cell therapies. JAMA. 2001 Feb 7;285(5):545-50.
- Kamijo T, Zindy F, Roussel MF et al. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell. 1997;91:649–59.
- Kanafi MM, Rajeshwari YB, Gupta S, et al. Transplantation of islet-like cell clusters derived from human dental pulp stem cells restores normoglycemia in diabetic mice. Cytotherapy. 2013 Oct;15(10):1228-36.
- Kang SK, Lee DH, Bae YC, et al. Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. Exp Neurol. 2003 Oct;183(2):355-66.
- Kern S, Eichler H, Stoeve J, et al. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells. 2006;24(5):1294–1301.
- Kido A, Ascher SM, Hahn W, et al. 3 T MRI uterine peristalsis: comparison of symptomatic fibroid patients versus controls. Clin Radiol. 2014 May;69(5):468-72.
- Kim J, Park S, Kang HM, et al. Human insulin secreted from insulinogenic xenograft restores normoglycemia in type 1 diabetic mice without immunosuppression. Cell Transplant. 2012;21(10):2131-47.

- Kim KH, Blasco-Morente G, Cuende N, et al. Mesenchymal stromal cells: properties and role in management of cutaneous diseases. J Eur Acad Dermatol Venereol. 2017;31(3):414–423.
- Kitaya K, Yasuo T. Leukocyte density and composition in human cycling endometrium with uterine fibroids. Hum Immunol. 2010 Feb;71(2):158-63.
- Koenig M, Hoffman EP, Bertelson CJ, et al. Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell 1987.50, 509–517.
- Kong QF, Sun B, Bai SS, et al. Administration of bone marrow stromal cells ameliorates experimental autoimmune myasthenia gravis by altering the balance of Th1/Th2/Th17/Treg cell subsets through the secretion of TGFbeta. J Neuroimmunol 2009; 207: 83–91.
- Koniaras K, Cuddihy AR, Christopoulos H, et al. Inhibition of Chk1-dependent G2 DNA damage checkpoint radiosensitizes p53 mutant human cells. Oncogene. 2001;20:7453–63.
- Korn T, Bettelli E, Oukka M, et al. IL-17 and Th17 Cells. Annu Rev Immunol. 2009;27:485-517.
- Kundrotas G, Gasperskaja E, Slapsyte G, et al. Identity, proliferation capacity, genomic stability and novel senescence markers of mesenchymal stem cells isolated from low volume of human bone marrow Oncotarget. 2016;7: 10788–802.
- Kunkel LM, Monaco AP, Hoffman E, et al. Molecular studies of progressive muscular dystrophy (Duchenne). Enzyme 1987.38, 72–75.
- Kurita T, Cooke PS, Cunha GR. Epithelial–stromal tissue interaction in paramesonephric (müllerian) epithelial differentiation. Developmental Biology, vol. 240, no. 1, pp. 194–211, 2001.
- Kyrgiou M, Athanasiou A, Kalliala IEJ, et al. Obstetric outcomes after conservative treatment for cervical intraepithelial lesions and early invasive disease. Cochrane Database Syst Rev. 2017 Nov 2;11:CD012847
- Kyurkchiev D, Bochev I, Ivanova-Todorova E, et al. Secretion of immunoregulatory cytokines by mesenchymal stem cells. World J Stem Cells. 2014;6: 552–70.
- Le Guiner C, Montus M, Servais L, et al. Forelimb treatment in a large cohort of dystrophic dogs supports delivery of a recombinant AAV for exon skipping in Duchenne patients. Mol Ther 2014;22(11):1923–1935.
- Le Guiner C, Servais L, Montus M, et al. Long-term microdystrophin gene therapy is effective in a canine model of Duchenne muscular dystrophy. Nat Commun. 2017 Jul 25;8:16105.
- Lee KD, Kuo TK, Whang-Peng J, et al. In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology. 2004 Dec;40(6):1275-84.
- Lima MI, Tafuri A, Araújo AC, et al. Cervical intraepithelial neoplasia recurrence after conization in HIV-positive and HIV-negative women. Int J Gynaecol Obstet. 2009;104(2):100–104.

- Liverani CA, Di Giuseppe J, Clemente N, et al. Length but not transverse diameter of the excision specimen for high-grade cervical intraepithelial neoplasia (CIN 2-3) is a predictor of pregnancy outcome. Eur J Cancer Prev. 2016 Sep;25(5):416-22.
- Lodi CT, Michelin MA, Lima MI, et al.Factors associated with recurrence of cervical intraepithelial neoplasia after conization in HIV-infected and noninfected women. Arch Gynecol Obstet. 2011;284(1):191–197.
- Luhn P, Walker J, Schiffman M, et al. The role of co-factors in the progression from human papillomavirus infection to cervical cancer. Gynecol Oncol. 2013;128(2):265–270.
- Iwahashi M, Muragaki Y, Ikoma M. et al.Immunohistochemical analysis of collagen expression in uterine leiomyomata during the menstrual cycle. Exp Ther Med. 2011 Mar;2(2):287-290. .
- Ma S, Xie N, Li W, et al. Immunobiology of mesenchymal stem cells. Cell Death Differ. 2014 Feb;21(2):216-25. .
- Magimaidas A, Hoffman B, Liebermann D. Gadd45b deficiency impairs G2/M cell cycle progression leading to premature senescence. Cancer Research. 2014;74:2252.
- Malvasi A, Cavallotti C, Nicolardi G,et al. NT, NPY and PGP 9.5 presence in myomeytrium and in fibroid pseudocapsule and their possible impact on muscular physiology. Gynecol Endocrinol. 2013 Feb;29(2):177-81.
- Maruyama T, Miyazaki K, Masuda H, et al. Review: human uterine stem/progenitor cells: implications for uterine physiology and pathology. Placenta. 2013 Mar;34 Suppl:S68-72..
- Mas A, Cervelló I, Fernández-Álvarez A et al. Overexpression of the truncated form of high mobility group a proteins (HMGA2) in human myometrial cells induces leiomyomalike tissue formation. Mol Hum Reprod. 2015 Apr;21(4):330-8.
- Mas A, Cervello I, Gil-Sanchis C, et al. Current understanding of somatic stem cells in leiomyoma formation. Fertil Steril. 2014 Sep;102(3):613-20..
- Mauro A. Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol 1961;9:493–495.
- McCredie MR, Sharples KJ, Paul C, et al. Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study. Lancet Oncol. 2008 May;9(5):425-34.
- McNally EM. New approaches in the therapy of cardiomyopathy in muscular dystrophy. Annu. Rev. Med. 2007. 58, 75–88.
- McWilliams MM, Chennathukuzhi VM. Recent Advances in Uterine Fibroid Etiology. Semin Reprod Med. 2017 Mar;35(2):181-189.
- Medzhitov R. Inflammation 2010: new adventures of an old flame. Cell 2010; 140: 771–776.
- Modugno F, Ness RB, Chen C, et al. Inflammation and endometrial cancer: a hypothesis. Cancer Epidemiol Biomarkers Prev. 2005 Dec;14(12):2840-7.
- Monaco AP, Bertelson CJ, Liechti-Gallati S, et al. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics 1988.2, 90–95.

- Moscicki AB, Ma Y, Wibbelsman C, et al. Rate of and Risks for Regression of CIN-2 in adolescents and young women. Obstet Gynecol. 2010; 116: 1373–80.
- Motamedi M, Böhmer G, Neumann HH, et al. CIN III lesions and regression: retrospective analysis of 635 cases. BMC Infect Dis. 2015 Nov 21;15:541.
- Mouly V, Aamiri A, Pe´ rie´ S, et al. Myoblast transfer therapy: is there any light at the end of the tunnel? Acta Myol 2005;24(2):128–133.
- Munk AC, Kruse AJ, van Diermen B, et al. Cervical intraepithelial neoplasia grade 3 lesions can regress. APMIS. 2007; 115: 1409-14.
- Naghdi M, Tiraihi T, Namin SA, et al. Transdifferentiation of bone marrow stromal cells into cholinergic neuronal phenotype: a potential source for cell therapy in spinal cord injury. Cytotherapy. 2009;11(2):137-52.
- Nakahara H, Dennis JE, Bruder SP, et al. In vitro differentiation of bone and hypertrophic cartilage from periosteal-derived cells. Exp Cell Res. 1991;195(2):492–503.
- Nance ME, Hakim CH, Yang NN, et al. Nanotherapy for Duchenne muscular dystrophy. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2018 Mar;10(2).
- Narayan G, Arias-Pulido H, Koul S, et al. Frequent promoter methylation of CDH1, DAPK, RARB, and HIC1 genes in carcinoma of cervix uteri: its relationship to clinical outcome. Mol Cancer. 2003;2:24.
- Nauseef WM. How human neutrophils kill and degrade microbes: an integrated view. Immunol Rev. 2007 Oct;219:88-102.
- Negroni E, Bigot A, Butler-Browne GS, et al. Cellular Therapies for Muscular Dystrophies: Frustrations and Clinical Successes. Hum Gene Ther. 2016 Feb;27(2):117-26.
- Nemeth K, Keane-Myers A, Brown JM, et al. Bone marrow stromal cells use TGF-beta to suppress allergic responses in a mouse model of ragweed-induced asthma. Proc Natl Acad Sci USA. 2010;107: 5652–5657.
- Nemeth K, Mezey E. Bone marrow stromal cells as immunomodulators. A primer for dermatologists. J Dermatol Sci. 2015;77(1):11–20.
- Noh H, Ahn H-J, Lee W-J, et al. The molecular signature of in vitro senescence in human mesenchymal stem cells. Genes Genom. 2010;32:87–93.
- Ono M, Qiang W, Serna VA et al. Role of stem cells in human uterine leiomyoma growth. PLoS One. 2012;7(5):e36935. .
- Ono M, Yin P, Navarro A et al. Paracrine activation of WNT/β-catenin pathway in uterine leiomyoma stem cells promotes tumor growth. Proc Natl Acad Sci U S A. 2013 Oct 15;110(42):17053-8..
- Ono M, Yin P, Navarro A, et al. Inhibition of canonical WNT signaling attenuates human leiomyoma cell growth. Fertil Steril. 2014 May;101(5):1441-9.
- Orciani M, Caffarini M, Biagini A, et al. Chronic Inflammation May Enhance Leiomyoma Development by the Involvement of Progenitor Cells. Stem Cells Int. 2018 May 13;2018:1716246.
- Orciani M, Caffarini M, Lazzarini R, et al. Mesenchymal stem cells from cervix and age: new insights into CIN regression rate. Oxid Med Cell Longev. 2018 Dec 2;2018:1545784.

- Orciani M, Campanati A, Caffarini M, et al. T helper (Th)1, Th17 and Th2 imbalance in mesenchymal stem cells of adult patients with atopic dermatitis: at the origin of the problem. Br J Dermatol. 2017 Jun;176(6):1569-1576.
- Orciani M, Lazzarini R, Scartozzi M, et al. The response of breast cancer cells to mesenchymal stem cells: a possible role of inflammation by breast implants. Plast Reconstr Surg. 2013 Dec;132(6):899e-910e..
- Orciani M, Sorgentoni G, Olivieri F, et al. Inflammation by breast implants and adenocarcinoma: not always a bad company. Clin Breast Cancer. 2017 Jul;17(4):286-292..
- Orciani M, Sorgentoni G, Torresetti M, et al. MSCs and inflammation: new insights into the potential association between ALCL and breast implants. Breast Cancer Res Treat. 2016 Feb;156(1):65-72..
- Panepucci RA, Siufi JL, Silva WA Jr, et al. Comparison of gene expression of umbilical cord vein and bone marrow-derived mesenchymal stem cells. Stem Cells. 2004;22(7):1263-78.
- Papoutsis D, Rodolakis A, Mesogitis S, et al. Regeneration of uterine cervix at 6 months after large loop excision of the transformation zone for cervical intraepithelial neoplasia. BJOG 2012;119:678-84.
- Park CW, Kim KS, Bae S, et al. Cytokine secretion profiling of human mesenchymal stem cells by antibody array. Int J Stem Cells. 2009 May;2(1):59-68.
- Parker WH. Etiology, symptomatology, and diagnosis of uterine myomas. Fertil Steril. 2007 Apr;87(4):725-36..
- Partridge TA, Morgan JE, Coulton GR, et al. Conversion of mdx myofibres from dystrophin negative to -positive by injection of normal myoblasts. Nature 1989;337(6203):176–179.
- Pe´ault B, Rudnicki M, Torrente Y, et al. Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. Mol Ther 2007;15(5):867–877.
- Pe´rie´ S, Trollet C, Mouly V, et al. Autologous myoblast transplantation for oculopharyngeal muscular dystrophy: a phase I/IIa clinical study. Mol Ther 2014;22(1):219–225.
- Petrof BJ, Shrager JB, Stedman HH, et al. Dystrophin protects the sarcolemma from stresses developed during muscle contraction. Proc Natl Acad Sci U S A. 1993; 90:3710–3714.
- Plumas J, Chaperot L, Richard MJ, et al. Mesenchymal stem cells induce apoptosis of activated T cells. Leukemia. 2005 Sep;19(9):1597-604.
- Practice Committee of the American Society for Reproductive Medicine. Myomas and reproductive function. Fertil Steril. 2006 Nov;86(5 Suppl 1):S194-9. Review.
- Protic O, Toti P, Islam MS, et al. Possible involvement of inflammatory/reparative processes in the development of uterine fibroids. Cell Tissue Res. 2016 May;364(2):415-27.
- Purohit P, Vigneswaran K. Fibroids and Infertility. Curr Obstet Gynecol Rep. 2016;5:81-88.
- Rackow BW, Taylor HS. Submucosal uterine leiomyomas have a global effect on molecular determinants of endometrial receptivity. Fertil Steril. 2010 Apr;93(6):2027-34.
- Rafei M, Campeau PM, Aguilar-Mahecha A, et al. Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th17 T cells in a CC chemokine ligand 2-dependent manner. J Immunol 2009; 182: 5994–6002.

- Ramzi S Cotran; Vinay Kumar; Tucker Collins; Stanley L Robbins. Robbins Pathologic Basis of Disease. Philadelphia : Saunders, ©1999.
- Reza M, Laval SH, Roos A, et al. Optimization of internally deleted dystrophin constructs. Hum Gene Ther Methods 2016;27(5):174–186.
- Roberts RG, Gardner RJ, Bobrow M. Searching for the 1 in 2,400,000: a review of dystrophin gene point mutations. Hum. Mutat.1994. 4,1–11.
- Rocheteau P, Gayraud-Morel B, Siegl-Cachedenier I, et al. A subpopulation of adult skeletal muscle stem cells retains all template DNA strands after cell division. Cell 2012;148(1–2):112–125.
- Ryan A. Denu and Peiman Hematti Effects of Oxidative Stress on Mesenchymal Stem Cell Biology Oxid Med Cell Longev. 2016; 2016: 2989076.
- Safford KM, Hicok KC, Safford SD, et al. Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun. 2002 Jun 7;294(2):371-9.
- Sahin E, Depinho RA. Linking functional decline of telomeres, mitochondria and stem cells during ageing. Nature 2010; 464: 520–8.
- Sanjabi S, Zenewicz LA, Kamanaka M, et al. Anti-inflammatory and pro-inflammatory roles of TGF-beta, IL-10, and IL-22 in immunity and autoimmunity. Curr Opin Pharmacol. 2009;9:447-53.
- Sellheyer K, Krahl D. Skin mesenchymal stem cells: prospects for clinical dermatology. J Am Acad Dermatol. 2010;63(5):859–865.
- Serrano AL, Muñoz-Cánoves P. Regulation and dysregulation of fibrosis in skeletal muscle. Exp Cell Res. 2010 Nov 1;316(18):3050-8.
- Sheridan C. Duchenne muscular dystrophy drugs at the crossroads, as newer agents advance. Nat Biotech 2016; 34: 675–76.
- Shervington A, Lu C. Expression of multidrug resistance genes in normal and cancer stem cells. Cancer Invest. 2008 Jun;26(5):535-42.
- Shi C, Zhu Y, Su Y, et al. Stem cells and their applications in skin-cell therapy. Trends Biotechnol. 2006;24(1):48–52.
- Silva MT, Correia-Neves M. Neutrophils and macrophages: the main partners of phagocyte cell systems. Front Immunol. 2012 Jul 4;3:174.
- Sinclair DC, Mastroyannis A, Taylor HS. Leiomyoma simultaneously impair endometrial BMP-2-mediated decidualization and anticoagulant expression through secretion of TGF-β3. J Clin Endocrinol Metab. 2011 Feb;96(2):412-21
- Song T, Seong SJ, Kim BG. Regeneration Process After Cervical Conization for Cervical Intraepithelial Neoplasia. Obstet Gynecol. 2016;128:1258-64.
- Sotiropoulou PA, Perez SA, Gritzapis AD, et al. Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells. 2006 Jan;24(1):74-85.
- Soundararajan M, Kannan S. Fibroblasts and mesenchymal stem cells: Two sides of the same coin? J Cell Physiol. 2018 Dec;233(12):9099-9109.
- Sozen I, Arici A. Interactions of cytokines, growth factors, and the extracellular matrix in the cellular biology of uterine leiomyomata. Fertil Steril. 2002 Jul;78(1):1-12.

- Spaggiari GM, Capobianco A, Abdelrazik H, et al. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood. 2008 Feb 1;111(3):1327-33.
- Spaggiari GM, Capobianco A, Becchetti S, et al. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood. 2006 Feb 15;107(4):1484-90.
- Spencer MJ, Tidball JG. Do immune cells promote the pathology of dystrophin-deficient myopathies? Neuromuscul Disord. 2001 Sep;11(6-7):556-64.
- Stewart EA,.Uterine fibroids. Lancet. 2001 Jan 27;357(9252):293-8..
- Stock P, Brückner S, Winkler S, et al. Human bone marrow mesenchymal stem cell-derived hepatocytes improve the mouse liver after acute acetaminophen intoxication by preventing progress of injury. Int J Mol Sci. 2014 Apr 22;15(4):7004-28.
- Sunderkötter C, Nikolic T, Dillon MJ, et al. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. J Immunol. 2004 Apr 1;172(7):4410-7.
- Tabebordbar M, Zhu K, Cheng JKW, et al. In vivo gene editing in dystrophic mouse muscle and muscle stem cells. Science 2016;351(6271):407– 411.
- Tang XM, Dou Q, Zhao Y, et al. The expression of transforming growth factor-beta s and TGF-beta receptor mRNA and protein and the effect of TGF-beta s on human myometrial smooth muscle cells in vitro. Mol Hum Reprod. 1997 Mar;3(3):233-40.
- Tocci A, Greco E, Ubaldi FM. Adenomyosis and 'endometrial-subendometrial myometrium unit disruption disease' are two different entities. Reprod Biomed Online. 2008 Aug;17(2):281-91.
- Tonlorenzi R, Dellavalle A, Schnapp E, et al. Isolation and characterization of mesoangioblasts from mouse, dog, and human tissues. Curr Protoc Stem Cell Biol. 2007 Dec;Chapter 2:Unit 2B.1.
- Toyserkani NM, Christensen ML, Sheikh SP, et al. Adipose-derived stem cells: new treatment for wound healing? Ann Plast Surg. 2015;75(1):117–123.
- Troyer DL, Weiss ML. Wharton's jelly-derived cells are a primitive stromal cell population. Stem Cells. 2008;26(3):591–599.
- Ullah I, Subbarao RB, Rho GJ. Human mesenchymal stem cells - current trends and future prospective. Biosci Rep. 2015 Apr 28;35(2). pii: e00191.
- van Deutekom JC, Janson AA, Ginjaar IB, et al. Local dystrophin restoration with antisense oligonucleotide PRO051. N Engl J Med 2007; 357: 2677–86.
- van Riggelen J, Buchwalter G, Soto U, et al. Loss of net as repressor leads to constitutive increased c-fos transcription in cervical cancer cells. J Biol Chem. 2005;280:3286-94.
- Vasiljević N, Scibior-Bentkowska D, Brentnall A, et al. A comparison of methylation levels in HPV18, HPV31 and HPV33 genomes reveals similar associations with cervical precancers. J Clin Virol. 2014 Mar;59(3):161-6..
- Villalta SA, Rinaldi C, Deng B, et al. Interleukin-10 reduces the pathology of mdx muscular dystrophy by deactivating M1 macrophages and modulating macrophage phenotype. Hum Mol Genet. 2011 Feb 15;20(4):790-805.

- Virmani AK, Muller C, Rathi A, et al. Aberrant methylation during cervical carcinogenesis. Clin Cancer Res 2001. 7(3):584–589.
- Vulin A, Barthe´le´my I, Goyenvalle A, et al. Muscle function recovery in golden retriever muscular dystrophy after AAV1-U7 exon skipping. Mol Ther 2012;20(11):2120–2133.
- Wallach EE, Vlahos NF. Uterine myomas: an overview of development, clinical features, and management. Obstet Gynecol. 2004 Aug;104(2):393-406. Review.
- Wang Y, Liu XH, Li YH, et al. The paradox of IL-10-mediated modulation in cervical cancer. Biomed Rep. 2013;1:347-351
- Waterman RS, Tomchuck SL, Henkle SL, et al. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. PLoS One 2010; 5: e10088.
- Wegienka G. Are uterine leiomyoma a consequence of a chronically inflammatory immune system? Med Hypotheses. 2012 Aug;79(2):226-31.
- Wei LH, Kuo ML, Chen CA et al. Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. Oncogene. 2003;22:1517-27.
- Weiss G, Goldsmith LT, Taylor RN, et al. Inflammation in reproductive disorders. Reprod Sci. 2009 Feb;16(2):216-29.
- Welford SM, Giaccia AJ. Hypoxia and senescence: the impact of oxygenation on tumor suppression. Mol Cancer Res. 2011;9:538–44.
- Widschwendter A, Gattringer C, Ivarsson L, et al. Analysis of aberrant DNA methylation and human papillomavirus DNA in cervicovaginal specimens to detect invasive cervical cancer and its precursors. Clin Cancer Res. 2004; 10(10):3396–3400.
- Yin P, Ono M, Moravek MB et al. Human uterine leiomyoma stem/progenitor cells expressing CD34 and CD49b initiate tumors in vivo. J Clin Endocrinol Metab. 2015 Apr;100(4):E601-6.
- Yoshino O, Nishii O, Osuga Y, et al. Myomectomy decreases abnormal uterine peristalsis and increases pregnancy rate. J Minim Invasive Gynecol. 2012 Jan-Feb;19(1):63-7.
- Zhang P, Andrianakos R, Yang Y, et al. Kruppel-like factor 4 (Klf4) prevents embryonic stem (ES) cell differentiation by regulating Nanog gene expression. Journal of Biological Chemistry 2010;285:9180-9.
- Zhang P, Zhang C, Hao J et al. Use of X-chromosome inactivation pattern to determine the clonal origins of uterine leiomyoma and leiomyosarcoma. Hum Pathol. 2006 Oct;37(10):1350-6..
- Zhang Q, Shi S, Liu Y, et al. Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation- related tissue destruction in experimental colitis. J Immunol 2009; 183: 7787–7798.
- Zhao W, Wang Y, Wang D, et al. TGF-beta expression by allogeneic bone marrow stromal cells ameliorates diabetes in NOD mice through modulating the distribution of CD4þ T cell subsets. Cell Immunol 2008; 253: 23–30.
- Zhou L, Lu H. Targeting fibrosis in Duchenne muscular dystrophy. J Neuropathol Exp Neurol. 2010 Aug;69(8):771-6.

- Zuk PA, Zhu M, Ashjian P, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002;13(12):4279–4295.