Chapter 27

Skeletal Muscle Regeneration for Clinical Application

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Additional information is available at the end of the chapter

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1. Introduction

Comprising nearly 50% of the human body [1] skeletal muscles compose the machinery that sets the body in movement. When well-trained, muscles have the capability to protect joints and bones from daily waste and trauma [2]. They hold an intrinsic protective mechanism against cancer formation and metastasis settling [3] and are at the same time the main energy reservoir of the body storing more than 80% of our glycogen reserve [4]. Hence, muscle tissue is associated to several functions and networks with different parts of the body. It is composed of muscle fibers, the contractile units, which are bound together by connective tissue. Most importantly, skeletal muscles display an astonishing regenerative capacity [5]. Due to resident stem cells, one week after severe trauma new myotubes are already being formed, and within 28 days after trauma muscle regeneration is almost complete [6]. These intrinsic features turn the skeletal muscle into a very interesting topic of study in regenerative medicine. Taking advantage of the regenerative potential of stem and precursor cells, skeletal muscle is constantly renewed in response to injury, damage or aging. It is this natural process that researchers are about to harness in order to help patients with many muscle diseases and diseases that causes weakness or destruction of the muscle - for instance stress urinary incontinence (SUI), muscular dystrophy. In this chapter, the focus will be on the regeneration of the skeletal muscle and especially in the case of incontinence. Urinary incontinence is the involuntary loss of urine and is a major medical problem affecting millions of people worldwide. It impairs the quality of life of patients and involves high healthcare costs. The main reason provoking SUI is the damage of the sphincter muscle due to childbirth, surgical treatments (as prostatectomy) or as an effect of aging. Current treatment encompasses behavioral training, pelvic floor exercising, drugs, medical devices and surgery. Unfortunately, all these options permit only limited recovery: short-term relief and are often accompanied with complications. The ultimate goal will be to prevent disease progression and to restore the tissue and its functions.

Stem cell therapy as a treatment for skeletal muscle diseases is becoming a reality and it represents a promising alternative for muscle regeneration and for treating SUI in a more complete and definitive manner.

In this chapter, the homeostasis and maintenance of skeletal muscle is explained in order to understand the basis behind muscle regeneration. As different types of stem cells have been demonstrated to form fibers and to develop into skeletal muscle, cell sources for a muscle cell therapy is discussed. Some of them have also been applied successfully in preclinical and clinical studies that are going to be described. Finally, we are going to highlight the parts important for the translational effort into clinics including biomaterials, cell delivery, imaging, regulatory affairs, and manufacturing.

2. Muscle homeostasis

2.1. Satellite cells are the secret of skeletal muscle regeneration

The secret of skeletal muscle staggering regenerative capacity is found in the specific components of its cell niche. The muscle tissue is composed of long and slender cells that form muscle fibers grouped in bundles (Figure 1). Adjacent to these myofibers, a heterogeneous pool of subsarcolemmal progenitor and stem cells known as muscle satellite cells (SC), respectively committed to myogenic differentiation or to self-renewal, guarantee a fast and efficient regenerative process after trauma [7]. These cells, activated by injury [8], work hierarchically to maintain the *in situ* pool of cells (Figure 1) and to reconstruct damaged tissue in less than one month by differentiating into new myotubes.

2.2. Injury and inflammation — The role of inflammation

After trauma an inflammatory infiltrate can be observed when neutrophils, macrophages, satellite cells and later myoblasts work chronologically together cleaning up damaged fibers and reconstructing new functional myotubes. Neutrophils are the first cells to arrive at the site of injury, followed by macrophages three hours after damage [6]. Through the combined action of free radicals, growth factors and chemotactic factors these inflammatory cells contribute both to injury and repair [9]. Without the neutrophil-related oxidative and proteolytic modifications of damaged tissue, phagocytosis of debris would not be possible [10]. Macrophages are the major housecleaners that remove remaining debris of fibers. Furthermore, macrophages produce proteases to lyse the sarcolemma membrane, which allows activation and proliferation of SC [11]. Dismantling of the extracellular matrix is key to SC activation, and the up-regulation of metalloproteinase is required for muscle regeneration [12]. Macrophage infiltration is also important for SC activation and proliferation by activating NF-κB via TWEAK ligand [13]. Quiescent SCs are still found between the basal membrane and sarcolemma until the third day after injury. Subsequently, they are slowly replaced by cells with large nuclei, nucleoli, and cytoplasmatic processes filled with ribonucleoprotein granules. These myoblasts display an initial exponential growth phase and after the seventh day they start to form myotubes with centrally placed nuclei and peripheral my-

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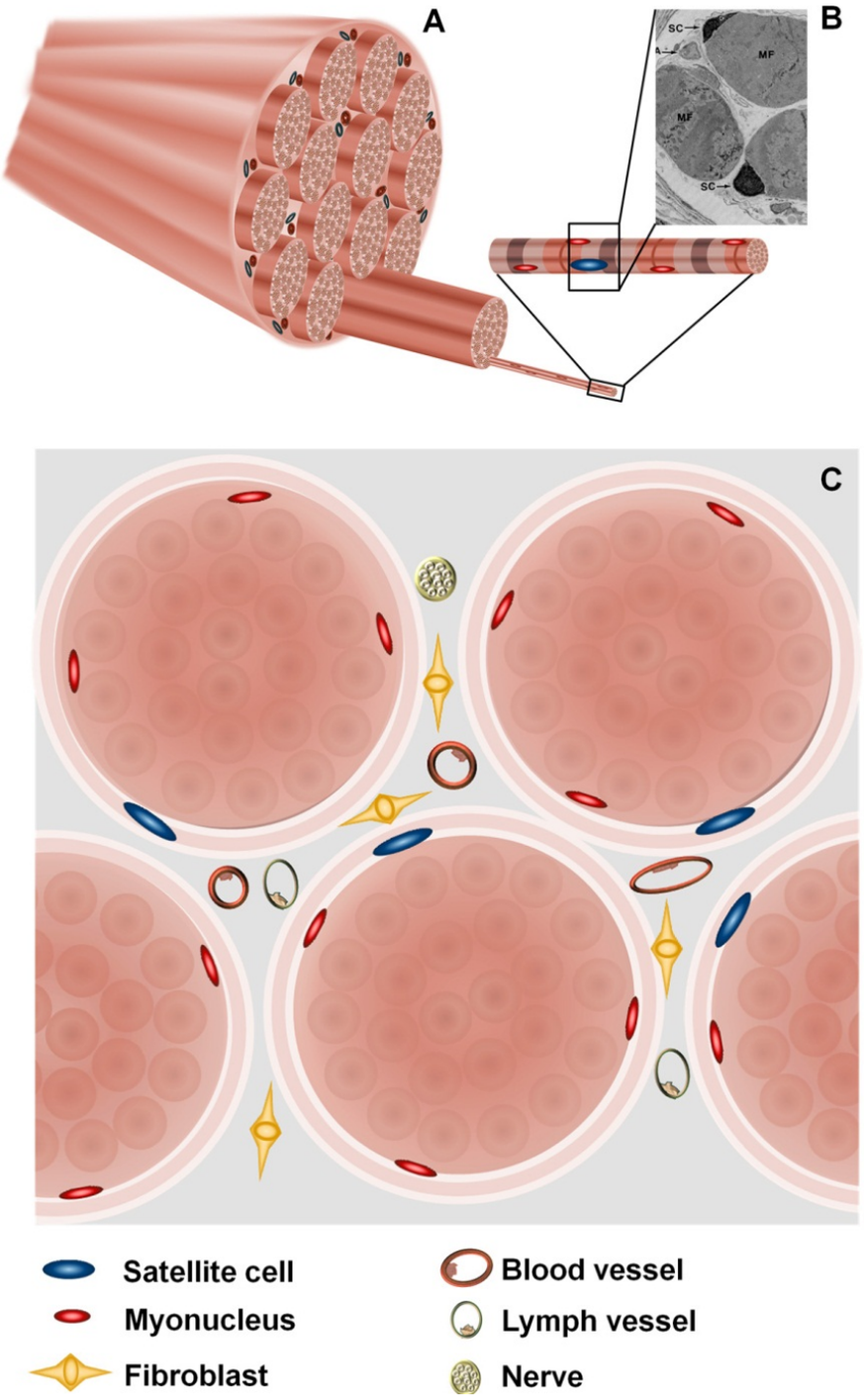

Figure 1. The muscle niche is the secret of skeletal muscle astounding regenerative capacity. Attached to bones, skeletal muscle are organs composed of skeletal muscle tissue, connective tissue, nerves and blood vessels. Each individual skeletal muscle is composed by hundreds or thousands bundles of muscle fibers that are single cylindrical muscle cells. (A) The connective tissue surrounding each muscle is called epimysium, and its projections that separe muscle bundles are called perimysium. (B) The connective tissue between single muscle fibers is called endomysium and servers as the muscle satellite cells (SCs) niche. SCs are subsarcolemmal cells that can be activated to regenerate new muscle fibers. (C) Skeletal Muscle tissue is not only formed by muscle fiber, but also by acellular matrix, cellular components, blood and lymphatic vessels and nerves. Altogether, these muscle niche components play a distinct role on muscle regeneration and on muscle progenitor cell regulation.

ofibrils. On the periphery of these newly formed myotubes a new population of subsarcolemmal quiescent cells replenishes the SC pool [6]. Finally, mature myofiber nuclei do not display mitotic figures throughout the regeneration process, demonstrating that the damaged fiber cannot heal itself without the activation of satellite cells.

2.3. The role of the muscle niche in muscle regeneration

Components of the muscle niche are important for skeletal muscle regeneration and satellite cell activation. The basal lamina is the common anatomic site of satellite cells and also contributes to cell fate. The basal lamina is rich in $\alpha 7\beta 1$ integrin which acts directly in the anchorage, adhesion and quiescence of satellite cells [14]. These integrin functions also comprise the migration and proliferation of developing myoblasts [15], the formation and integrity of neuromuscular junctions [16], as well as the binding of muscle fibers. Another integrin, VLA-4, is expressed as myotubes form and influences the alignment and fusion of myoblasts [17]. Finally, the calcium-dependent cell adhesion protein M-cadherin is a morphoregulatory molecule facilitating myoblast fusion and cell adhesion to its adjacent myofibers [18, 19].

The surrounding acellular matrix (ACM) contains a number of components that can influence the behavior and regulate the growth of muscle progenitor cells. The ACM is a source of hepatocyte [20] and fibroblast [21] growth factors, which act on the activation of satellite cells, proliferation and inhibition of differentiation. Another factor produced by the ACM is the endothelial growth factor, which promotes satellite cell activation and cell survival after injury [22]. Finally, the aged ACM is capable of impairing the regenerative potential of satellite cells and inducing fibrosis by activating the Wnt signaling pathway [23].

Fibroblasts are the main source of collagen in the muscular interstitial space [24]. They continuously promote the formation of the basal lamina during myogenesis [25] and after muscle injury proliferate hand in hand with Pax7 positive satellite cells, orchestrating the fine balance between muscle reconstruction and fibrosis formation [26]. These fibroblasts prevent premature activation and differentiation of muscle progenitor cells, thereby avoiding depletion of the pool of satellite cells. Accordingly, satellite cells are sufficient to regulate the ingrowth of fibroblasts and fibrosis formation [26]. Fibroblasts are also involved in myosin switch from fetal to adult muscle, specially promoting Myosin Heavy Chain type 1 expression (slow twitch) in several limb muscles in the fetal mouse and in the soleus in the adult muscle [27].

Circulating and locally produced soluble factors participate in the signaling pathway that regulates satellite cell activity. During exercise and stretching muscle fibers liberate hepatocyte growth factor (HGF) through nitric oxide stimulation and induce activation of satellite cells [28]. HGF can also activate satellite cells by activating the sphingolipid signaling cascade upon disruption of the laminin-integrin adhesion in the event of trauma [29]. Furthermore, the insulin-like growth factor 1 (IGF-1), a potent mitogen produced locally during muscle hypertrophy and injury, can induce activation, proliferation and differentiation of satellite cells [8, 30]. In contrast, mysotatin, a growth differentiation factor and member of the TGF-beta protein family secreted by adult skeletal muscle, is capable of inhibiting activation and self-

renewal of quiescent cells [31]. Finally, a hormone produced by the thyroid gland and responsible for inducing hypercalcemia named Calcitonin [32], has been associated with delay of satellite cell activation [33]. Together all these components and products of the muscle niche are key regulators of all the development and regeneration processes of skeletal muscle.

2.4. Satellite cells are also required for exercise related muscle turn–over

Exercise is capable of activating muscle gene transcription within seconds and these molecular responses can last for hours even after exercise cessation [34]. During endurance exercise, muscle consume large amounts of oxygen to generate energy by breaking down carbohydrates and posteriorly fat [35]. Muscle fibers are not in a smooth continuous muscle contraction during exercise, but rather act as a series of small groups of fibers contracting at the same time [36]. This occurs due to stimulation of neuromuscular junctions of terminal branches of axons whose cell body is in the anterior horn of the spinal cord. Altogether, these nerve and muscle components comprise the motor unit [37] and conduce impulses that enable sharp muscle contraction within milliseconds [38]. A signaling pathway is then activated by rapamycin kinase (mTOR) leading to hypertrophic changes in muscle mass [39]. The opposing effect is found during starvation when the AMP-activated protein kinase (AMPK) is switched on to up-regulate energy-conserving processes and ultimately induce muscle atrophy [39]. However, exercise is sufficient to increase the pool of stem cells reversing the effects of atrophy after prolonged limb immobilization [40].

After a trauma or during exercise nitric oxide is liberated and modulates the activation of satellite cells [41, 42]. Another evidence of this cell addition during exercise is the decrease of telomeres length detected in marathon runners, which correlates to their running hours [43]. Endurance exercise has been reported to stimulate the production of free radicals like nitric oxide [44], which has been shown to again induce activation of satellite cells thereby increasing muscle turn-over [28]. On the other hand, during muscle atrophy caused by limb immobilization an apoptotic decrease of myonuclei occurs [45] associated with a decrease in mitotic activity of satellite cells [46]. These findings underline the involvement of satellite cells in the regulation of muscle mass during exercise.

2.5. Markers for satellite cells

A transcriptional network controls progression of both embryonic and adult muscle stem cells [47]. Quiescent muscle embryonic progenitor cells can be identified by the co-expression of the paired-domain transcription factors Pax3 and Pax7 (Figure 2) and are maintained as a self-renewing proliferative population [48]. During embryogenesis Pax3 is required to maintain muscle progenitor cells in the somite and further induce cell migration to the required site of skeletal myogenesis [49]. Indeed the normal expression of Pax3 seems to be decisive for the development of normal muscle, and its mutation promotes malignant growth and induces tumorigenesis in alveolar rhabdomyosarcoma tumor cells [50]. However, its down-regulation is necessary for final cell commitment to myogenesis and leads to rapid and robust entry into the myogenic differentiation program [49]. The expression of transcription factor Pax7 is detectable in cells starting from the embryonic

muscle progenitor to the quiescent and activated satellite cells (Figure2). Its induction in muscle-derived stem cells induces satellite cell specification by restricting alternate developmental programs [51].

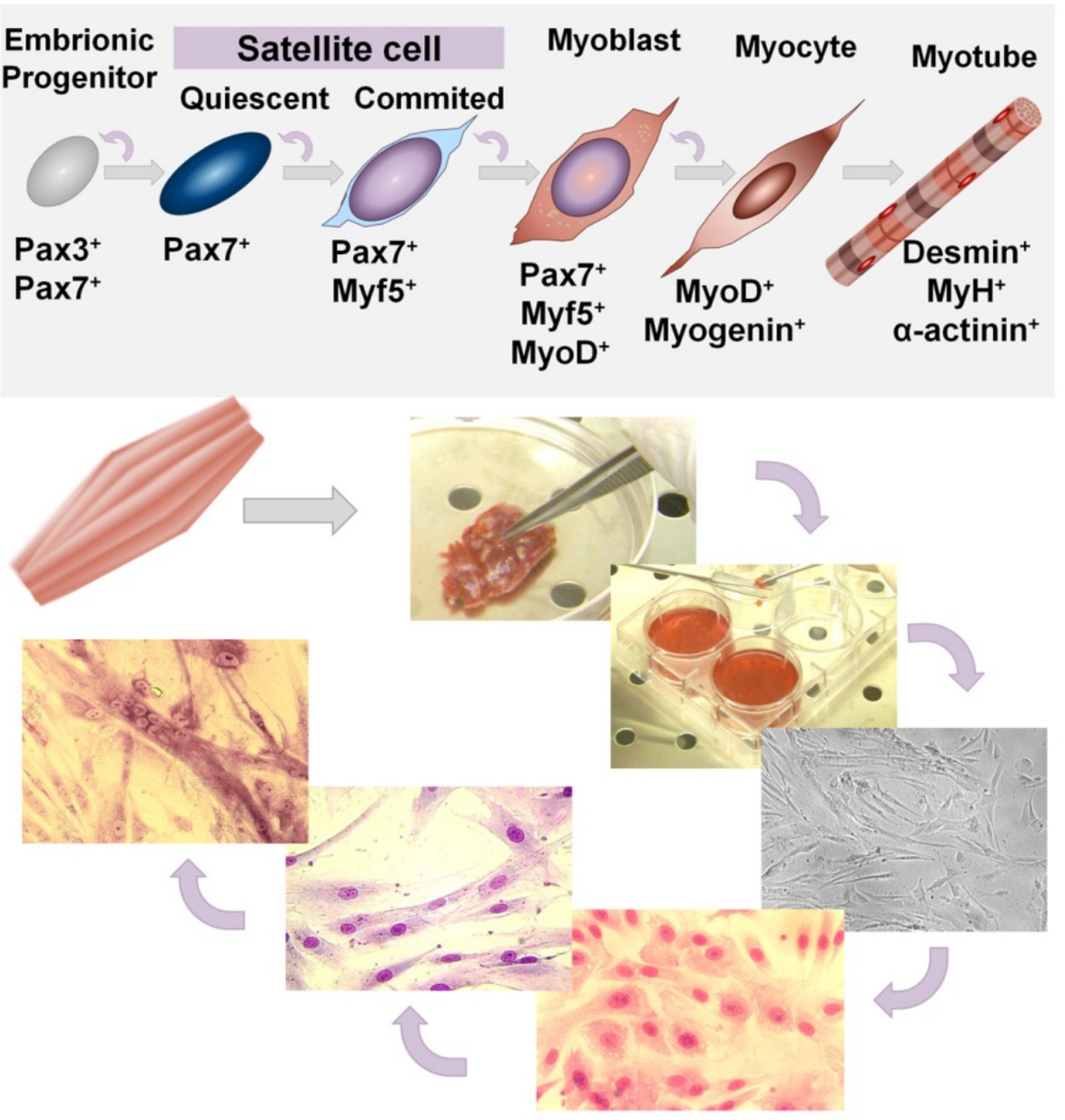

Figure 2. Myogenic cell characterization and culture. Myogenic cell lineage can be identified in each differentiation state and pursue tightly regulated proliferation and differentiation cycles. From the embryonic state until the terminal differentiation into muscle fibers an intricate network of transcription factors regulates the fate of muscle progenitor cells. These cells can be isolated from any skeletal muscle tissue, grown in culture and reimplanted into a damaged muscle to promote muscle regeneration.

Specific molecular markers have been demonstrated to distinguish between activated and quiescent SC. Quiescent satellite cells express the transcription factor Pax7, after activation in

co-expression with MyoD [52]. This dual expression is followed by a proliferative phase, down-regulation of Pax7 and terminal differentiation. If Pax3 and Pax7 down-regulation do not occur *in vitro* differentiation is blocked [53, 54]. In this context microRNAs (miRNAs) play a regulatory role conferring robustness to developmental timing by posttranscriptional repression of genetic programs of progenitor and satellite cells [55]. They allow rapid gene program transitions from proliferation to differentiation, blocking PAX3 [56] and Pax7 [57] activity in progenitor and satellite cells.

This interplay during development is required to ignite the commitment of satellite cells to the myogenic program, to activate the myogenic regulatory factors Myf-5 and MyoD and to promote terminal muscle differentiation [55] [58] [59], which are decisive to subsequent myoblast cell cycle progression or exit into differentiation. Through the action of the myogenic regulatory factors (MRFs), Myf5 and MyoD, the muscle progenitor cells ($Pax3^+$) and quiescent satellite cells ($Pax3^+/Pax7^+$) become muscle lineage committed and activated myoblasts [60]. They express *Myf5* and *Mrf4* and rapidly give rise to $Desmin^+$ cells, whose differentiation is regulated by myogenin, MyoD and MRF4 [61]. Completing these regulatory features, MyoD is also a main player in the intricate epigenetic cascade that controls skeletal myogenesis [62].

3. Muscle regeneration

3.1. Cell sources for skeletal cell therapy

Several types of cell populations have been identified as potentially efficient in muscle regeneration, especially in cell therapy. They are able to self-renew, proliferate and form muscle fibers. Among these cells some are muscle derived and some are from other origin.

3.2. Muscle–derived cells

Muscle satellite cells, which are squeezed between the plasma membrane and basement membrane of muscle fibers, are the natural source of muscle regeneration during homeostasis or after injury in postnatal stages [63]. They are specifically expressing the paired box transcription factor pax7 [51] and have been shown to be efficient in the muscle regeneration process. One study illustrated that as little as seven satellite cells were able to generate more than 100 muscle fibers in irradiated muscle [64]. Though, satellite cells isolated from different muscles are not equivalent: they produce muscle fibers with variable contractile abilities depending on the muscle of origin [65]. This can be explained by the fact that a satellite cell pool does not seem to consist of a homogeneous population of cells [66-70]. Once activated, satellite cells are triggered toward proliferation and differentiation by giving rise to muscle precursor cells that fuse and form skeletal muscle fibers [71]. The two techniques used to isolate muscle precursor cells are selection of single fibers that are cultured or mechanical processing of muscle biopsies and enzymatic treatment with a mixture of collagenase and dispase [64, 72-74]. The first method is claimed to be less aggressive and to better preserve the cells.

Another type of cells is isolated from muscle biopsies through a series of preplating stages. These cells are also recognized to have a myogenic profile and are capable to fuse and form skeletal muscles fibers. They are known as muscle-derived stem cells (MDSC) with characteristics of non-committed progenitor cells [75, 76] and are most probably originating from blood vessel walls [77]. Similarly, other cells types isolated from the muscle compartment such as mesoangioblasts and pericytes are involved in the muscle regeneration but are of non-myogenic origin. These are vessel-associated progenitors, not expressing myogenic markers such as Myf5 and MyoD even though they can differentiate to myotubes and fuse to form fibers [78-80]. More cell types with non-myogenic profile are found in the skeletal muscle and have recently been demonstrated to form fibers. Hence, skeletal myogenic precursors or muscle stem cells sorted by FACS are capable to reconstitute fibers in rodent models [72, 81]. The first type of cells is characterized by expression of β1-integrin (adhesion protein) and CXCR4 (SDF-1 receptor), the second type by α7-integrin (adhesion protein) and CD34 markers. Side populations are also isolated from muscle tissues and are expressing specific surface markers [82]. They are distinct from satellite cells and have been used successfully in muscle regeneration in rodent models [83-87]. Surprisingly, more types of cells of the skeletal muscle tissue can contribute to muscle regeneration. In fact, recently, a new type of myogenic cells, localized in the area of the interstitium between muscle fibers, has been characterized and is known as PW1-interstitial cells (PICs). They are characterized as positive for cell stress mediator PW1 but negative for Pax7; though they possess myogenic profile *in vitro* and lead to muscle regeneration *in vivo*, which includes the generation of satellite cells [88]. Hence, various types of cells isolated from skeletal tissue either mechanically or by flow cytometry are capable to regenerate muscle. In addition to the muscle there are more sources of stem/precursor cells isolated from other compartments.

3.3. Sources outside of the skeletal muscle compartment

Mesenchymal stem cells (MSC) are procured from bone marrow biopsies and are multipotent stem cells that give rise also to skeletal muscle fibers and participate to restore the satellite cell niche [89]. These cells are well characterized and involved in many different applications due to their multipotency as it is the case for adipose-derived stem cells (ADSC). The latter are easily harvested by liposuction, cultured *in vitro* and injected to restore muscle in the case of SUI [90, 91]. Embryonic stem cells, induced pluripotent stem cells and umbilical cord blood have been demonstrated to be good alternatives for skeletal muscle regeneration [74, 92, 93]. However, precaution should be taken when these types of cells are considered for further development in clinics, as different types of viruses are used during the process of myogenic induction. In addition, there are still potential tumorigenicity issues with this sort of cells that need to be solved before further clinical application.

Hence, the sources of stem/progenitor cells for skeletal muscle regeneration are large. Though, several important factors need to be considered when choosing the optimal source for treating patients. Autologous cell therapy avoids immunogenic reaction and therefore complications after the implantation procedure. Therefore, autologous satellite/muscle precursor cells are advantageous for muscle regeneration. They are committed to muscle restoration and

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therefore the most convenient cells for applications in cell therapy. Their dedication to one lineage offers an advantage over other, previously discussed sources, which are multipotent and hence differentiate also into non-muscular tissue cells. Furthermore, satellite / muscle precursor cells can be isolated in a simple procedure and are easily expanded in a GMP facility. They produce enough cells to be injected after 2-3 weeks, which is much faster than the 5 - 6 weeks required for muscle derived stem cells. For allogenic application, mesenchymal and adipose derived stem cells represent valid alternatives when satellite cells cannot be extracted from the skeletal muscle.

4. Preclinical and clinical studies

Several animal studies have been addressing the problem of stress urinary incontinence (SUI) and different strategies have been tested to restore continence, either by applying pharmacological therapies, bulking agents, sling surgical procedures or cell-based therapies [94]. Until now, the first three strategies mentioned above are commonly applied in clinics. However, the outcomes are associated with adverse events and limited effectiveness in middle and long terms [95-97]. Therefore, cell-based therapies are aiming to bring new solutions to the treatment of SUI. Numerous preclinical studies have been implementing stem/progenitor cell injections for restoration of muscle contraction in SUI. Animal models that mimic SUI are crucial for the understanding of effects and benefits of the different therapies options.

4.1. Animal model

To stimulate SUI in animals, various methods were applied. The goal is to injure one or several aspects of the urinary continence mechanisms to provoke incontinence as found in patients. The methods comprise the compression of the muscular and neurological system involved in continence by vaginal distension [98, 99], crush of the pudental nerve [100], damaging of anatomic supports such as fascia and pubourethral ligament [101-104] or destruction of intrinsic urethra by periurethral cauterization, urethral sphincterectomy, pudendal nerve transection and botulinum toxin periurethral injection [105-112]. One has to note that vaginal distension or pudendal nerve injury are relatively limited models due to the fact that the injury is naturally recovered after 2 weeks and thereby does not mimic an irreversible SUI. Eberli et al. have been describing a large animal model for SUI that was followed for 6 months. In this study, the sphincter muscle of dogs has been irreversibly damaged by surgically removing part of it. During the follow-up, the dogs were permanently affected by this procedure with long term decrease in sphincter pressures [107].

4.2. Preclinical studies

Rats are the preferred animal models for studying safety and efficacy of several cell types for treatment of SUI (table.1).

Cell type	Animal model	SUI model	Injection Target organ	Time point Weeks	Reference	Year
MDSC	SCID mice / Rats	Cryoinjury bladder	Bladder	1 to 4	Huard et al.	2002
MPC	Mice	Noxetin	Urethral	2 to 4	Yiou et al.	2002
MDSC	Rats	Sciatic nerve section	Urethral	4	Lee et al.	2002
MDPC	Rats	Sciatic nerve section	Urethral	2	Cannon et al.	2003
MPC	Rats	Electrocoagulation	Urethral	0.7 to 4	Yiou et al.	2003
MDSC	Rats	Pudendal nerve section	Urethral	12	Lee et al.	2004
MDC	Rats	Electrocauterization	Urethral	2 to 6	Chermansky et al.	2004
MDSC	Rats	Sciatic nerve section	Urethral sling	2	Cannon et al.	2005
MDC fibroblasts	Rats	Sciatic nerve section	Urethral	4	Kwon et al.	2006
Myoblasts	Rats	Cryoinjury / noxecin	Urethral	1 to 6	Praud et al.	2007
Myofibers	Pigs	Urethral injury	Myofiber implantation	4	Lecoeur et al.	2007
MDSC_FACS sorted	Rats	Nerve transection / sphincter injury	Urethral	4 to 12	Hoshi et al.	2008
ADSC	Rats	Vaginal dilatation	Urethral	4	Lin et al.	2010
BMSC	Rats	Sciatic nerve section	Urethral sling	4 to 12	Zou et al.	2010
BMSC	Rats	urethrolysis / cardiotoxin	Urethral	13	Kinebuchi et al.	2010
MDSC	Rats	Pudendal nerve section	Urethral	1 to 4	Xu et al.	2010
Myoblasts / ADSC	Rats	Vaginal dilatation	Urethral	4	Fu et al.	2010
UCBSC	Rats	Electrocauterization	Urethral	2 to 4	Lim et al.	2010
BMSC	Rats	Pudendal nerve section	Urethral	4 to 8	Corcos et al.	2011
BMSC	Rats	Pudendal nerve section	Urethral	4	Kim et al.	2011
BMSC	Rabbits	Cryoinjury	Urethral	1 to 2	Imamura et al.	2011
ADSC	Rats	Pudendal nerve section	Periurethral	3 to 4	Wu et al.	2011
ADSC	Rats	Pelvic nerve section	Periurethral	2 to 4	Watanabe et al.	2011
ADSC	Rats	Pudendal nerve section	Periurethral	8	Zhao et al.	2011
MPC	Dogs	Urethral sphincterecomy	Periurethral	24	Eberli et al.	2012
BMSC	Rats	Pudendal, other nerves section	Urethra and baldder neck	1 to 8	Du et al.	2012
MSC	Rats	Vaginal dilatation	intravenously	0.6 to 1.4	Cruz et al.	2012

Abbreviations: ADSC, adipose-derived stem cells; BMSC, bone-marrow mesenchymal stem cells; MDC, muscle-derived cells; MDSC, muscle-derived stem cells; MPC, muscle precursor cells; MSC, mesenchymal stem cells; UCBSC, umbilical cord blood stem cells;

Table 1. Animal studies for treating stress urinary incontinence based on cell therapy.

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Muscle derived cells were the first cells to be used for urethral regeneration and to demonstrate that cell therapy might represent an option for the treatment of SUI. Hence, MDSC, myoblasts, MPCs or muscle fibers injected around the injured area were surviving, participating in fiber formation and re-establishing muscle contractility [113-118]. In rat models, it was shown that the injected MDSC – isolated by preplating procedures or FACS-sorted - were participating actively in muscle regeneration for up to 3 months [119, 120]. Interestingly, in a dog study, a rare large animal model for SUI, transplanted MPCs were efficiently restoring the sphincter pressure to 80% of normal values during a half year follow-up period [121]. Concerning the speed of regeneration, Cannon et al. noticed 87% recovery after only 2 weeks post-injection and Chermansky et al. a full recovery after 4 weeks with myoblast and 6 weeks with MDSC [113, 116, 122]. Hence, muscle derived cells are able to incorporate the urethral structure and help recovering continence by reconstructing new fibers and connections with the surrounding cells – nerves, Schwan cells, vessels etc. [119, 121]. However, they are not the only kind of cells facilitating this cell therapy. Bone marrow derived mesenchymal stem cells, adipose-derived stem cells, umbilical cord blood stem cells (UCBSC) have been proven to also restore continence in animal SUI models [123-135]. In fact, different studied showed that these cells are contributing to the formation of fibers and contractile muscles which permit to control urinary leakage. When compared to common procedures for treatment of SUI such as the injection of collagen bulking agent, ADSC cell therapy provided better results [132]. Moreover, the association of cells with biomaterials seems to enable further improvements as observed with BMSC and sling systems or MDSC with fibrin glue [135, 136]. To improve this cell therapy model, Zhao et al. took account of the fact that muscle regeneration is an interaction process involving paracrine factors produced by surroundings cells and combined with ADSC the nerve growth factors. This method stimulated muscle regeneration and demonstrated that combining different cell types could be beneficial for muscle restoration in SUI.

4.3. Clinical trials

Several clinical trials applying cell therapy in SUI have been conducted in the last decade (table.2).

Safety and efficacy of this strategy have been proven with several types of cells in women and men. As the procedures differ from one trial to another straight comparisons between them are difficult. Additionally, the recruited patients suffered from different levels of SUI - from moderate to severe- and some studies even included complementary therapies such as electrical stimulation or exercises to improve the results. Nevertheless, the results were highly promising and have demonstrated that a solution for patients suffering of SUI is within reach. Surprisingly, the first cells to be used in a clinical trial for SUI was not muscle derived cells but chondrocytes isolated from auricular cartilage that were expanded in culture before injection in female patients. Out of 32 treated patients 26 had an improved situation and 50% were continent after one year [137]. This was the only clinical study using chondrocytes for voiding dysfunction. Muscle derived cells (myoblasts and MDSC) are the most frequently used cells in muscle regeneration for SUI in both genders. Myoblasts have been used in many trials and were injected in or around the external urethral sphincter. The efficiency was stated to be

Cell type	Source	Patients / n	Injection	Target organ	Delivery biomaterial	Time point Months	Outcomes Measurements	Reference	Year
Chrondrocytes	autologous	Women / 32	Trans/peri-urethral	Bladder neck	calcium alginate	12	81% improved 50% continent	Bent et al.	2001
Myoblasts and fibroblasts	autologous	Woman / 123	Transurethral	Urethra	autologous serum collagen	12	79% continent 13% improved	Mitterberger et al.	2007
Myoblasts and fibroblasts	autologous	men / 63	Transurethral	Urethra	autologous serum collagen	12	65% continent 17% improved	Mitterberger et al.	2008
MDSC	autologous	Women / 8	Trans/peri-urethral	External sphincter	none	3 to 24	63% improved 13% continent	Carr et al.	2008
Myoblasts	autologous	Boys / 7 Girls / 1	Transurethral	External sphincter	none autologous serum	12 to 18	88% improved 38% continent	Kajbafzadeh et al.	2008
UCBSC	allogenic	Women / 39	Transurethral	Submucosa	none	12	72% improved 9% continent	Lee et al.	2010
Myoblasts	autologous	Women / 12	Transurethral	External sphincter	none	12	50% improved 25% continent	Sèbe et al.	2011
MDC	autologous	Men / 222	Transurethral	External sphincter	none	at least 12	12% continent 42% improved 46% no efficacy	Gerullis et al.	2012
ADSC	autologous	Men / 3	Transurethral	External Urethra sphincter Submucosa space	none	6	improvement	Yamamoto et al.	2012
Myoblasts	autologous	Women / 38	Intrasphincteric	External Urethra sphincter	none	1.5	78.4% improved 13.5% cured 8.1% unchanged	Blagange et al.	2012

Abbreviations: MDC, muscle-derived cells; MDSC, muscle-derived stem cells; UCBSC, umbilical cord blood stem cells.

Table 2. Clinical trials for treating stress urinary incontinence based on cell therapy.

between 50% and 88% in a follow-up of 12 months [138-141]. Even if the designs between studies differ, the combination of cell therapy with electrical stimulation or/and pelvic floor exercises may explain the variation between the values. In fact, a cell therapy with the application of myoblasts alone seems to provide a 50% improvement [139, 141], improving to 78.4% if electrical stimulation is added [138] and reaching 88% with pelvic floor exercises [140]. This approximate comparison can encourage future clinical studies to combine other therapies and exercises with cell therapies in order to optimize the outcome. Myoblasts have also been combined with fibroblasts mixed in a collagen solution. The results were impressive: 79% of treated women and 65% of the men reached continence [142, 143]. As a Lancet publication of this group was retracted, these results should be handled with precaution and should be confirmed by other groups [144]. Other muscle-derived cells have been injected in patients with SUI. Since 2008, MDSC have been applied in several clinical trials [145, 146] with improvement rates of 53% after 1-year follow-up with 10 million cells injected, 63% with 20 million and 67% with 50 million. The efficiency of the cell therapies seems to be dose-dependent. This was confirmed by Kaufman et al. in a 6-month dose escalating study, where improvements increased with the dose of injected cells. The best results were obtained with 200 million MDSC injected [146]. Interestingly, no serious adverse effects were observed even when numbers of UCBSC as high as 400 million were applied [147]. In this latter case, 72% of

39 patients were more than 50% satisfied 12 months post-injection. This represents another type of cells that is suitable for SUI treatment. Although the cell therapy with UCBSC is allogenic, no immunosuppressive effects were observed during this cure. As a source of multipotent stem cells, ADSC were trusted in recovering the contractility of the sphincter muscle in patients [148]. Certainly, the encouraging preclinical studies enabled transplantation of ADSC in patients suffering from SUI. However, only 3 patients were treated so far. Peri-urethral injection of ADSC seems to be safe and showed improvement of the sphincter contraction after 6 months follow-up. The use of total nucleated cells associates with lysates seems to be another good option for treating SUI. This type of cells significantly helped all treated patients in the study: 100% noticed improvement in their situation and 88% reached complete continence after 6 months. Hence, these clinical trials show that different sources of cells were able to improve the continence level of patients suffering from SUI.

5. Non–biological translational work

The ability to regenerate muscle tissue from patient derived cells would have profound impact on many human diseases. Cell therapy is within reach as a novel treatment option for incontinence, reflux, vocal cord dysfunction and other muscle-related pathologies. However, the carrier used for cell delivery and the techniques used to inject the cells are still being optimized.

5.1. Cell delivery

It has been demonstrated for more than a decade that cells injected in a saline solution carrier are able to ectopically form contractile muscle [149]. However, further studies have reported very poor cell survival rates (5-20%) associated with myogenic cell implantation without embedding into protein based carriers that support cell settling into their new niche [150, 151].

Species-specific cues play an important role in cell affinity to carriers. A previous study demonstrated advantages using collagen rather than matrigel coated dishes, boosting cell growth and differentiation potential [73]. In contrast, another study with porcine satellite cells demonstrated cell preference to matrigel coated dishes and growth decrease on collagen layers[152]. Moreover, three-dimensional (3D) matrigel coated PLGA (poly lactic-co-glycolic acid) scaffolds were capable of improving cell survival when compared to direct cell injection [153]. However, the same study failed to demonstrate a comparative improvement of matrigel coated PLGA with other cell carriers. Furthermore, matrigel has not presented advantage *in vivo a*s a carrier for myogenic cells when compared to hyaluronic acid-photoinitiator (HA-PI) complex. It rather downgraded the quality of muscle structure and decreased the total number of new myofibers after cell injection [154].

Collagen is a main component of the natural extracellular matrix of skeletal muscle, it is therefore expected that satellite cells would have their functionality up-scaled in a collagen rich environment [155]. Combined with electrical stimulation collagen induces three-dimensional expansion of muscle precursor cells *in vitro* and in syngeneic recipient muscle [156]. Cell cycle analyses of engrafts implanted into a 3D collagen sponge highlighted the increment of

cell fractions in proliferating phases, with 80% of cell survival [157]. In addition, the use of parallel aligned collagen nanofibers yielded good proliferation and enabled the generation of aligned cell layers [158]. Finally, grafts of myoblasts seeded into three-dimensional collagen scaffolds and implanted into injured sites in mice demonstrated improvement in muscle healing, innervation and vascularization [159]. Altogether these recent studies confirm that collagen is a very promising matrix for satellite cell ingrowth and an ideal carrier for the transplantation of myogenic cells.

5.2. Imaging techniques for guided cell implantation in vivo

The success of cell transplantation into a specific site *in vivo* is directly dependent of 3 key points: cell source, cell carrier and injection technique. The first two were previously discussed in this chapter. We dedicate this section to the discussion of injection techniques used so far to inject myogenic cells into a specific injury site. The application of myogenic cells was already used for the treatment of male and female patients suffering from urinary incontinence, the involuntary loss of urine that represents a hygienic and social problem [160]. Transurethral ultrasound guided injections of autologous cells isolated from limb skeletal muscle biopsies were so far the method of choice [161, 162]. This method is also standard for the injection of bulking agents like collagen in the clinical practice [163]. Finally, ultrasound guidance was also used to monitor percutaneous trans-coronary-venous transplantation of autologous myoblasts in infarcted myocardium [164, 165].

Recently magnetic resonance imaging (MRI) has gained attention as a useful tool for guidance during injection of drugs and potentially of cells [166]. Pulsed focused ultrasound is a new ultrasound technique that associated with magnetic resonance guidance was recently suggested as a new imaging modality that may be utilized to target cellular therapies by increasing homing to areas of pathology [167]. It has also been demonstrated to increase drug uptake into a specific target in the prostate [168] and brain [169]. This same technique has been shown to facilitate the delivery of neural stem cells into a specific site in the brain [170]. Overall, the most successful deliveries of myogenic cells have been done either operatively in 3D scaffolds or in collagen carrier that facilitates cell settling into the new cell niche. Ultrasonography is still the most adaptable and widely used imaging technique allowing visualization of the injury zone and real time needle guidance. However, new approaches combining MRI and ultrasonographic pulses are very promising methods that need to be further studied and adapted for cell injection in different anatomic sites. Moreover, MRI is used in tracking stem cells after injection [171, 172]. In fact, it is important not only to inject the cells at the right place but also to ensure that cells are not migrating to other parts and pursuing their role in regenerating the tissue of interest. Additionally, developments in MRI technology, especially in scanning technics, offer the possibility to follow the differentiation process of injected MPCs and their fate in making fibers [173].

5.3. Regulation and guidelines

The application of cell-based therapies is not only advancing scientifically but also regulations are adapting and including the new scientific discoveries for clinical use. The relevant health

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agencies all around the world are creating committees that are modifying the regulations in order to take account of these new categories of products that are cell-based. Stem cell based therapies are part of advanced therapies, which are therapies based on genes, or cells, or tissues [174]. Concerning this emerging branch of medicinal products, the regulations are new and still in development. They have their own classification, distinct from chemical and biologic drugs, transplantation organs and medical devices. Though, they can be sometimes included in these categories. In Europe, the European Medicines Agency (EMA) is in charge of improving the standards and reviewing the applications for stem cell based therapies, which are part of Advanced Therapy Medicinal Products (ATMP), and they are found in regulation (EC) N° 1394/2007 [175]. The Committee for Advances Therapies (CAT) is the body responsible within EMA of this new field of science and its approval for marketing. The goals are to protect the patient from contaminated tissues/cells, to avoid the inappropriate handling of tissues/cells and to guaranty safety and efficacy of therapies. The documents are providing a regulatory framework that is coherent with existing ones, specific to biological and chemical entities for instance. Hence, before starting any clinical trial on human, several requirements are to be fulfilled. The cell-based product needs to be grounded on a sound and solid scientific work that is confirmed in pre-clinical studies, which show its quality, safety and efficacy. During this preparation phase, CAT is available for giving advice in preparing all the relevant files for obtaining clinical trials authorization or latter for marketing authorization. Guidelines are specifying aspects of pharmacovigilance, risk management planning, monitoring, labeling, safety, efficacy follow-up and traceability. The submission process should comply with these requirements in order to receive the green light for starting clinical trials or entering the market. During product development and clinical investigations guidelines have also been adapted by CAT for stem-cell based therapies for specifications on Good Manufacturing Practice (GMP) and Good Clinical Practice (GLP) [176]. In the US, the Office of Cellular, Tissue, and Gene Therapies (OCTGT) - part of the Center for Biologics Evaluation and Research (CBER) in FDA- is responsible of the cellular therapies products [177]. They are regulated by human cells, tissues, and cellular and tissue-based products (HCT/Ps) under the authority of Section 361 of the Public Health Services (PHS) Act as well as Title 21 of the Code of Federal Regulations (CFR) part 1271 [178]. The OCTGT are making sure that the cell-based products meet safety, purity, potency and effectiveness qualifications. EMA and FDA are collaborating closely together in the Advanced Therapies Medicinal Product cluster. The development of regulatory frameworks is not equal in all countries and is independent from a state to another state. However, at the international level, regulatory agencies are working together in sharing and harmonizing the regulatory frameworks for cellular therapy products through the International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use (ICH), the Pan-American health Organization (PAHO), WHO and Asia-Pacific Economic Cooperation (APEC). This global interaction facilitates the development of the cellular therapy field and prepares in bringing the products to the markets. As the experience is right now limited in this field, this discussion panels permits to cover the different applications and cases among the countries and therefore increase the knowledge levels among the participants and the regulatory boards. In addition, it creates convergence in the develop-

ment of the regulations and guidelines concerning different aspects: manufacturing, quality assurance, quality control and pre-clinical studies [179].

Therefore, the regulations and guidelines have been reviewed and adapted for some of them in order to be applied in the field of cell therapy. This paves the road for regenerating the sphincter muscle by using stem cells.

5.4. Production of cell–therapies

Besides, chemical drugs, medical devices and biotechnology drugs, advanced therapies are developed and offer tailored solutions for patients. These therapies are based on genes, cells or tissues.

Cell therapy for skeletal muscle is one of many therapies that are in translational phase and can be applied in near future on treating patients. As it is involving individuals' health and the cell product is delivered to human, safety concerns are raised. In fact, cell therapy product – as an investigational or marketed one- needs to meet requirements as any medicinal product or medical device. The goal is to deliver a consistent, safe, good quality and well-defined product. Therefore, Good Manufacturing Practice (GMP) is requested for the development of cell-based product, or its production for the market, and it consists on guidelines and regulations that advertise quality principles for manufacturing biological products. These rules are covering all the processes from the biopsy up to the final product. It involves several aspects:

Quality management, buildings and facilities, the equipment, the personnel, the documentation, the materials management, the processes in production, the monitoring, the packaging and labeling, the storage and distribution, the laboratory controls.

Advanced therapies are new technology. Hence, protocols, guidelines and regulations that are used for existing medicinal product cannot be transposed literally for cell therapies and need adaptations. However, the goals stay the same: safety, quality and efficacy.

5.5. Manufacturing process

In cell therapy, the starting material represents a critical part that takes account of donor eligibility criteria including age, tissue quality, source accessibility and viral testing. For skeletal muscle cell therapy, as described above, the sources are multiple and the efficiency of most of them is good in regenerating muscle in the case of SUI.

As soon as the biopsy is received in the manufacturing site, the GMP requirements have to be followed. Hence, quality management should be applied at all production steps: processing, testing, release, storage and transport.

Manufacturing cell product necessities safe and certified raw materials and components for cell culture and preparation. In addition, upon reception to the GMP facility, the materials need to be tested in-house regarding quality and safety. Only then, the products can be released and accepted into the production area by the responsible for quality in the facility. It is highly recommended by the regulations to use supplements – as cytokines and growth factors- from human origin and therefore some adaptations are needed in the production protocols coming

from the research laboratories. One of the major problems in the cell culture is to replace the fetal bovine serum (FBS). Most of the protocols are still based on this animal derived product. Recently, some efforts have been made to work with xeno-free medium by replacing FBS with human serum and platelet lysate [180]. In the case of MPCs, one of the major sources of cells for muscle cell therapy as described above, pooled human platelet lysate was demonstrated to be a good alternative to FBS [181]. Other factors are important and must be controlled as cell seeding, growth rate, differentiation process, markers expression, potency of the cells in making contractile fibers. The protocols for each step - from receiving the biopsy up to the final product -must be standardized and approved by local authorities before starting clinical trials. Standardization means that clear and details protocols should be written and followed without deviation or modifications. Quality controls are done not only for starting materials but also at critical steps in production. Quality is a key parameter that applies to all levels of the cell therapy production: building and facilities environments, equipment, production, labeling, storage and distribution. The quality unit performs all the controls to show the purity of the products, the cleanness of the environment, the maintenance of the equipment and the respect of the specifications set for obtaining a safe, effective and potent cell product. In muscle cell therapy, the cell population should have a pure or a very high percentage of cells expressing markers of skeletal cell as described above.

All the stages and elements related to the GMP facility or the production process should be documents to insure traceability of every single action. The documents should be prepared, reviewed, approved and distributed as specified in established and written procedures. All these demanding steps require qualified personnel, well-trained in working in GMP facilities. It includes good sanitation and health habits and the right skills to accomplish the work with products for cell therapy. Finally, internal and external audits are conducted regularly to verify the respect of the GMP regulations and guidelines as validated by the GMP facility and the authorities.

6. Outlook: Concerns and improvements

6.1. Effects of age

A decline of approximate 30% in muscle strength and 40% in muscle volume occurs between the second and seventh decades of life [182]. Also the total number of MPCs and their proliferation potential in culture gradually decrease in an age-dependent manner [183] due to apoptosis [184]. Additionally cell fate is tightly defined by the interactions with the microenvironment and the host age is of key importance, as the stem cell regenerative capacity reduces in aged niches [185]. We have reported that although human MPCs can be successfully isolated and grown from patients of all ages and genders (figure 3), both elderly and male donors provide unstable and slower growing cells *in vitro* with decreased contractile output *in vivo* [186]. Hence, a combination of stem cell and gene therapy might be needed in older patients [187, 188].

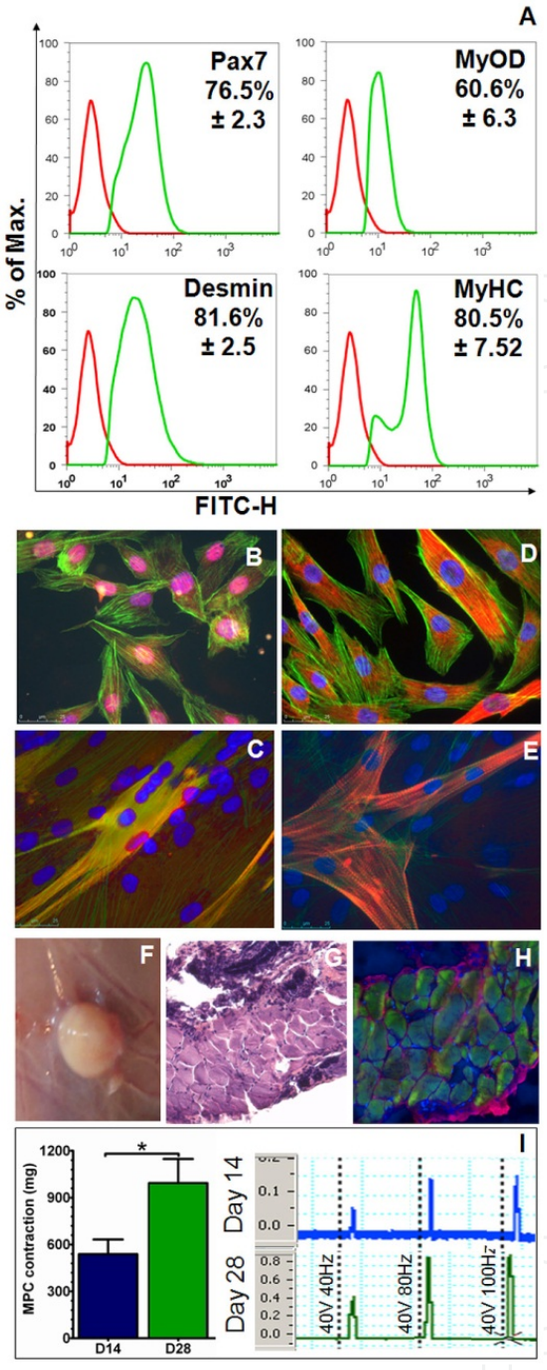

Figure 3. Muscle progenitor cells identification *in vitro* and muscle formation after transplantation *in vivo*. Myogenic cells isolated from the *Rectus abdominis* of patients undergoing abdominal surgery, grown in culture and characterized by FACS, immunohistochemistry *in vitro*. Tissue formation was evaluated *in vivo* by Hematoxilin and Eosin staining and immunohistochemistry. Function was assessed by electromyography. A: FACS analyses of cells in P2 expressing Pax 7, MyOD, desmin and upon differentiation induction Myosin Heavy Chain (MyHC). An IgG Isotype control (red curve) was used to determine the background, whereas positive cells are plotted as a green curve. Immunocytochemistry of cells in culture expressing, MyOD (B), MyHC (C), desmin (D), sarcomeric α-actinin (E) (green -Phalloidin 488, blue – DAPI, red - mM anti-IgG Cy3). Muscle cells injected subcutaneously in nude-mice revealed muscle formation *in vivo* (F, G, H) and contraction upon eletrical stimulation (I). HE stained (G) and labelled with sarcomeric α-actinin-Cy3 and PKH67 (H). Muscle function significantly improved over time (I), with contraction strength still increasing after 4 weeks.*p=0.015

6.2. Overcoming pitfalls by reactivating muscle metabolism, tissue vascularization and innervation

In the context of muscle reconstruction, gene therapy is not aimed at rectifying a genetic mutation, but at boosting the myogenic potential and ultimately the muscle functionality of the injected autologous muscle cells. Two key factors have been demonstrated to improve the quality of satellite cells for transplantation: a better vascularization [189] and endurance exercise [190]. We have previously described that an angiogenic modification of muscle precursors can overcame some of the limitations of aged muscle cells [189]. For future application expanding the knowledge produced on this study and therapeutically combining it with the intrinsic adaptation effects of endurance exercise would be of major interest. In this context, studies using muscle-specific PGC-1α transgenic animals demonstrated that ectopic expression of PGC-1α in muscle seems sufficient to evoke a trained phenotype avoiding muscle atrophy [191]. Upon activation, PGC-1α in turn controls many, if not all of the adaptations of skeletal muscle to endurance exercise [192]. Hereafter, PGC-1α muscle-specific transgenic animals exhibit high endurance, oxidative muscle fibers, an increase in mitochondrial biogenesis and oxidative metabolism, augmented muscle capillarization and a remodeling of the neuromuscular junction [193, 194].

Although innervation of the newly implanted tissue is also essential to engineer a functional muscle tissue there is few approaches that could effectively promote nerve ingrowth after transplantation. Some studies described a spontaneous nerve ingrowth from the neighbor tissues into the newly transplanted sites [195, 196], but non-invasive methods to induce nerve ingrowth after newly formed muscle engrafts are still to be investigated. We have recently proposed that magnetic stimulation supports regeneration of injured muscle with activating resident stem cells or supporting integration of newly implanted myoblasts [197, 198]. Exposition of injured limb and co-cultures of muscle cells and neurons to magnetic fields was sufficient to trigger synapses, induce acetylcholine receptors clustering and cause typical muscular metabolic adaptations verified during endurance exercise [197]. Notwithstanding, magnetic stimulation mimicked the effects of exercise inducing PGC-1α up-regulation, induces myogenic cells differentiation and increases nerve fibers and acetylcholine receptor clustering after cell transplantation [198]. New efforts in establishing functional innervation, proper vascular network and the development of a high endurance resistance muscle are going to be the three main pillars supporting future translational studies and bringing myogenic cell transplantation from bench to bedside.

7. Conclusion

Regeneration of skeletal muscle for SUI is becoming a reality and the cell therapy may soon be available to patients. Tremendous progresses have been made to understand the science behind the natural process of skeletal muscle regeneration that involves primarily satellite cells and their progenitors, MPCs. In addition, these cells are now well-characterized with several markers at different stages of proliferation and differentiation. They are also interacting

actively with their environment, which is composed of different types of cells. These neighboring cells have a significant influence on the environment and on stimulating the factors that trigger satellite cells renewal, proliferation and differentiation into myofibers. The process is complicated and involves cocktails of factors and cells. However, the interaction between these parameters is better-understood and applied in research and preclinical studies to ameliorate lack of early vascularization and innervation. In clinical trials, the first results are promising and many patients with SUI were treated successfully. The cell sources are important for a successful skeletal muscle cell therapy but they must be accompanied by a set of tools to ensure the safety and the quality of the process: culture medium, biomaterials, imaging for injection and follow-up. The advances have been made and the solutions are ready, even at the regulatory level. Although, there is not yet a standardized cell therapy for SUI, the solutions and the first results are encouraging. The cell therapy for SUI treatment will be certainly part of the choices that urologists will adopt very soon in hospitals.

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