Advanced Drug Delivery Reviews 146 (2019) 60–76

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/addr

Muscle fibrosis in the soft palate: Delivery of cells, growth factors and anti-fibrotics

Johannes W. Von den Hoff [a,*], Paola L. Carvajal Monroy [b], Edwin M. Ongkosuwito [a], Toin H. van Kuppevelt [c], Willeke F. Daamen [c]

[a] Department of Orthodontics and Craniofacial Biology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, P.O. Box 9101, 6500HB Nijmegen, The Netherlands
[b] Department of Oral and Maxillofacial Surgery, Special Dental Care and Orthodontics, Erasmus Medical Center, P.O. Box 2060, 3000CB Rotterdam, The Netherlands
[c] Department of Biochemistry, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, P.O. Box 9101, 6500HB Nijmegen, The Netherlands

ARTICLE INFO

Article history:
Received 28 February 2018
Received in revised form 29 June 2018
Accepted 6 August 2018
Available online 11 August 2018

Keywords:
Cleft palate
Skeletal muscle
Scar
Anisotropic scaffolds
Small molecules
Decorin
miRNAs
Myofibroblast
Transforming growth factor-β

ABSTRACT

The healing of skeletal muscle injuries after major trauma or surgical reconstruction is often complicated by the development of fibrosis leading to impaired function. Research in the field of muscle regeneration is mainly focused on the restoration of muscle mass while far less attention is paid to the prevention of fibrosis. In this review, we take as an example the reconstruction of the muscles in the soft palate of cleft palate patients. After surgical closure of the soft palate, muscle function during speech is often impaired by a shortage of muscle tissue as well as the development of fibrosis. We will give a short overview of the most common approaches to generate muscle mass and then focus on strategies to prevent fibrosis. These include anti-fibrotic strategies that have been developed for muscle and other organs by the delivery of small molecules, decorin and miRNAs. Anti-fibrotic compounds should be delivered in aligned constructs in order to obtain the organized architecture of muscle tissue. The available techniques for the preparation of aligned muscle constructs will be discussed. The combination of approaches to generate muscle mass with anti-fibrotic components in an aligned muscle construct may greatly improve the functional outcome of regenerative therapies for muscle injuries.

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* Corresponding author at: Department of Orthodontics and Craniofacial Biology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, P.O. Box 9101, 6500HB Nijmegen, The Netherlands.
E-mail addresses: Hans.Vondenhoff@radboudumc.nl (J.W. Von den Hoff), P.CarvajalMonroy@erasmusmc.nl (P.L. Carvajal Monroy), Edwin.Ongkosuwito@radboudumc.nl (E.M. Ongkosuwito), Toin.vanKuppevelt@radboudumc.nl (T.H. van Kuppevelt), Willeke.Daamen@radboudumc.nl (W.F. Daamen).

https://doi.org/10.1016/j.addr.2018.08.002
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1. Introduction

Skeletal muscle fibrosis often occurs after major muscle trauma or extensive surgical reconstructions [1–4]. The fibrotic process seems to be induced by the loss of a large volume of muscle tissue and leads to reduced muscle function and disability. The gold standard for treatment of volumetric muscle loss is free standing muscle flaps but these are hampered by donor site morbidity and a low success rate [5,6]. Limited muscle injuries such as crush injuries or lacerations generally heal without any functional problems indicating the high regenerative capacity of muscle tissue. In these cases, satellite cells and other local stem cells are readily available and the original instructive niche and structural cues are maintained. However, volumetric muscle loss surpasses the regenerative capacity of the tissue [1]. Crucial regenerative components such as the satellite cells (SCs), niche factors and structural cues are lost, which precludes functional muscle regeneration. This requires the therapeutic delivery of these key components to promote healing. Also, in some congenital conditions such as cleft palate and diaphragmatic hernia, similar approaches are required to restore muscle function [3,4,7].

In the last decades, research within the field of regenerative medicine has provided many strategies to supply muscle volume in order to restore muscle function. However, up to now, the results have been highly variable [8]. This seems to be caused by complications such as a poor survival of the applied cells and a lack of knowledge about growth factors and other niche components that are required to stimulate muscle regeneration. These components can be delivered to the affected muscle tissue in several ways. SCs or other stem cells can be injected into the tissue or combined with a carrier material and then surgically implanted. We will focus on the latter approach as it is more suitable for larger muscle defects. The carrier material can be a scaffold or a hydrogel composed of either synthetic or biological materials. In addition, suitable niche components can be added to the carrier material to stimulate myofiber formation. Generally, the carrier material should also contain structural cues to guide the regenerating myofibers into the desired orientation. Recent developments allow the construction of such scaffolds [9–11].

A major complication in larger muscle injuries is the development of muscle fibrosis, which limits the regeneration of myofibers and the restoration of function. Volumetric muscle loss may be the result of reconstructive surgery after trauma or tumor resection. Up to now fibrosis prevention has received far less attention in research than the regeneration of new myofibers. Similar to fibrosis in other organs, muscle fibrosis is mainly caused by myofibroblasts that contract the tissue and deposit large amounts of collagen and other extracellular matrix (ECM) components [12]. Anti-fibrotic therapies generally aim to reduce the differentiation of myofibroblasts. These cells can arise from normal fibroblasts but also from other tissue cells as described for liver, kidney and lung fibrosis. Myofibroblast differentiation can be targeted by the delivery of compounds that reduce the activity of transforming growth factor β1 (TGFβ1). In muscle fibrosis, the fibrotic process is further enhanced by the action of myostatin, another member of the TGFβ superfamily [3]. This growth factor inhibits the activity of SCs and the proliferation of myoblasts. In organ fibrosis, many small molecules are being investigated that target TGFβ signaling.

A perfect example of a condition that requires both the regeneration of new myofibers as well as the prevention of fibrosis is cleft lip and/or palate (CLP) [3]. In this congenital disorder, the muscles of the soft palate and the lip are often affected in addition to the bony part of the upper jaw and the palate. The muscle mass in the soft palate is reduced because of the developmental disorder and a lack of function. In addition, the surgical closure of the cleft at an early age induces muscle fibrosis, which has a negative impact on speech development [3]. The fibrotic tissue limits the formation of new myofibers in the wound area. Research into this specific disorder is limited but a suitable rat model for muscle fibrosis in the soft palate has recently been developed [13]. The design of regenerative therapies for the soft palate may be inspired by ongoing research in the field of regenerative medicine for other (skeletal) muscle defects, and the development of anti-fibrotic therapies.

In summary, research in the field of muscle regeneration is mainly focused on the restoration of muscle mass, while the prevention of fibrosis is receiving far less attention. Therefore, the aim of this review is to discuss the available options within the field of regenerative medicine for various debilitating skeletal muscle disorders by the delivery of cells, growth factors and anti-fibrotic components in suitable carriers. We will pay special attention to approaches for the prevention of fibrosis. A disorder requiring a combined approach is the reconstruction of the muscles in the soft palate in CLP patients. Hence, we will start with a concise overview of CLP focusing on the defects in the muscles of the soft palate. Subsequently, the available approaches for muscle regeneration and suitable delivery systems will be discussed with special focus on anti-fibrotic components and scaffold directionality. To conclude we will present the most promising approaches to improve muscle regeneration in CLP and other muscle disorders.

2. Cleft lip and/or palate

Cleft lip and/or palate (CLP) is the most common congenital facial malformation in humans (Fig. 1). Worldwide, every three minutes a child with an orofacial cleft is born. Especially in developing countries this constitutes a major problem where millions of children are suffering from untreated clefts [14]. CLP can be associated with other congenital malformations and is then considered to be part of a syndrome. CLP is generally divided into two groups based on both genetic and embryological grounds; clefts involving the anterior structures (lip and primary palate) and clefts involving only the soft palate or extending into the hard palate (secondary palate) [14–16]. Hence CLP is a highly heterogeneous group of disorders affecting the upper lip, the nose, and the oral cavity [16]. CLP is known to be caused by both genetic and environmental factors [17]. The treatment of these patients is complex and lasts until adulthood involving a multidisciplinary team of specialists. Treatment includes multiple surgical reconstructions, speech therapy, hearing control/management, and dental and orthodontic treatment [18]. In addition, children may develop psychosocial problems due to esthetic and speech issues.

2.1. Muscles in the lip and soft palate

The orbicularis oris is the major muscle of the lip and it is responsible for oral closure and puckering of the lip [19,20]. Recent studies show the complexity of the functional anatomy of the upper lip including

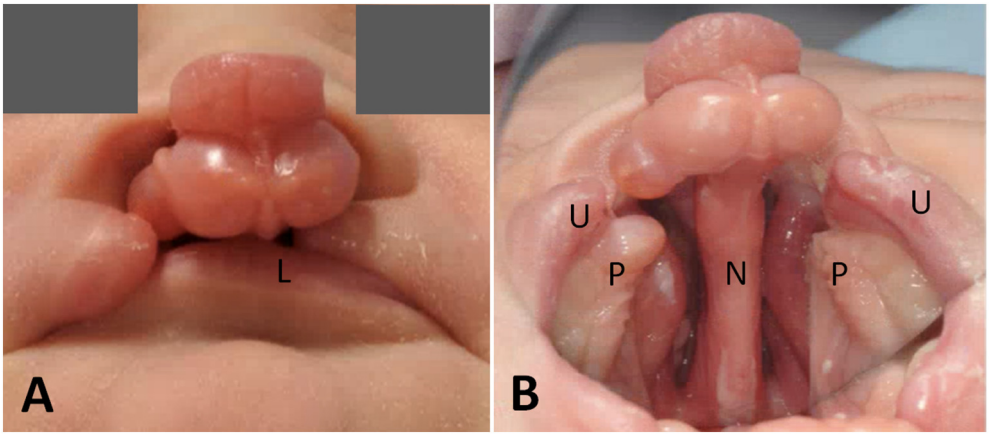

Fig. 1. Complete bilateral cleft lip and palate. A. Frontal view of the bilateral cleft lip with the premaxilla protruding outward (L = lower lip). B: Intraoral view of the cleft in hard and soft palate (P = palate, N = nasal septum, U = upper lip).

elevators of both the upper lip and the commissure [21]. In a cleft of the lip, the orbicularis oris inserts abnormally into the underlying bone and the anterior nasal septum on the medial side as well as into the nostrils and the periosteum of the piriform aperture on the lateral side [22]. It causes distorted growth of the soft tissue of the nose and its underlying bone. The soft palate is formed by an interweaving of muscles from the skull base and the pharynx (Fig. 2A). All muscles extend from nearby bony structures and are inserted into the aponeurosis located in the center of the soft palate. In the cleft palate (Fig. 2B), the muscles are attached to the posterior border of the hard palate. The abnormal insertions of the muscles, in particular the levator veli palatini (LVP), prevent normal functioning of the soft palate.

The origins of the head muscles are highly heterogeneous, but both the lip and the soft palate muscles originate from the pharyngeal mesoderm of the pharyngeal arches [23]. Both slow and fast fibers are present in the lip and soft palate muscles in similar amounts. Slow fibers are highly resistant to fatigue, with a low activation threshold, whereas fast fibers are more fatigable, with a higher activation threshold. In CLP, both lip and soft palate muscles are predominantly composed of fast fibers [24,25]. In addition, these muscles have a reduced capillary supply and are mostly atrophic and disorganized at the border of the cleft [26–28]. The atrophic muscles often have only half of the thickness of normal muscles, which may result from reduced function [27–29]. In summary, fiber type composition, reduced capillary supply and atrophy may contribute to muscle dysfunction in CLP.

2.2. Surgical reconstruction

There is a wide range of surgical protocols for CLP, but both lip and soft palate repair generally take place during the first year after birth [30,31]. Several surgical techniques have been described for both lip and soft palate repair [22,32]. In all cases an appropriate reconstruction of the muscle anatomy constitutes a crucial step during surgery [33,34]. Lip repair aims to improve facial symmetry, whereas soft palate repair mainly aims at optimal function of the velopharyngeal sphincter and speech development [33–35]. For the lip, surgical techniques are oriented to preserve or correct the vertical height of the lip and maintain the anatomy of the cupid's bow and philtrum. In this case, the muscle is carefully dissected from the underlying skin and mucosa and freed from its abnormal attachments along the nose and maxilla until it can be rotated freely and be repaired assuring the functional continuity of the orbicularis oris muscle [22]. Lip repair is considered an important factor that restrains maxillary growth in individuals with a repaired cleft [36]. When reconstructing the soft palate, the abnormal attachments of the soft palate muscles are dissected and the levator veli palatini (LVP) muscle sling is reconstructed. In spite of surgical reconstruction of the soft palate, 10 to 30% of the patients is still not able to achieve complete closure of the connection between the nasal and oral cavity after surgery [37]. This results in hypernasal speech and other functional deficits such as nasal air escape and articulation disorders that require an additional surgical correction in 25% of these

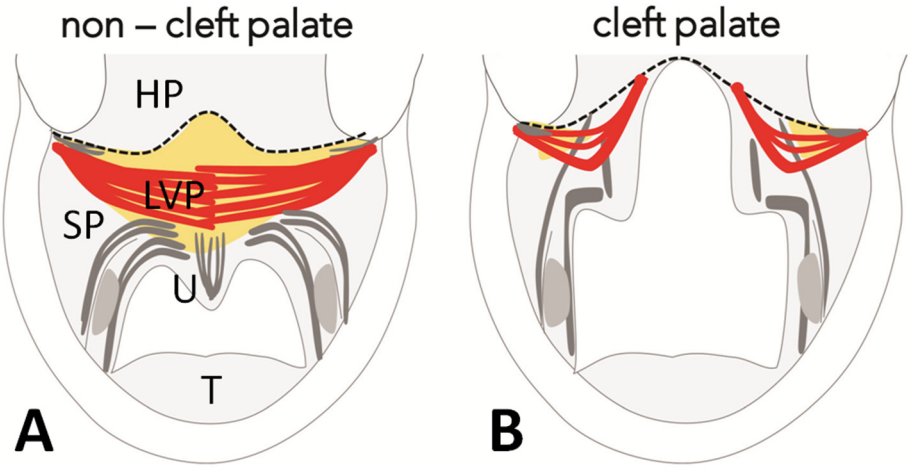

Fig. 2. Intraoral view of the muscles in the soft palate. A. Non-cleft palate. The main muscle in the soft palate is the levator veli palatini (LVP, red) running from the lateral sides to the median. The LVP moves the soft palate upwards and backwards to close the connection between the oral and nasopharyngeal cavities during speech. The minor muscles are indicated in grey (HP = hard palate, SP = soft palate, LVP = levator veli palatini, U = uvula, T = tongue). B. Cleft palate with aberrant muscle anatomy.

patients [37]. In summary, function and esthetics are greatly improved by surgical repair but functional problems remain due to incomplete muscle regeneration and fibrosis.

2.3. Fibrosis in the soft palate

Muscles possess a strong ability to regenerate after injury. Satellite cells (SCs) [38] are the primary muscle stem cells, and are responsible for postnatal muscle growth, maintenance, and repair [39]. The formation of scar tissue may prevent proper muscle regeneration [40]. Freeze or crush injuries in the masseter muscle, another muscle derived from the pharyngeal mesoderm, regenerate slower than similar injuries in limb muscles [41]. Moreover, much more fibrous connective tissue is formed in the damaged area. Also, the masseter muscle seems to contain less SCs than limb muscles [42]. As mentioned before, cleft palate muscles predominantly contain fast fibers unlike normal soft palate muscles. As the percentage of SCs in fast muscle fibers is significantly lower than in slow muscle fibers [43,44], this may further decrease the SC number in cleft muscles. In addition, SCs within atrophic muscles display a reduced function [45,46].

Regeneration of the lip and soft palate muscles after surgical repair is hampered by the development of fibrosis. Fibrosis represents a pathologic excess of the normal tissue repair process [47]. In general, muscle fibrosis is induced by transforming growth factor-β1 (TGF-β1) and myostatin, both members of the TGF-β super family of growth factors [48]. TGF-β1 plays a significant role in the initiation of fibrosis by inducing myofibroblast differentiation [48], while myostatin reduces the migration, proliferation and differentiation of SCs [49,50]. Fibrosis in the lip restricts maxillary growth [36,51], whereas fibrosis in the soft palate impairs the function of the velopharyngeal sphincter and speech development [3]. We developed the first *in vivo* model for soft palate muscle regeneration in rats by making a full-thickness defect in the soft palate [13,52]. After muscle injury, extensive fibrotic tissue developed in the wound area with little or no formation of new myofibers (Fig. 3). This model can be used to develop specific regenerative therapies for the soft palate [53].

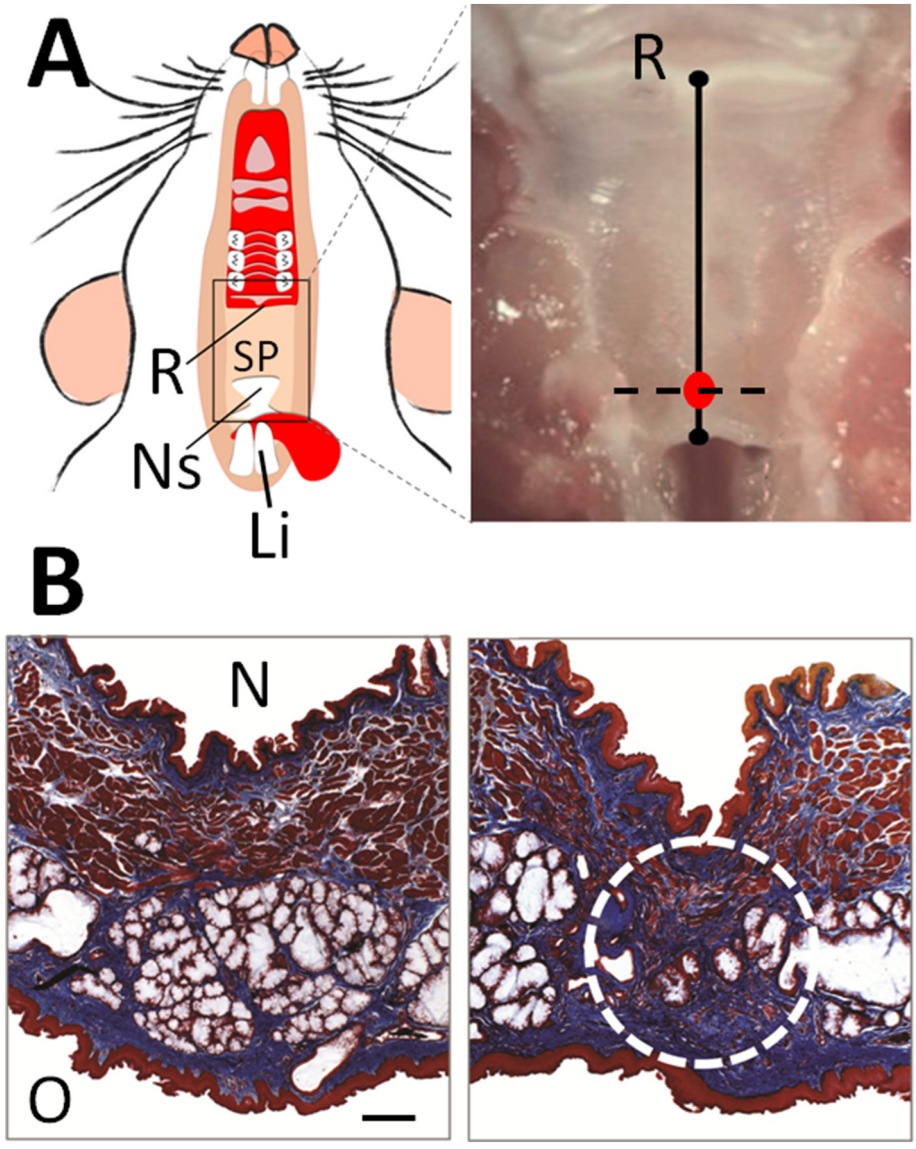

Fig. 3. Model for muscle regeneration in the soft palate of the rat. A) Intraoral view of the palate of the rat (SP = soft palate, R = rugae, Ns = nasopharyngeal sphincter, Li = lower incisors). The right picture shows a clinical view of the soft palate of the rat. The red circle indicates the location of the experimental wound. The dashed line indicates the level of sectioning. B) The left picture shows Azan staining of the normal soft palate with muscle in red and connective tissue in blue (N = nasal cavity, O = oral cavity, bar = 200 μm). The right picture shows the soft palate 56 days after wounding. The dashed circle indicates massive fibrosis.

In summary, the lower regenerative capacity of muscles originated from the pharyngeal mesoderm, and the specific properties of cleft muscles may compromise muscle regeneration following surgical repair in cleft patients. In CLP, the muscles of the soft palate are reduced in mass, which impairs functional recovery after surgery. In addition, surgical reconstruction induces fibrosis, which limits muscle regeneration and their function in speech and swallowing. The outcome of reconstructive surgery may be improved by the delivery of cells, growth factors and anti-fibrotic components.

3. Regenerative strategies

The treatment of volumetric muscle loss may be improved by the delivery of cells, growth factors and other regenerative components in a suitable carrier or scaffold in order to generate new muscle mass. Extensive research is being conducted in this field, which has been reviewed in an excellent way by others [8,54,55]. In this section, we will give a short overview of the state-of-the-art in this field of regenerative medicine based mainly on recent reviews, and then focus on some promising developments. Next, we will discuss research that is being performed specifically to prevent fibrosis, which is a common complication in volumetric muscle loss and other muscle disorders but has received far less attention. Therefore, we will also include approaches that are being developed for the treatment of fibrosis in other organs such as the liver, the kidney and the skin. The combination of these anti-fibrotic approaches with regenerative strategies for volumetric muscle loss may greatly advance the treatment of muscle defects (see Fig. 4).

3.1. Generation of muscle mass

Initially, attempts have been made to treat muscle defects by the injection of myoblasts cultured from isolated SCs or freshly isolated SCs. The latter seem to perform better since cultured SCs partly lose their regenerative potential and die after implantation. However, also freshly isolated SCs have lost their instructive niche, which appears to be crucial to fully maintain their regenerative properties [8]. This is highlighted by data showing that the implantation of intact myofibers containing SCs induces even more new myofibers than freshly isolated SCs [56]. Also other muscle-derived stem cells such as mesangioblasts and pericytes are able to differentiate into myofibers *in vivo*, as do hematopoietic stem cells (HSC) [57]. Especially for muscle dystrophies, gene therapy strategies are being developed that aim to correct the genetic defect *in situ* or to deliver corrected (induced) pluripotent stem cells to the affected muscles, which is discussed elsewhere [58–60]. Anti-fibrotic strategies for muscular dystrophies are discussed in Section 3.2.4. Here we will focus on the delivery of cells in a carrier material or construct suitable for larger muscle defects. The ideal muscle construct for implantation, however, could be cell-free but attract local precursor cells from the surrounding tissue (*in situ* tissue engineering [61]). Such a muscle construct would have to contain all the necessary properties to allow the precursor cells to proliferate, differentiate and fuse into aligned muscle fibers as discussed in the following sections. Larger muscle defects such as volumetric muscle loss require a delivery material that can temporarily fill up the wound site. A wide range of biological and synthetic materials or combinations thereof are being developed for the delivery of cells or to allow local precursors to migrate into the wound area. Muscle constructs generally require directionality to obtain aligned myofibers as in the original tissue. We recommend that these constructs should be further improved by the addition of antifibrotic agents (Section 3.2). The available materials and the main production methods for aligned carrier systems are discussed in detail in Section 4. In addition, the construct should contain niche factors and growth factors to guide the proliferation and differentiation of cells into new myofibers.

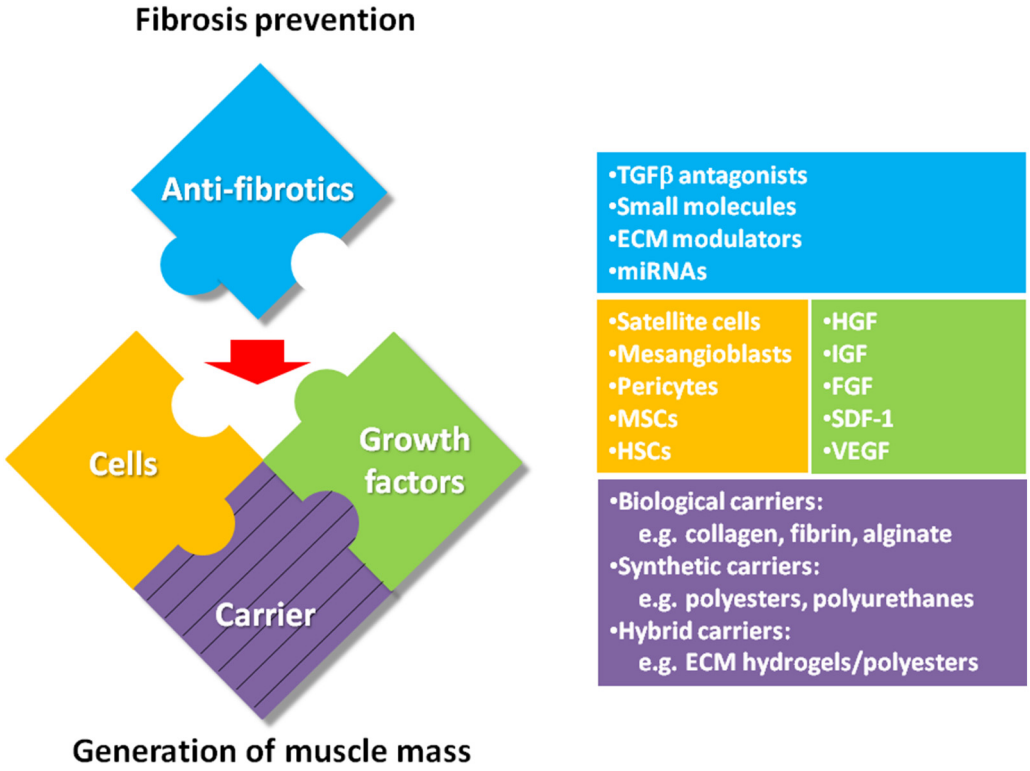

Fig. 4. Combined approach to generate muscle mass and prevent fibrosis. Supplementation with anti-fibrotics will improve the efficacy of the current approaches for muscle regeneration in volumetric muscle loss and other muscle defects. The parallel lines indicate the directionality of the carrier. TGFβ: Transforming growth factor-β, ECM: extracellular matrix, MSC: mesenchymal stem cell, HSC: hematopoietic stem cell, HGF: hepatocyte growth factor, IGF: insulin-like growth factor, FGF: fibroblast growth factor, SDF-1: stromal-derived growth factor-1, VEGF: vascular endothelial growth factor. For clarity, specific niche factors such as vascular and neuronal factors, and laminin, collagen IV and entactin are not indicated (see Section 3.1.1).

3.1.1. Niche factors and growth factors

The muscle niche consists of all factors in the microenvironment that regulate the migration, proliferation and differentiation of precursor cells. Crucial factors are the available adhesion sites and growth factors but also the vasculature and innervation may be essential. In addition, the biophysical properties of the tissue contribute to cell behavior.

In the native tissue, adhesion sites are provided by ECM molecules in the cellular microenvironment. The SCs are located between the sarcolemma of the myofiber and the surrounding basal lamina [57]. The basal lamina contains type IV collagen, laminin and heparan sulfate proteoglycans. The SC adheres to laminin in the basal lamina through the linker protein entactin via integrins (e.g. α7β1). At the opposite side the SC connects to the sarcolemma through M-cadherin. Biological or synthetic carriers can be functionalized by the inclusion of factors that offer adhesion sites such as RGD-containing peptides, laminin, fibronectin or collagen [54,62,63].

A complex network of stimulatory and inhibitory growth factors is involved in muscle regeneration [57,64]. These factors are produced upon injury by immune cells such as neutrophils, macrophages and T-cells but are also released from platelets. In addition, many growth factors are liberated from the disrupted ECM. The nearby vasculature and motor neurons may provide additional growth factors. Factors, chemokines and cytokines that stimulate myogenic proliferation and differentiation include hepatocyte growth factor (HGF), fibroblast growth factor 2 and 6 (FGF-2, FGF-6), vascular endothelial growth factor (VEGF), platelet-derived growth factor AA and BB (PDGF-AA, PDGF-BB), stromal-derived growth factor 1 (SDF-1), granulocyte-colony stimulating factor (G-CSF), interferon γ (IFNγ) and insulin-like growth factor 1 and 2 (IGF-1, IGF-2). These factors often have synergistic effects. We will briefly discuss the main stimulatory growth factors here. IGF-1, and to a lesser extent also IGF-2, stimulates myoblast proliferation and differentiation by regulating the expression of myogenic regulatory factors. Myoblast proliferation is also stimulated by G-CSF and IFNγ. HGF induces proliferation of SCs after injury and their migration to the wound site. In addition, SDF-1 acts as a powerful chemoattractant for stem cells. In a later phase, HGF inhibits the fusion of myoblasts into myotubes. During the healing process, VEGF in conjunction with PDGF stimulate angiogenesis to provide nutrients and oxygen to the regenerating tissue.

The main inhibitory growth factors in muscle regeneration belong to the TGFβ superfamily. These include TGFα, TGFβ1, myostatin and bone morphogenetic proteins (BMPs). Myostatin is produced by SCs and myoblasts and maintains SC quiescence. In conjunction with TGFβ1 it also reduces myoblast recruitment and differentiation. Next to this, TGFβ1 stimulates ECM production and remodeling and thus is one of the main factors inducing fibrosis, which will be discussed more extensively later. BMPs are generally known for their inhibition of stem cell proliferation. Several of the stimulatory growth factors have been used in muscle constructs to promote regeneration [61,62,64]. Apart from adhesion sites and growth factors the biophysical properties of the matrix are also important to stimulate the differentiation of precursor cells into new myofibers.

3.1.2. Biophysical factors

The biophysical properties of the carrier material are also crucial for the performance of the muscle construct. These include the stiffness of the material but also geometrical aspects such as porosity, pore interconnectivity and alignment. The stiffness of the matrix is a key factor for the differentiation of stem cells [65]. Mesenchymal stem cells differentiate along the osteogenic lineage when exposed to substrates with high stiffness, while a low stiffness induces adipogenic differentiation. The stiffness of muscle tissue is in the order of 12 kPa. Interestingly, the stiffness of tissue culture plastic is many times higher (>>100 kPa), which may explain why isolated SCs gradually lose their myogenic properties after prolonged culture [8]. As the fibrotic tissue has a higher stiffness than healthy muscle, it may also be one of the reasons why fibrosis impairs the regeneration of myofibers [66]. Cells attach to the matrix and continuously tug it to sense its stiffness. Signals are transduced to the nucleus via integrins and intracellular pathways, where they regulate gene expression and differentiation [65,67].

The outgrowth of seeded cells within the construct and the migration of host cells into a construct further require that the carrier material has a porous structure. The specific geometry of muscle tissue requires that the carrier material guides the newly formed myofibers into an aligned arrangement [6,68]. This can be achieved in several ways as discussed in Section 4. The pores will also allow ingrowth of blood vessels and nerves, crucial for cell survival within the construct. Vascularization of the construct can either be induced *in vitro* by inclusion of vascular cells or *in vivo* by host cells instructed by suitable chemical and structural cues [5,54]. For the *in vitro* approach endothelial cells, endothelial precursor cells and vascular smooth muscle cells have been used, generally seeded in a construct with structural cues. With time a vascular network forms in culture. However, remodeling and maturation of the vascular network after implantation will always occur. The *in vivo* approach relies on the attraction of precursor cells from the host to create a vascular network. Chemoattractants such as VEGF or SDF-1 may be included in the scaffold or produced by a population of seeded (stem) cells. It seems to be favorable for integration when a preformed vascular architecture is already present [5].

Cellular constructs may be further improved by mechanical or electrical conditioning *in vitro*. Mechanical stimuli have been shown to activate SCs and promote myofiber formation, maturation and alignment as well as force production after implantation [69,70]. However, this highly depends on the exact regimen of mechanical stimulation as also negative effects have been observed. In addition, the optimal regimen may be different for the specific type of muscle that is the therapeutic target [70]. This may especially be relevant for craniofacial muscles as these differ from trunk/limb muscles mainly with respect to embryonic origin, architecture and molecular regulation [67]. Similar effects have been reported for electrical stimuli but also here the optimal regimens are unclear [70]. Considering the crucial role of mechanical and electrical stimuli in muscle physiology, the general idea is that some form of preconditioning is crucial for optimizing cellular muscle constructs.

3.1.3. Clinical translation

At present, decellularized ECM scaffolds seem to be the most promising option for rapid application in the clinic for volumetric muscle loss [5,54]. Decellularized ECM already contains many of the abovementioned niche factors and structural cues for tissue regeneration and integration in the host tissue. The group of Badylak made use of decellularized porcine small intestinal submucosa in the form of powder pillow constructs to treat volumetric muscle loss in the mouse tensor fasciae latae muscle [71]. Although the host response was similar to the non-treated group, angiogenesis, muscle ingrowth, and the number of nerve fibers had increased. At 56 days post-surgery, islands of desmin-positive striated skeletal muscle fibers were observed [71]. The cells involved in regeneration were shown to be CD146-positive perivascular stem cells. Upon electrical nerve stimulation 180 days after surgery, ECM-treated defects gave better results than untreated muscle, although less than uninjured muscles. In a clinical trial with 5 patients with 58–90% tissue deficit, treatment with this material resulted in increased force production in 3 patients and improved functional tasks in 4 patients. Muscle biopsies showed angiogenesis, $CD146^+$/neurogenin-2^+ perivascular stem cells that had migrated out of their niche and $desmin^+$ cells, suggesting *de novo* skeletal muscle formation [72]. In a follow-up study with 8 patients (including the previous five), 5 patients showed improved electrophysiological function [73]. Wolf *et al.* state that critical determinants in the use of decellularized tissue are the recruitment of endogenous stem cells, modulation of the innate immune system (towards the macrophage M2 phenotype), and scaffold degradation. Chemical crosslinking of the ECM seems to inhibit both degradation and release of biological factors,

leading to impaired regeneration [74]. However, some concerns are raised on the availability of donor tissue and organs, and the efficacy of the decellularization process, which might yield undesirable immune reactions [5,54]. These concerns justify the further development of constructs prepared from purified ECM components, synthetic materials or hybrids thereof that mimic the instructive muscle environment.

Although a large area of research is dedicated to finding the most suitable precursor cells for the population of cellular constructs, the ideal construct for implantation might be acellular and should contain all the necessary cues to direct host cells into the correct differentiation pathway and alignment. This requires a construct with biophysical properties similar to muscle tissue that attracts the proper cell types, induces their proliferation and differentiation, and guides growing myofibers into the correct orientation. A crucial factor for clinical success we would like to discuss in detail here is the prevention of fibrosis.

3.2. Anti-fibrotic strategies

In volumetric muscle loss, but also in other muscle disorders, tissue regeneration is often counteracted by the occurrence of fibrosis. Next to muscle, fibrosis can occur in other organs such as kidney, liver, lung and heart [12,75]. In general, the fibrotic response is triggered by some form of acute or chronic injury to the tissue. This could be physical trauma as in volumetric muscle loss but also injury by toxic compounds or an infection. Fibrosis always leads to compromised structure and function of the organ or tissue. The key cells in the fibrotic response are the myofibroblasts that can differentiate from normal tissue fibroblasts but also from pericytes and stellate cells as in liver fibrosis. In addition, myofibroblasts can be generated by epithelial-to-mesenchymal or endothelial-to-mesenchymal transition [75]. Myofibroblasts contract the tissue and deposit large amounts of ECM, mainly collagen, which disturbs normal tissue function. Myofibroblasts differentiate during the wound healing process as a result of changes in the mechanical condition of the tissue and the release and activation of cytokines, specifically TGFβ1. Myofibroblast differentiation involves the expression of α-smooth-muscle actin (αSMA), which is required for contraction of the ECM. Large focal adhesions are formed that transmit the contractile forces to the ECM. Newly expressed αv integrin connects the actin cytoskeleton of the myofibroblast to latent TGFβ1, which under tension causes its activation. Myofibroblasts persist in the wound area through the continuous activation of latent TGFβ by increased stiffness of the ECM [12,75]. The myofibroblast characteristics offer targets for therapeutic strategies. Up to now, research specifically dedicated to muscle fibrosis is rather limited. In this section, an overview is given of experimental approaches for fibrosis in muscle but also in other organs, which may be applicable to muscle tissue. They include approaches specifically targeting myofibroblast differentiation but also approaches that target the ECM, which should be combined with current strategies to generate muscle mass in volumetric muscle loss in order to improve treatment outcome (Fig. 4).

3.2.1. Small molecules

In view of its central role in the differentiation of myofibroblasts, TGF-β1 is an obvious target for anti-fibrotic therapies. Modulators of the renin-angiotensin system such as the small molecule losartan have been shown to block the activation of latent TGF-β1 through inhibition of thrombospondin-1 (TSP-1). TSP-1 is a key regulator of the activation of latent TGFβ1 [76]. In several animal models for muscle injury losartan reduces fibrosis and improves muscle regeneration, which may be enhanced by the simultaneous delivery of stem cells [77–80]. However, the functional recovery of the muscles after losartan treatment may be limited [81]. Losartan also seems to reduce fibrosis in mouse models for muscle dystrophies [82,83]. Anti-fibrotic therapies in muscular dystrophy are discussed more extensively in Section 3.2.4. In a rat model for renal fibrosis induced by urethral obstruction, losartan reduced αSMA expression and collagen deposition possibly by reduced phosphorylation of STAT3 [84]. Also in diabetic cardiomyopathy in rats, interstitial fibrosis was reduced by losartan through reduced JAK/STAT signaling [85]. Cardiac fibrosis induced by endurance training was reduced by losartan and accompanied by a reduced expression of TGFβ1 [86]. Hepatic fibrosis induced in rats by carbon tetrachloride (CCl_4) can be improved by losartan [87], which was further enhanced by combined treatment with atorvastatin, a statin with anti-inflammatory properties. CCl_4 injection is commonly used to induce hepatic fibrosis in animal models. Reduced fibrosis was indicated histologically and by a reduction in αSMA and hydroxyproline, a marker for collagen. Histology showed a clear reduction in the fibrous expansion of the portal tract. In addition, serum levels of TGFβ1 were reduced. In a mouse model for hepatic fibrosis induced by thioacetamide/alcohol, intravenous delivery of losartan incorporated in hyaluronic acid micelles resulted in a larger anti-fibrotic effect than oral administration of losartan [88]. In partial thickness burn wounds in pigs, losartan reduced the number of myofibroblasts and contraction [89]. Several studies in rat models for lung fibrosis showed the efficacy of losartan as an anti-fibrotic agent [90–93]. In animal models for organ fibrosis, angiotensin receptor antagonists such as losartan seem to reduce the TGFβ1-mediated fibrotic pathway. However, in a small clinical pilot study for lung fibrosis, the effects of losartan were inconclusive. In a small clinical study for liver fibrosis, beneficial effects were found in specific cases only [94].

The review by Nanthukamar *et al.* discusses many other small molecules that are in various stages of research for anti-fibrotic therapies [94]. It is noteworthy that, up to the publication of this review in 2015, only two small molecules were approved for treatment of fibrosis, more specifically idiopathic pulmonary fibrosis, a disease with high incidence and mortality. Since then, only a few additional small molecules with a similar mechanism of action have been approved for the treatment of fibrosis [95]. The first molecule, pirfenidone, is also in early clinical trials for systemic sclerosis and diabetic kidney disease. The exact mechanism of action of pirfenidone is unknown, but it seems to affect TGFβ signaling, inflammatory cytokines and p38 mitogen-activated protein kinases. The second molecule, nintedanib, acts as a tyrosine kinase inhibitor that targets VEGF, FGF and PDGF signaling. In general, many of the small molecules being studied target processes that are related to myofibroblast differentiation, including the inhibition of peroxisome proliferator-activated receptors (PPARs), the modulation of growth factor signaling, and the inhibition of αSMA expression by Rho-associated protein kinase inhibitors and focal adhesion kinase inhibitors. Of special interest may be the small molecules that specifically inhibit TGFβ activation and signaling by binding to αv integrins and Smad3, respectively. Using another approach, small molecules may prevent fibrosis by targeting the synthesis, crosslinking and posttranslational modification of collagen and other ECM proteins [12,94]. Considering the complex etiology of fibrotic diseases, a therapy with a combination of small molecules that target different processes may be most promising.

3.2.2. Decorin

Another approach to reduce TGFβ1 activity in the tissue is by applying decorin. This small leucine-rich proteoglycan can bind TGFβ and other growth factors. Decorin was shown to prevent TGFβ signaling after injection in the tibialis anterior muscle in mice. This was evidenced by reduced expression of connective tissue growth factor (CTGF) and fibronectin, which depend on TGFβ [96]. In old, spontaneously hypertensive rats, cardiac fibrosis was prevented by systemic delivery of the decorin gene in a recombinant adeno-associated viral vector via the tail vein. This resulted in stable expression of decorin and reduced cardiac fibrosis while improving cardiac function [97]. In thioacetamide-induced liver fibrosis, human mesenchymal stem cells (MSCs) infected with a decorin-expressing adenovirus improved several fibrosis parameters [98]. In this study, infected MSCs or control MSCs were injected directly into the liver. Decorin-expressing MSCs improved liver function

and reduced liver collagen content, α-smooth-muscle actin, TGFβ1 expression and Smad3 phosphorylation. This indicates that effects were mediated through suppression of TGFβ signaling. In CCl_4-induced liver fibrosis, decorin seems to improve fibrotic parameters as evidenced by a reduction in collagen content, TGFβ1 and αSMA [99]. Several studies indicate that decorin can also reduce fibrosis in the eye. In a rabbit model for corneal fibrosis, decorin gene therapy with an adeno-associated virus was applied topically to the eye. This resulted in a reduction of corneal haze, αSMA and fibronectin while the therapy was non-toxic and non-immunogenic [100]. In a rat model for trabecular meshwork fibrosis in the rat eye induced by TGFβ, decorin was injected directly into the eye. Decorin reduced ECM deposition, increased MMP2 and MMP9 levels while TIMP2 expression was reduced, making it a potential anti-fibrotic for open-angle glaucoma [101]. Finally, animal models indicate that decorin therapy may also be beneficial for peritoneal and epidural fibrosis [102,103].

3.2.3. MicroRNAs

A completely different but promising approach is based on the modulation of post-transcriptional gene expression by microRNAs (miRNAs or miRs). These small RNA molecules bind to mRNAs in the cell after which the complex is degraded leading to reduced protein expression. The binding of a miRNA to mRNA does not require perfect complementarity so one miRNA can recognize multiple target mRNAs [104]. *Vice versa*, the level of one mRNA can be regulated by many different miRs. In order to reduce the mRNA level, miRNA mimics can be delivered (miRNA replacement therapy). This can also be achieved by the delivery of miRNA precursor mimics in a viral vector, which may have the advantage of more sustained miRNA expression. In miRNA inhibition therapy, the repression of protein expression by miRs is blocked to enhance the protein level. This can be achieved by the delivery of anti-miRNA oligonucleotides, which can be chemically modified to enhance their efficacy. Approaches targeting miRNAs and their application in regenerative medicine are extensively discussed elsewhere [104–106]. Up to now, the number of studies on the application of these techniques for fibrotic diseases is rather limited, among which only a few are specifically aimed at muscle fibrosis. We will discuss some of these studies here.

A rat study used a combination of miRNAs (miR-1 and miR-206) and small interfering RNA (siRNA) against myostatin to improve the healing of chemically injured tibialis anterior muscles [107]. Both miRNAs are known to enhance myoblast differentiation, while myostatin is a profibrotic factor in muscle regeneration. This cocktail was directly injected into the injured area of the muscle. Ten days after treatment, the number of new myofibers and force production had increased. Histologically, the fibrotic area within the muscle tissue was smaller. However, long-term effects of the treatment are not available. MiR-1 was also used in a study on cardiac hypertrophy induced by left ventricular pressure overload in rats [108]. The miRNA was delivered in an adeno-associated virus expressing miR-1 and injected into the tail vein. Treatment led to reduced left ventricular hypertrophy and improved functional parameters. At 7 weeks post-treatment, the fibrotic area and the expression of the profibrotic mediators TGFβ1 and CTGF were reduced.

Also in animal models for myocardial infarction induced by ligation of a coronary artery, miRNAs have been used to reduce cardiac fibrosis. In a mouse study, miR-199a-3p and miR-590-3p were delivered with lipofectamine by a single cardiac injection [109]. With respect to safety and technical issues, this may be advantageous over viral delivery. Both miRNAs improved cardiac function and reduced hypertrophy up to 8 weeks. In addition, the infarct size had significantly reduced compared to controls. In a similar model in rats, adenovirus-mediated overexpression of miR-101a markedly improved cardiac performance after 4 weeks [110]. Also, interstitial fibrosis and expression of TGFβ1 were reduced.

Renal fibrosis is a common complication in diabetic nephropathy. In diabetic db/db mice, renal fibrosis was reduced by ultrasound-mediated gene transfer of a miR-29b containing vector. The vector was injected via the tail vein after which the ultrasound probe was placed on the skin near both kidneys to obtain gene transfer. miR-29b reduced the expression of the profibrotic markers TGFβ1, type I and IV collagen and fibronectin, due to a reduced activation of the TGFβ1/Smad3 signaling [111]. In streptozotocin-induced diabetic mice, the inhibition of miR-192 also reduced renal fibrosis [112]. MiR-192 is known to be upregulated by TGFβ1 in the kidneys of diabetic mice and induces profibrotic markers. Levels of miR-192 were reduced by repeated subcutaneous injections of the inhibitor LNA-anti-miR-192. The expression of TGFβ1, collagen and fibronectin was reduced up to 17 weeks. Renal fibrosis induced by unilateral ureteral obstruction in mice was attenuated by the delivery of miR-146a using polyethylenimine nanoparticles injected into the tail vein [113]. These non-viral delivery techniques may have advantages over viral delivery with regard to long-term safety. The miR-146a study showed a reduction in the fibrotic area in the kidneys along with reduced collagen I and αSMA expression induced by inhibition of the TGFβ1/Smad4 signaling pathway.

Finally, two studies show that miR-29b may reduce scar formation in the skin after injury. A recent study on thermal wounds in mice showed that subcutaneously injected miR-29b mimics reduced scarring. MiR-29b diminished collagen deposition and the expression of profibrotic marker genes by inhibition of TGFβ1/Smad signaling [114]. A 2014 study delivered miR-29b in a crosslinked collagen scaffold in full thickness skin wounds in rats [115]. At 28 days after wounding, miR-29b slightly but significantly reduced wound contraction. Protein and mRNA expression data indicated that ECM remodeling was increased but the data on TGFβ1 expression were inconclusive. The authors suggest that miR-29b targets ECM remodeling by modulating post-transcriptional gene expression of TGFβ1 target genes. Interestingly, this study also indicates that the incorporation of miR-29b in a collagen scaffold increases gene transfer *in vitro*. The *in vitro* data indicate that the release of RNA from a collagen scaffold can be controlled by tuning the crosslink ratio.

In summary, animal models for multiple types of organ fibrosis, including muscle fibrosis, show the efficacy of anti-fibrotic strategies. The main target in these strategies generally is the TGFβ/myofibroblast axis. A few approaches using small molecules have already reached the stage of clinical application but many other small molecules have shown positive results in preclinical research. More recent approaches using miRNAs have also yielded promising results up to now. The approaches that are being developed for fibrosis in other organs may also be suitable to prevent muscle fibrosis after VML or other muscle injuries. An area that requires further research is the development of optimal delivery methods for anti-fibrotic components. Preferably, anti-fibrotic components should be delivered locally to avoid systemic side effects. Some data indicate that this can be achieved by incorporating the components into a suitable scaffold. To allow optimal regeneration of muscle tissue, these scaffolds should also include the proper niche factors, biophysical cues and an aligned structure as discussed in Section 3.1. In Section 4, the available techniques to prepare aligned scaffolds are discussed. The next section will give an overview of research into the congenital muscular dystrophies, which are also characterized by the development of fibrosis.

3.2.4. Anti-fibrotic strategies in muscular dystrophy

In contrast to volumetric muscle loss or surgically induced muscle trauma, reduced muscle function in muscular dystrophies is caused by a genetic defect. The congenital muscular dystrophies are a heterogeneous group of rare genetic disorders that are characterized by progressive muscle wasting and secondary fibrosis, which further compromises muscle function as well as the efficacy of therapy [116–118]. The most well-known muscular dystrophy is Duchenne muscular dystrophy (DMD), which affects 1 in 3,500 male births and is generally fatal within the second or third decade of life [117]. DMD is caused by defects in the cytoskeletal dystrophin gene, which is crucial for the stability and

function of myofibers. The other CMDs are generally caused by defects in ECM-related components such as type VI collagen or defects in intracellular and nuclear proteins such as lamin A/C and selenoprotein [116]. Up to now there is no cure for these disorders but the clinical symptoms can be improved by long-term treatment with corticosteroids. However, the significant side effects of corticosteroid treatment warrant further research into alternative, more specific therapies. Most of the preclinical research focuses on DMD but similar approaches may be relevant for the other congenital muscular dystrophies [116]. At present, preclinical research into DMD aims at a combination therapy that corrects the causative genetic defect but also reduces the secondary features such as inflammation and fibrosis [117]. We will not go into the genetic approaches but will discuss the options to counteract the fibrotic process in DMD. The approaches to correct the genetic defects are extensively discussed elsewhere [59,60].

The preclinical testing of anti-fibrotic therapies for DMD is generally performed in genetic mouse models such as the *mdx* mouse. As in the fibrotic conditions we discussed above, many of the experimental approaches target the TGFβ/myofibroblast axis as this also seems to be the major fibrotic pathway in DMD [116]. In a direct approach, neutralizing antibodies against TGFβ or CTGF were shown to reduce fibrosis in *mdx* mice [116]. More indirectly, small molecule inhibitors targeting the renin-angiotensin system such as losartan and lisinopril also seem to reduce fibrosis through inhibition of TGFβ signaling [116]. Similar effects were reported for the small molecule PDGF receptor α inhibitors imatinib and nilotinib [116]. Other experimental therapies in DMD targeting TGFβ employ antineoplastic drugs such as suramin, the plant alkaloid halofuginone and the estrogen receptor modulator tamoxifen [118].

In DMD, the fibrotic process seems to be induced by the upregulation of inflammatory mediators. A crucial pathway in the inflammatory response is the NF-κB signaling. This pathway can be inhibited by corticosteroids, which is the treatment of choice at present but, as mentioned, can have severe side-effects. Several experimental strategies are aimed at attenuation of the inflammatory process by inhibition of NF-κB or the neutralization of inflammatory mediators such as TNFα using antibodies (inflixamib, [118]).

Several studies indicate that therapies aimed at promoting muscle regeneration also lead to a reduction of fibrosis. This has been shown in *mdx* mice for genetic or antibody-mediated inhibition of myostatin. Early phase clinical trials using these antibodies are underway [118]. Similar results have been reported for IGF-I, which is a key mediator of skeletal myoblast differentiation and increases muscle mass along with an anti-fibrotic effect. Many other *in vitro* and preclinical mouse studies employing small molecules or miRNA modulation are being conducted [116–118].

To counteract fibrosis in DMD, approaches are partly similar to the other fibrotic diseases we discussed. The combination with techniques to correct the causative genetic defect may improve the efficacy of treatment. Generally, application is by local or systemic injection instead of delivery in a construct as in volumetric muscle loss. For volumetric muscle loss, techniques are developed to prepare aligned constructs that can be functionalized with compounds to generate muscle mass and limit fibrosis. The available techniques to prepare aligned constructs are discussed in the next section.

4. Delivery of therapeutic components

The vehicle used to deliver active components to the injured site is of major importance for the final result. A wide range of biological and synthetic materials are being developed for the delivery of cells or to allow local precursors to infiltrate into the wound area. Among the biological materials, the advantage of decellularized muscle tissue is that it already contains many of the niche factors native to muscle such as ECM components, growth factors, biophysical properties and cues for myofiber alignment [62,119]. In addition, this material already provides a conduit for the ingrowth of blood vessels and nerves. On the other hand, the availability and consistent quality of decellularized tissue may be problematic, and there is a risk of rejection and disease transmission. This stimulated the use of purified ECM components such as type I collagen, fibronectin, fibrin and many others [62]. Collagen and other ECM components can easily be prepared into sheets and 3D scaffolds containing suitable porosity and geometry for the alignment of myofibers. Alignment or directionality seems to be crucial for the regeneration of muscle tissue. The available techniques for creating aligned ECM scaffolds are discussed in detail below. Several of these components such as gelatin or fibrin can also be used to prepare hydrogels. In general, however, the stiffness of hydrogels is too low compared to muscle although it can be increased by crosslinking.

Next to ECM components, other biological materials such as chitosan, silk fibroin or alginate are being used for tissue engineering. Several types of synthetic materials such as polypropylene, polyesters (polylactic acid, poly-glycolic acid and poly-ε-caprolactone) and their copolymers, and many types of polyurethanes have been used for muscle tissue engineering [62]. The reliable composition and availability, and tunable properties such as stiffness and degradability are a potential advantage of these materials. Synthetic materials for muscle regeneration are generally prepared in the form of foams, hydrogels or electrospun scaffolds. Some of these materials also allow the establishment of directionality. Hybrid scaffolds prepared from ECM components and synthetic materials may offer the best of both worlds; biocompatibility and tunable stiffness, respectively. Delivery materials all require specific components to guide the cells into the formation of new myofibers, collectively designated as “niche factors”.

We here discuss the use of scaffolds with a directional pore structure that mimic the anisotropic organization of the extracellular matrix as observed in skeletal muscle, the use of aligned nanofibrous scaffolds that support the correct orientation of cells, and injectable alternative approaches, like hydrogels, that can be delivered without surgery. From these studies, it is irrefutably clear that biomaterials in the field of skeletal muscle regeneration have focused on the increase of muscle mass instead of the attenuation of fibrosis. Therefore, only few studies on fibrosis are discussed below. Please note that we did not incorporate micropatterned or microgrooved materials as these merely represent a 2-dimensional surface that is not easily translated to a 3D construct.

4.1. Scaffolds with directional pores

Skeletal muscle regeneration of volumetric muscle loss may be promoted by the application of biomaterials. As skeletal muscles possess an anisotropic orientation, it seems rational to use scaffolds that have a similar architecture. Anisotropic scaffolds with directional pores can be produced using various approaches, but we will here discuss methodologies that apply decellularization, lyophilization or 3D bioprinting.

4.1.1. Top-down approach: decellularized muscle

A straightforward option to create a scaffold with unidirectional pores is to decellularize muscle tissue, leaving the extracellular matrix without the cells (Fig. 5). This is a top-down approach as it starts with the intact tissue, which is then decellularized to obtain only the extracellular matrix in a reverse engineering design. Decellularization procedures generally make use of detergents like Triton X-100 or sodium dodecyl sulfate, enzymatic approaches by endonucleases to degrade RNA and DNA, dispase to digest adherent cells and limited trypsin digestions to disrupt protein interactions, as well as physical methods like freeze-thawing to lyse the cells. In 2004, Borschel *et al.* decellularized extensor digitorum longus muscles from adult mice and cultured immortalized C2C12 myoblasts on top of the decellularized tissue for two weeks to obtain a muscle construct. The obtained construct exhibited longitudinal force generation upon electrical stimulation [120]. Decellularized rat abdominal muscles cultured with and without autologous myoblasts have been used for abdominal muscle repair. The cell-

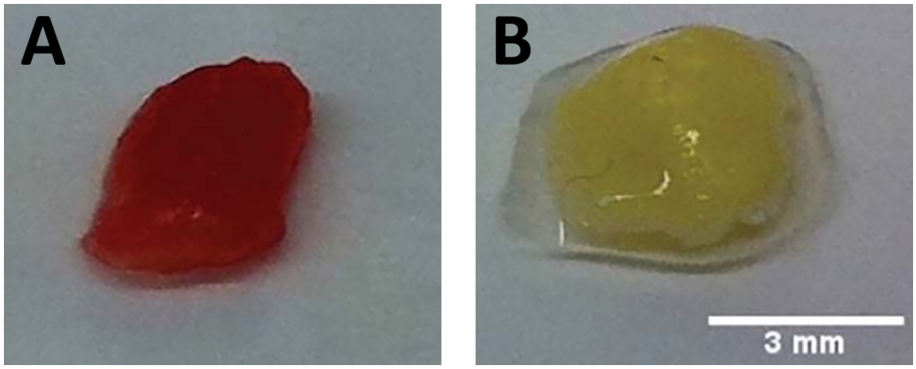

Fig. 5. Macroscopic view of human skeletal muscle A) before and B) after decellularization. Reproduced with permission from the publisher [126].

seeded scaffolds integrated into the host tissue and temporarily generated electrical potentials, whereas non-seeded scaffolds resulted in fibrosis [121]. Whole decellularized mouse tibialis anterior was used to replace the same muscle in a host animal where it supported the formation of skeletal muscle tissue, even more so when the mice were treated with an immunosuppressant [122]. Porcine rectus abdominis muscle was decellularized by perfusion, leaving the vascular bed intact as assessed by vascular corrosion casts [123]. *In vivo*, the material enhanced neovascularization, myogenesis and recellularization in partial-thickness abdominal wall defects in rats. Abdominal rectus muscles from human cadavers have also been decellularized to obtain a muscle scaffold. In rabbits, this decellularized material integrated well with native muscle, although it was mostly replaced with neovascularized fibrous connective tissue [124]. Murine decellularized skeletal muscle tissue supported the formation of muscle fibers at an orthotopic location *in vivo*, but no muscle fibers were observed upon heterotopic implantation in the renal capsule or the xiphisternum [125]. This indicates that the specific cells infiltrating the scaffold are an important aspect of the regeneration process as well. An advantage of decellularized tissue is that the 3D architecture is preserved and handling of the obtained materials is generally easy. In the described studies, decellularized muscle tissue generally enhanced muscle formation compared to controls, although it does not (yet) result in optimal regeneration. However, the methodology comes with several drawbacks, as (donor) organs are needed for the preparation of the scaffold, the exact composition of the decellularized tissue is not known, and batch-to-batch variation may be rather large, so this material requires extensive quality control. In the next approach, the quality of created scaffolds can be controlled at multiple levels in the production process Fig. 5 [126].

4.1.2. Bottom-up approach: directional scaffolds

Another possibility to mimic the directional muscle architecture is by building a scaffold from individual purified components, therefore called a bottom-up approach. The advantage of this approach is that it offers the possibility for extensive quality control of the individual components and the end product alike. In addition, many different combinations of components can be prepared. The bottom-up approach can be performed with molecules from synthetic [127–129] or biological origin, and combinations thereof. In this review, we will focus on biological (extracellular matrix-based) components.

4.1.3. Lyophilization

Scaffolds from biological components are generally prepared by freezing and lyophilization. Collagen is often used as a biological component for such a scaffold [130]. When a collagen suspension in aqueous acetic acid is frozen, phase separation occurs as the water crystallizes into ice first, which increases the concentration of collagen and acetic acid in the non-frozen fluid and pushes it away from the pure ice crystals. In the presence of a temperature gradient, ice crystals nucleate at the coldest site and grow along the temperature gradient [131]. Pot *et al.* identified four steps in the formation of unidirectional collagen scaffolds on a wedge-shaped metal block (Fig. 6): 1) formation of an ice dendrite network, 2) development of vertical protrusions/nuclei, 3) evolutionary selection, and 4) unidirectional (mainly cellular) ice crystal growth [11]. During the lyophilization step, the ice is removed by sublimation leaving the entrapped collagen as an imprint of the ice crystals Fig. 6 [132].

Several types of lyophilized directional scaffolds have been evaluated to enhance skeletal muscle regeneration. These include chitosan scaffolds with a unidirectional orientation that promoted the formation of ~50-μm-diameter myotubes [133]. Collagen scaffolds with a unidirectional pore structure containing C2C12 cells were used to replace the anterior tibial muscle of immunodeficient mice, which resulted in the formation of aligned muscle fibers *in vivo* [9]. Collagen scaffolds with a radial pore orientation [10] were used in a rat model for diaphragmatic hernia. The diaphragm is composed of a central tendon plate with radially oriented skeletal muscle fibers. Implantation of cell-free scaffolds showed increased infiltration of cells and alignment of smooth-muscle-actin positive cells compared to scaffolds with randomly oriented pores [134]. The use of directional scaffolds prepared from purified ECM components for skeletal muscle applications is still rather limited, but it provides clear advantages over decellularized tissues as the composition can easily be adapted to various applications, and the quality of the individual components can be checked prior to preparing the scaffold. With a myriad of potential scaffold materials, however, the main question may be what the optimal composition is for a specific application. Another method for the preparation of scaffolds with unidirectional pores is bioprinting, also termed additive manufacturing.

4.1.4. 3D bioprinting

Next to lyophilization, 3D printing can be used to prepare biomaterials for skeletal muscle regeneration. This technique also allows for the preparation of scaffolds with unidirectional pores [135]. Multiple techniques exist for 3D printing such as droplet-based methods (inkjet bioprinting), microextrusion-based methods and laser-based methods (stereolithography and forward transfer) [136,137]. Collagen, gelatin, hyaluronic acid, fibrin and alginate are natural polymers that are often used for 3D bioprinting. Currently, most approaches are aimed at cell-laden bioprinting techniques instead of first printing the 3D scaffold and then seeding the cells, as cell printing allows for an even distribution of cells throughout the construct. Cells can be bioprinted using bioinks, which are mostly based on hydrogels. Here, some examples of 3D bioprinting in the context of alignment of skeletal muscle are discussed. The reader is referred to additional papers for a more elaborate review of the 3D bioprinting process [138–140].

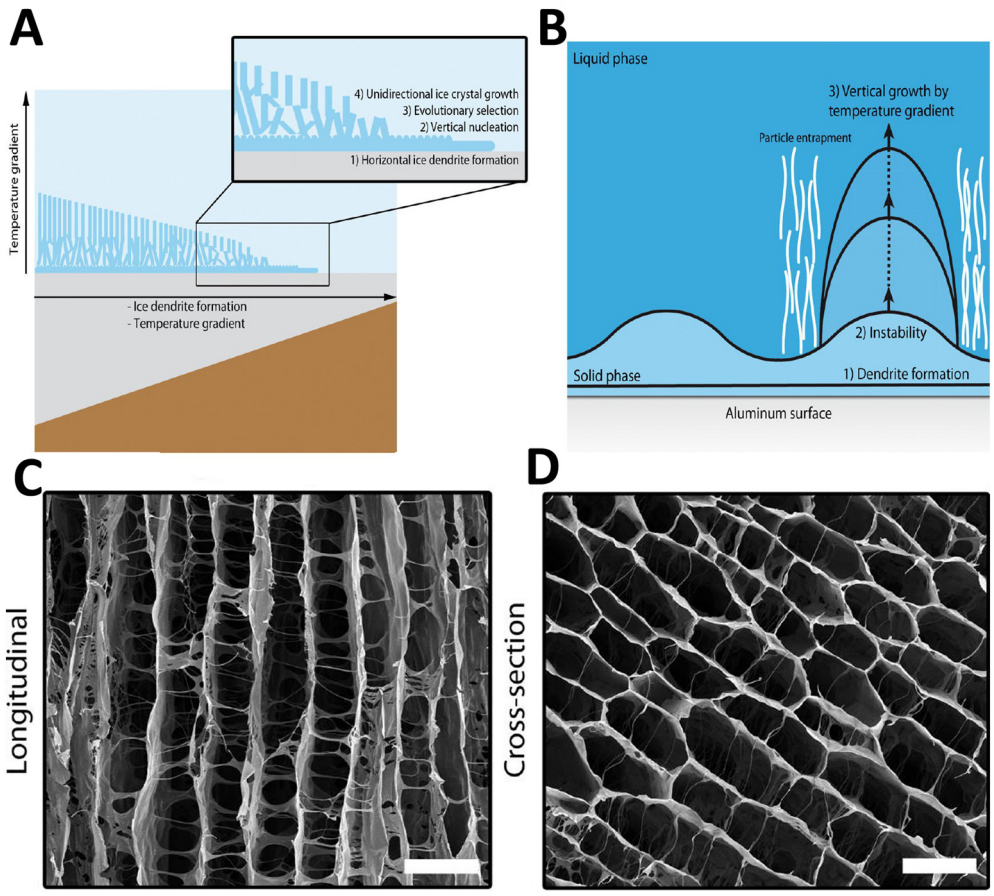

Fig. 6. Proposed mechanism for the formation of collagen scaffolds with aligned pores and scanning electron micrographs of such scaffolds. A) A small horizontal temperature gradient results in a controlled solidification area for ice dendrites. Next, nucleation sites merge into ice crystals by evolutionary selection. A larger vertical temperature gradient facilitates the vertical growth of the ice crystals. B) Physical disturbances in the freezing media result in an unstable surface. Ice crystals grow out of these instabilities and are further guided by the vertical temperature gradient, where collagen fibrils become entrapped between the growing ice crystals. SEM of a C) longitudinal and D) cross-section of the scaffolds. Adapted with permission from the publishers [11,132], copyright 2015 American Chemical Society.

Direct-write bioprinting of C2C12 cells in a solution of natural alginate with the thermosensitive block copolymer Pluronic resulted in alignment of the cells in the direction of deposition (Fig. 7) [141]. Constantini *et al.* used a bioink based on photocurable PEG-fibrinogen with alginate to print C2C12 cells. In this system, a co-axial needle extruder was used to simultaneously print the cells and temporarily crosslink the alginate gel using Ca^{2+} ions, while in a next step the PEG-fibrinogen in the gel was UV-crosslinked and the alginate was washed away. In culture, myosin heavy chain expression increased in time and striated myofibers were observed at day 21. Twenty-eight days after subcutaneous implantation in SCID mice, bioprinted implants showed more myofibers and better alignment compared to non-bioprinted (bulk) implants [142]. Pati *et al.* used an alternative approach by printing three-dimensional tissue analogues from cells in

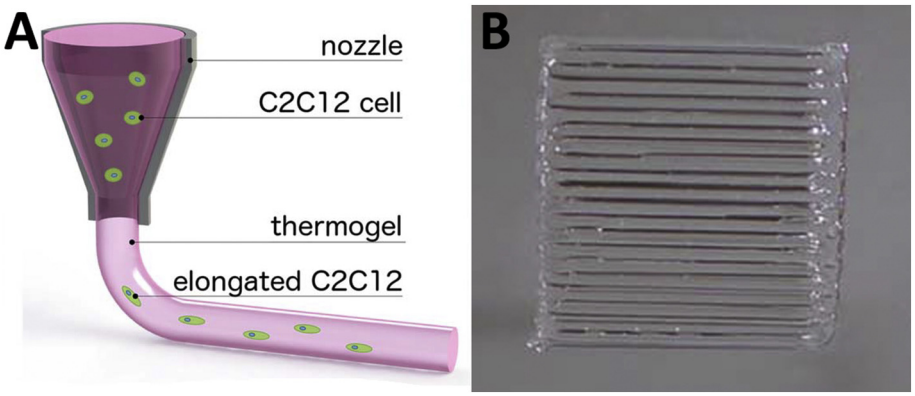

Fig. 7. Direct-write bioprinting. A) Overview of the process. B) Macroscopic view of a multilayered material. Figure reproduced with permission from the publisher [141].

decellularized and solubilized extracellular matrix bioink obtained from adipose, cartilage and heart tissue, although only decellularized heart ECM was printed without a supporting framework of polycaprolactone (PCL) [143]. In a recent study using solubilized decellularized skeletal muscle, the same group showed that this material enhanced *in vitro* cellular differentiation compared to collagen bioink [144].

Kang *et al.* presented an integrated tissue-organ printer to produce human-scale tissue constructs, which was used to prepare a skeletal muscle construct using murine myoblasts. For this, myoblasts in composite hydrogels (containing fibrinogen, gelatin, hyaluronic acid, and glycerol) were printed interspersed with Pluronic hydrogel and supporting PCL pillars. After reaction with thrombin and washings with medium, only fibrin and PCL remained in the construct. Constructs that had differentiated for 7 days were then implanted subcutaneously in nude rats, while embedding the dissected common peroneal nerve in the construct. Two weeks after implantation, myosin heavy chain positive and α-bungarotoxin binding cell clusters were observed, also displaying neurofilament contacts. The construct showed functionality as the action potential was a third of control muscle [145]. An *in vitro* study using printed C2C12 muscle and 3T3 fibroblast cell lines showed the potential of combining tissues (skeletal muscle-tendon) using integrated organ printing [146].

Another method to influence the directionality of cells is by aligning the individual fibrils or fibers in a biomaterial. This is different from the preparation of biomaterials through lyophilization and 3D printing, in which directionality is mostly effectuated by influencing the pore direction.

4.2. Aligned fibrous scaffolds

For the preparation of anisotropic scaffolds with aligned fibers, electrospinning is the most widely applied technique. Electrospinning can be applied for the preparation of biological scaffolds and results in fibers in the nanometer to micrometer range, similar to fibers in the extracellular matrix. Electrospinning makes use of an electrical field (kV range) to attract a protein jet to a grounded target. When the jet travels to the target, the solvent evaporates and a dry fiber is collected.

A 2008 paper of Choi *et al.* showed the superior alignment and myotube formation of human skeletal muscle cells on electrospun unidirectionally oriented PCL/collagen nanofiber scaffolds compared to randomly oriented nanofibers [164]. However, mostly polymers that are being electrospun anisotropically are used for skeletal muscle applications, including polyesterurethane [147,148], poly(L-lactide) [149,150], poly(ε-caprolactone) [151–154], poly(L-lactide-co-glycolide)) [155], and poly(vinylidene fluoride) [156], as well as combinations of poly(ε-caprolactone)/polyaniline [157,158], poly(urethane)/growth factors [159], poly(L-lactide-co-glycolide)/superparamagnetic iron oxide nanoparticles [160], and polymers with multi-walled carbon nanotubes [161]. Polymers are applied because of their ease of handling and compatibility with solvents that are used in the process. Natural materials, like proteins may be affected by the applied solvents [162].

Natural materials that have been electrospun for skeletal muscle applications in an aligned mode include chitosan (with PCL) [163,164], silk fibroin [165] (also with PCL [166]), gelatin (with multi-walled carbon nanotubes [167] or polyaniline [168]) and collagen [169] (also with PCL [170,171]). Multi-walled carbon nanotubes were incorporated to improve both the mechanical properties and conductivity of the nanofibers, which resulted in an increased elastic modulus and larger C2C12 myotubes *in vitro*, which increased even more upon electrical stimulation. Electrical stimulation also enhanced the contractility of the myotubes [167]. Another method to produce electrically conductive scaffolds is to incorporate a conductive polymer like polyaniline, which requires the presence of a doping agent, e.g. camphorsulfonic acid. On gelatin-polyaniline-dopant nanofibers, myotube formation and maturation were enhanced compared to gelatin or gelatin-dopant fibers [168]. Takeda *et al.* used electrospun glutaraldehyde crosslinked collagen to prepare microfibers onto a U-shaped jig. When rat myoblasts were cultured on this so-called string-shaped scaffold, it resulted in cell-dense muscle tissue with cross-striation [169]. Recently, aligned PCL-collagen nanofibers were also electrospun with diluted acetic acid as solvent, a solution generally used for the preparation of lyophilized collagen scaffolds, which does not denature the collagen. Process parameters like electrical field and flow rate proved critical in the formation of aligned nanofibers [171]. This approach may result in an improved biocompatibility of prepared nanofibers. Others have coated electrospun polymers with extracellular matrix proteins, like (non-aligned) poly(methyl methacrylate) nanofibers with laminin/collagen [172].

Alternative methods to align fibers/fibrils in fibrous scaffolds include lyophilization and co-extrusion. Please note that this differs from the lyophilization strategy above where the pores are aligned. When a suspension of insoluble collagen in diluted acetic acid was frozen while falling into liquid nitrogen propane and successively lyophilized, it contained longitudinally oriented fibrils [173] that may guide skeletal muscle cells in the correct orientation. Micropatterned 3D porous collagen scaffolds have also been prepared using lyophilization [174], albeit that this resulted in just one layer of grooves. *In vitro*, rat L6 myoblasts formed myotubes and multi-layered skeletal muscle bundles on the microgrooved scaffolds. Alignment of collagen fibers has further been pursued by the application of a magnetic field [175], an electric field [176] or shear flow [177]. Fibrin threads with a diameter in the micrometer range have been co-extruded from fibrinogen and thrombin/$CaCl_2$ solutions [178,179] and seeded with human myoblasts. Upon implantation in a partial-thickness central skeletal muscle defect in nude mice this reduced fibrosis, but this is one of only a few *in vivo* studies with anisotropic fibers.

For most applications of scaffolds that induce an increase in muscle mass, open surgery would be necessary. It would be advantageous in that respect to apply injectable materials that can be delivered with minimally invasive surgery. In the next section, injectables will be discussed with potential for skeletal muscle regeneration.

4.3. Injectable materials

Hydrogels are polymer networks with hydrophilic properties generally described as “polymeric material that exhibits the ability to swell and retain a significant fraction of water within its structure, but will not dissolve in water” [180]. For skeletal muscle regeneration, hydrogels have been extensively studied including the use of natural components like fibrin, gelatin, collagen, alginate, matrigel, laminin, hyaluronic acid and heparin [181–186]. In addition, hydrogels have been combined with growth factors (*i.e.* IGF-1, VEGF) to enhance skeletal muscle regeneration or to reduce fibrosis [183,187]. The use of hydrogels is especially attractive if these can be applied as an injectable material. Early results showed that regenerating agents (RGTA®s) such as carboxylated and sulfated dextrans that mimic heparan sulfates [188], enhance muscle mass and reduce muscle fibrosis. RGTAs bind growth factors in a similar fashion as heparan sulfates do, sulfate and carboxyl groups being the main structural elements for binding. OTR4120 (synonymous to RGTA11) was shown to bind and protect growth factors including FGF2 and VEGF [189,190]. Contrary to the natural heparan sulfates, RGTAs are resistant to enzymatic degradation by mammalian glycanases and heparanases due to the α1-6 carbon-carbon bond between sugar moieties [191], which will allow the compounds to persist in the body over time. When rat soleus muscle is crushed from tendon to tendon, this results in fibrotic tissue after repair. Intramuscular injection of OTR4120, however, accelerated and improved healing. Compared to the control, this RGTA reduced fibrosis, increased fiber density, augmented fiber maturation and improved electromyographic response [192]. In a more recent paper, the intramuscular injection of OTR4131 in a rat model for critical limb ischemia resulted in an increased number of myofibers that were also more mature. In addition,

an increased VEGF expression and microvessel density was observed for the OTR4131 group [193].

Hydrogels have also been prepared from decellularized skeletal muscle [144,194,195], but this destroys the original skeletal muscle 3D architecture. Several methods have been assessed to make anisotropic hydrogels that may guide the direction and alignment of skeletal muscle cells. Anisotropy is often accomplished by a specific production process or by an external force; e.g. by casting in a defined mold [196], by extrusion [197], by microfluidics [198,199], by tension [200], by dielectrophoresis [201] or by a magnetic field [202]. Not all of these methods allow for translation to *in vivo* injectable delivery, but a few do have potential for future patient applications. Liquid crystalline nanofibrous scaffolds based on peptide amphiphiles with a palmitoyl tail and amino acid cap improved the engraftment of FACS-sorted murine muscle stem cells when injected in muscle. Long-range alignment of nanofibers parallel to existing myofibers was achieved *in vivo* using a controlled pump [197]. De France *et al.* recently described an elegant method to prepare nanocomposite hydrogels that were both injectable and aligned magnetically [202], and consisted of cellulose nanocrystals in a poly(oligoethylene glycol methacrylate) hydrogel. The alignment of the cellulose nanocrystals persisted when the magnetic field was removed. When C2C12 myoblasts were encapsulated, the cells showed alignment in a magnetic field and enhanced myotube F-actin filaments upon differentiation. This may offer an injectable system that can be applied *in vivo*.

5. Conclusions

Many types of skeletal muscle defects do not only involve loss of muscle tissue but also the development of tissue fibrosis or scarring. Fibrosis often causes a severe impairment of function after the surgical treatment of a muscle defect. A large area of research has focused on the regeneration of muscle tissue, mainly for limb muscles. However, the prevention of muscle fibrosis after treatment has received little attention. As an example, we have presented the muscle defect in the soft palate of cleft lip and/or palate patients, which is characterized by a shortage of muscle tissue as well as the occurrence of fibrosis after surgical treatment. The treatment of this disorder therefore requires both the restoration of muscle volume and the prevention of fibrosis.

The main approaches for generating new muscle mass are based on biological or synthetic scaffolds, or hybrids of these materials. Generally, the scaffolds should have an aligned structure to guide new myofibers into the correct orientation. Techniques to obtain these anisotropic scaffolds include decellularization, freeze-drying, 3D bioprinting and electro-spinning. Researchers have also put effort in making injectable hydrogels with a unidirectional structure. The scaffold should also contain suitable niche factors and biophysical properties to stimulate the differentiation of precursor cells into new myofibers. Several types of precursor cells can be included in the scaffold to initiate the regeneration process, but in order to reduce preparation time and costs it may be more efficient to attract local precursor cells. This approach is called *in situ* tissue engineering and is exemplified by the use of decellularized extracellular tissue matrices, which already contain many of the required niche factors. Presently, this seems to be the most promising option for rapid translation into the clinic. However, for reasons of availability and safety, the further development of artificial scaffolds is warranted.

The prevention of muscle fibrosis is not extensively studied, especially not in combination with biomaterials, but interesting options are provided by research into fibrosis in other organs such as the lungs, the kidneys and the liver. Generally, these approaches target the TGFβ/myofibroblast axis by the delivery of small molecules, decorin or miRNAs. A few small molecules are already approved for clinical use in lung fibrosis, and several others have shown promising results in preclinical studies or clinical pilot studies. The application of miRNAs has not yet passed the preclinical stage but offers interesting possibilities to counteract the regulatory network in organ fibrosis. MiRNA-based therapy is an emerging field in several diseases including cancer [203]. Interestingly, we have recently shown that specific miRNAs appear to be dysregulated in cells from cleft palate patients [204]. The promising results of therapeutic anti-fibrotics in other organs may give direction to applications in muscle fibrosis. Local delivery of anti-fibrotics can be achieved by inclusion into a scaffold, which prevents undesired systemic side effects. In contrast, experimental anti-fibrotic strategies for muscular dystrophy require systemic delivery or local injection.

In summary, the ideal muscle construct should stimulate the formation of new myofibers, while preventing the development of fibrosis. This can be achieved by an aligned scaffold or carrier material that contains all necessary niche factors and one or more anti-fibrotic agents. We expect that this combinatorial approach will greatly improve the clinical outcome of surgical treatment for skeletal muscle defects.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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