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Skeletal Muscle Tissue Engineering: Present and Future

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Abstract

Introduction: Approximately 40% of every adult human body mass is composed of skeletal muscle. Skeletal muscle diseases range from the debilitating to crippling and may even end to death. The skeletal muscle stem cells, the satellite cells, are few in numbers. The ability to take a few cells from an adult human and produce a large mass of functional skeletal muscle would be of invaluable benefit to human kind.

Conclusion: As of today it seems that designing and constructing a skeletal muscle substitute with real functional properties is a little far out of reach; because there as yet exists an effective and clinically feasible method for engineering a skeletal muscle that would be large enough with mature and livable fibers aligned in the same direction.

Keywords: Skeletal Muscle Tissue Engineering, Satellite Cells, Niche, Scaffold, Growth Factors

Introduction

The self-repair capability of skeletal muscle is so limited that makes it unable to self-treat injuries such as trauma, mother-born deficiencies, tumor, long term denervation and different kinds of myopathies [1]. Disconnection between nerve and muscle for long periods of time leads to the elimination of the positive effects of nerve impulses and neurotropic factors, on the production of different proteins in skeletal muscle and eventually loss of a muscle mass [2]. In addition to these factors, ischemic injuries, which lead to reduced perfusion of the muscle, will result in cellular death and finally destruction of the body mass [3]. Moreover, aging paves the way for destroying the body mass and its power. Different factors such as decrease in protein synthesis, increase in apoptosis level and decrease in replacement of dead cells by live cells (cell turnover) play a role in destruction of skeletal muscle during aging [4, 5].

The activities of investigators have gone beyond the border of purification, preservation and cell proliferation to look for a structural and functional reconstruction of different body tissues, using tissue engineering principles. Many mistakenly assume that tissue engineering is the same as implanting prosthesis in human body (or in animals in research cases) to recover the lost ability to the person. Tissue engineering is, in fact, benefiting from the rules and principles of engineering in changing the behavior of injured but still living tissues; in a way that the tissue can regain its lost function. However, the construction of replacements for skeletal muscles, using tissue engineering is not as simple as constructing skin replacements. In the latter case where skin is produced and distributed by many business companies around the world, scientific breakthroughs have been greatly prominent. Perhaps one of its important reasons is the simplicity of skin tissue engineering in comparison with other tissues; needless to emphasize on the will of people towards beautification issues.

Today, the construction of any kind of muscle replacement (cardiac, smooth and skeletal muscle) is the focus of tissue engineers' attention. Success in the area of introducing a variety of stem cells into the ischemic or necrotic myocardium and their transformation into myocardial cells has happened in the medical world. Accordingly, there is high hope that in a near future, patients can use heart replacement to restore the pumping activity of an attacked heart. Perhaps it would be an ideal situation to obtain a three-dimensional image of a necrotic area of a diseased heart (by scanning and further software reconstruction), to prepare a scaffold made out of the host ECM in equal size and volume properties, into which autologue stem cells and growth factors are co-seeded. This outside-made 3-D muscle replacement is then ready to be implanted into the patient's myocardium via a surgical operation. We are not far away from practicing this process in day clinics in the decade commencing 2020.

Unfortunately, up to the turn of the millennium, the interest in creating Skeletal Muscle Substitute (SMS) has not been too much. The complexity of SMS has flared the situation and has held it back furthermore. Skeletal muscles, like other tissues, have residing stem cells called satellite cells (SC). These cells divide in times of muscle injuries and differentiate into mature

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cells. The main limitation about these cells is their low number. As such, biologists have tried to isolate and expand them in *in-vitro* environment. The proliferation of SCs in static culture medium, which are mainly rigid and inflexible, has encountered failure. In the past few years, the introduction of the variety of bioreactors, providing a dynamic environment, has facilitated further the multiplication of these stem cells. It seems that skeletal muscles, by lengthening and shortening due to nerve impulses, help to preserve and survive these SCs. In other words, keeping a muscle from contraction for long time not only atrophies the muscle fibers but also decreases the number of innate SCs.

Development of muscle fibers

Skeletal muscle is consisted of hundreds or thousands of muscle cells, all aligned in the same direction. This property of fibers lying parallel to each other in a three-dimensional space is quite critical in producing optimum tension and muscle power. Usually, fibers are stretched along the two poles of the muscle so that the force created is transferred to the bones (or other supports) by means of connective tissues (ligaments and tendons) and therefore activity or movement of body parts follow. Important structural and functional elements in skeletal muscles include muscle fibers, capillaries, motor nerves, extracellular matrix and growth factors. In addition to these, there are numerous determinant parameters within each aforementioned element that need be investigated. Assuming that all above elements are efficiently provided, the non-parallel status of emerging muscle fibers can upset all the endeavors.

As mentioned above, the recovery of multinucleated muscle fibers in mature mammalian skeletal muscle is limited. Satellite cells become active in response to factors released from the injured muscle fibers and go through a specific path for proliferation and differentiation to replace the injured fibers. The activation of SCs will lead to hypertrophy of cellular organelles, expansion of cytoplasm and changes in the shape of the cell. Satellite cells, when activated, produce myoblasts which would then express myogenic transcription factors and a specific filament protein called desmin [6].

The differentiation of myoblasts into multinucleated myofibers depends on four transcription factors, Myf5, myoD, myogenin and Mrf4. During differentiation process, mature sarcomere possess parallel Z disks, thin and thick filaments and bands of A, H, I and M. Nuclei become wide and stretched and travel to the myoplasm sidelines. Early thinner fibers merge together to create secondary thicker fibers [7]. With progress in differentiation, the cell membrane resting potential hyperpolarizes and an increase in the amplitude of the action potential and the speed and magnitude of calcium flux ensues. Sarcoplasmic reticulum and transverse tubules are matured simultaneously in order to create an appropriate and efficient coupling of excitation-contraction [8, 9]. Receptors of acetylcholine will appear on the end-plate on sarcolemma [10, 11]. This will lead to an increase in the electrical excitability of muscle fibers while maturing [2, 12].

Agrin, a growth factor stemmed from nerves, has attracted attention for more than two decades, due to its vast role in the development of structure and function of synapses both at neuromuscular junction and in the central nervous system and also for its role in immune system [13]. In skeletal muscles, agrin plays an important role in differentiation of post-synaptic membrane at neuromuscular junction and in particular in forming and establishing acetylcholine receptors [13].

The capacity of producing force and power by skeletal muscle not only depends on the structure and function of actin, myosin, tropomyosin and troponin but also on the extent of expression of proteins involved in power transfer from a single fiber to the whole muscle tissue. Muscles are divided into two categories in terms of tension transfer to the bones. In muscles whose tendons are located at the two poles, each muscle fiber spans the whole length of the muscle to enter the tendon. The other category includes shorter muscle fibers chained together sequentially before reaching the tendons at both ends. In this group, structures similar to tendons connect these shorter fibers and finally are responsible for tension transfer [14, 15, 16]. Several researches have shown that the costamere is a structural-functional component of striated muscle fiber which connects the sarcomere of the muscle to the cell membrane. Costameres are sub-sarcolemmal protein assemblies circumferentially aligned in register with the Z disks of peripheral myofibrils. They physically couple force-generating sarcomeres with the sarcolemma in striated muscle cells and are thus considered one of several Achillis heels of skeletal muscle, a critical component of striated muscle morphology which, when compromised, is thought to directly contribute to the development of several distinct myopathies.

Strategy to repair skeletal muscle

The number of satellite cells (SC) which are able to repair muscle is quite low in skeletal muscles (1 to

5%) depending on the age and the type of muscle fibers [18]. Therefore, the endogenous population of these cells is not enough to replace the large number of necrotic muscle fibers to regain the function of the injured muscle. At present, muscle autograft and myoblast transplantation are two treating strategies to reconstruct the injured muscle tissue. Autograft technique has had limited clinical output, partly due to unfavorable microenvironment of the injured region which modulate the function of the transplanted tissue and simultaneously, the recipient muscle will face problems leading to destruction of muscle mass and its function [1]. Myoblast Transfer Therapy (MTT) is proposed as one of medical strategies for muscular dystrophies. The intramuscular injection of healthy myoblasts to Duchenne Muscular Dystrophy (DMD) causes the recovery of expression of dystrophin and an increase in muscle tension [19]. DMD is a genetic disease in which the dystrophin gene located on X chromosome is affected. In any case, in clinics, MTT has not gained enough success due to the instant death of a large number of transferred cells immediately after injection, inappropriate distribution of injected cells and immunological responses related to allognic myoblasts [20]. The use of autologue myoblasts eradicates undesired immunological responses; however, the problems concerning low survivability and inappropriate distribution of injected cells still remain.

The science beyond the borderlines

To our knowledge, no laboratory has yet claimed the construction of skeletal muscle replacement. Since the crucial part of this construct is its cell component, this section tries to emphasize on the stem cells. Stem cells display two main functions; "self-renewal" and "differentiation into different cell lines". Stem cells are responsible for development and repair of tissues and limbs. Biochemical and biomechanical signals lead to their proliferation. For the first time, *Studitsky* demonstrated that skeletal muscle has a significant repair capacity and that the satellite cells (SCs) are responsible for this repair and muscle construction [21]. Myosatellite or satellite cells are small mononuclear progenitor cells with virtually no cytoplasm found in mature muscle. SCs are found sandwiched between the basement membrane and sarcolemma of individual muscle fibers, and can be difficult to distinguish from the sub-sarcolemmal nuclei of the fibers [22]. Myoblasts have two fates: they either join the existing myofibers to increase their number of nuclei, or fuse together to form new multinucleated myofibers [23]. The most important role of SCs in the postnatal period is the production of multinucleated myofibers in order to mature the parent skeletal muscle and to increase the number of muscle fibers. In a mature muscle, the role of SCs changes to increase the number of nuclei without an increase in the number of muscle fibers and as such, preserve muscle homeostasis and hypotrophy [24].

During fetal life in rats, 30 to 35% of sublaminal nuclei belong to SCs, but this proportion falls over time and by adulthood, only 1 to 4% represents SCs [25]. Heterogeneity of myoblasts derived from satellite cells is low and their myogenic markers are similar in most of their developmental stages. However, heterogeneity of satellite cells between different muscles is greater and more obvious [26]. For instance, the masseter muscle in the head regenerates poorly as compared with limb muscles but it is not fully evaluated if this effect is dictated by its own microenvironment. However, SCs within extraocular muscles remain proliferative and add nuclei into the uninjured myofibers [27]. This phenomenon may happen due to environmental cues. Perhaps the myogenic progenitors giving rise to different muscle types, reflecting their different ontogeny from body muscles. It is not still clear whether heterogeneity of SCs has anything to do with being multi-potent or not.

The cells isolated from muscle tissue are able to differentiate into neurogenic and myogenic lines as well [28]. These cells can also differentiate into endothelial cells [29]. Recently, it has been shown that under standard cultural conditions, these cells can differentiate into fat and bone cells.

The main challenges in this field are as follows: Do stem cells of satellite cells reside in muscle tissue? Perhaps, the self-renewal of SCs is not the only mechanism that preserves its population. When in a healthy and mature muscle, most SCs differentiate into myogenic cells. Thus, it is necessary to replace them by precursor cells which are not stemmed from satellite cells. In this scenario, it is possible that a precursor cell exists in a region outside satellite cell niche that would replace the satellite cell. Such a cell can be a multi-potent stem cell which is able to differentiate into several cell lines. Precursor endothelial cells and interstitial cells in vessels' smooth muscle are candidates for this replacement [30]. Stem cells derived from vessel during the course of development can occupy SC niche after transplantation [31]. Endothelial cells from infant rat vasculature and those from adult rat and also cells stemmed from vessel's smooth muscle can all be

transformed into myogenic cells [32, 32]. Therefore, it is hypothesized that SCs exist in these structures as well. In addition, satellite cells and endothelial cells express similar specific biomarkers (e.g., CD34) and myogenic and endothelial cells probably share a common embryonic progenitor [34, 35]. Although other cellular resources, apart from SCs, may not play a vital role in muscle repair in normal and natural conditions; however, under aging and disease conditions, they are mobilized and recruited to improve and synergize the function of SCs to preserve myofibers.

Satellite cells niche

The constituting components in niche affect the behavior of stem cells. Changes that take place in niche, due to aging, may also affect the function of stem cells in adult muscles. Environmental cues such as those borne from the vessels, nervous system and other existing cells in interstitial layer, all influence niche composition. Age-related alterations, like an increase in the number of fibroblasts in the interstitial layer, an increase in the extracellular matrix in tissue and changes in neuromuscular junction, directly or through niche components, affect the activity of a stem cell to some extent. Therefore, to improve the regenerative function of stem cells in an adult muscle, niche components are to be targeted [36].

Previously, it was believed that stem cells derived from adult tissues only turn into the cells of the same tissue. Recently, the researchers have shown that stem cells derived from mature tissue have the potential to differentiate into different types of cells. For example, it was shown that hematopoietic stem cells derived from bone marrow contribute to the regeneration of skeletal muscle, cardiac muscle, liver and multiple endothelial tissues [37].

Satellite cells in adult muscle remain inactive until external stimuli trigger their re-entry into the cell cycle. Their progeny, myoblasts, fuse to form new multinucleated myofibers. Cell surface markers associated with the SC phenotype, either in the quiescent or activated state, have been identified and include: M-cadherin, c-met, and CD34. Therefore, it is believed that SCs constitute a stable and self-renewing pool of stem cells in adult muscle where they participate in the growth and repair of the tissue [38].

In addition to satellite cells, skeletal muscle has at least two more sources of stem cells: 1) muscle-derived satellite cells (MDSC) with several differentiative abilities and self-renewal property, a candidate for being the predecessor of the SCs in skeletal muscles, and 2) side population or SP which actively excludes Hoechst 33342 dye [39]. Muscle SP cells expressing Sca-1, the hematopoietic stem cell marker, possess the ability to differentiate
into either hematopoietic cells, skeletal muscle fibers, or satellite cells following transplantation. Those SP cells expressing hematopoietic marker CD45 are capable of differentiating into hematopoietic and muscle cells. Thus it appears that these novel muscle stem cells have characteristics similar to those of hematopoietic stem cells, and can contribute to muscle regeneration [40]. In a muscle undergoing regeneration, the number of progenitor cells exceeds that of SCs and this indicates that undifferentiated progenitor cells are recruited from other sources than within the muscle itself. Furthermore, the capability of hematopoietic cells to enter myogenesis has increased number of investigation aiming at the embryological origin of the SCs. A few researches assume that at least a group of SCs derive from the embryonic vascular system. These evidences are indicative of existence of pluripotent stem cells in adult skeletal muscle [41].

Skeletal muscle tissue engineering research in Iran

In Iran, there has not been much research on tissue engineering of skeletal muscle or the construction of muscle replacement. The web searched articles are mostly concerned with differentiation of various stem cells into smooth and cardiac muscles. Since stem cells are the indispensable component of any tissue engineering efforts, along with appropriate scaffold and growth factors, any breakthrough in terms of transforming stem cells into muscle cell (of any kind) can be considered as an underpinning step in the process of muscle construct. It seems that the production of cardiomyocyte from stem cells has helped scientists to generate skeletal fibers, but the reader should acknowledge the very important fact that the diversity of structure and function within different types of muscles is as extensive as the difference between muscle and non-muscle cells!

Activities carried out in the field of cardiomyoctes

In Royan's Research Institute, the main focus is on the differentiation of embryonic stem cells (ESC) into cardiomyocytes. *Baharvand* et al. have done several studies concerning the capability of differentiating ESCs into mature and functional cardiomyocyte *in vitro* [42] and the role of fibroblast growth factor in differentiation of ESCs into cardiomyocyte in mouse [43]. They in a “comparative proteomic analysis of mouse embryonic stem cells and neonatal-derived

cardiomyocytes" claimed that a 95% striking similarity exists between proteins of ESC-derived cardiomyocytes and neonatal-derived cardiomyocytes [44].

In anatomy department of Tarbiat Modarres, a postgraduate University in Tehran, researchers have investigated the role of oxytocin and BMP-4 in differentiation of ESCs into cardiomyocyte. Their findings show that both compounds are capable of inducing differentiation; however, BMP-4 needs serum to play its role [45, 46]. Moreover, in Ilam Medical Sciences University, *Haghghani* et al. showed that mesenchymal stem cells from bone marrow are capable of differentiating into cardiomyocyte [47]. Furthermore, young investigators of Pasteur Institute of Iran reported that they could differentiate stem cells derived from umbilical veins into cardiomyocyte [48].

Investigation in the field of smooth muscle cell in Iran

The researchers in this field have advanced further to include appropriate scaffolds to repair smooth muscle. In Amir Kabir University, researchers in heart and vessel engineering group showed that the use of stretch can increase the reproduction capability, development and differentiation of bone marrow mesenchymal stem cells into smooth muscle cells of vessels *in vitro* [49]. Several similar *in vivo* activities are carried out by *Kajbafzade* et al. in Tehran University of Medical Sciences. In one of their reports they used pericardium as a natural scaffold to repair the bladder wall. After transplanting cells of smooth muscle of bladder on pericardium, they implanted the replacement between the mucous and the muscular layer of rabbit's bladder and observed (after eight weeks) that this replacement could well repair the smooth muscle of bladder [50]. They investigated the potential of different scaffolds for *in vivo* construction of bladder muscular and urothelial wall in another study [51]. Bladder wall was used as a bioreactor to create a model of the natural environment for cellular interactions, growth, and differentiation. They demonstrated the effective role of polyglycolic acid-coated tissue engineered pericardium as a potential scaffold for muscular and urothelial fragment seeding in bladder wall acting as a natural bioreactor [51].

Sharifi-Aghdas et al. tried to find a suitable and cost-effective technique for isolation and culture of muscle-derived stem cells (MDSCs) obtained from muscle biopsy in large quantities [52]. They collected a strip of rectus muscles from patients undergoing open abdominal surgery for any reason. The isolated satellite cells detached, migrated, and slowly divided. The MDSCs proliferated around the native myofiber and after 2 to 3 weeks, individual muscle cells appeared elongated and fused to create large multinucleated myotubes. They concluded that tissue explant method is a suitable and cost-effective technique for isolation and culture of MDSCs from muscle biopsy in large quantities [52].

Ai et al. hypothesized that endometrial stromal cells are an appropriate source of myogenic cells, in addition to their easy access and lesser isolating complications [53]. They claimed that due to the higher colonogenicity of these cells, in comparison with the bone marrow stem cells, the future of skeletal muscle tissue engineering will progress sooner in this course. *Mohyeddin-Bonab* et al. could isolate and proliferate bone marrow MSCs from eight patients and then injected them as autologues into the coronary artery (three patients) and into the cardiac scar tissue (five patients) during a bypass operation [54]. These patients were followed for six months; however, the article does not quantifies the results! Finally, *Haghighi-pour* and *Bayati* reported on differentiation of mesenchymal and adipose tissue stem cells into myogenic and like cells, using single-axed stretches and different mechanical stimulators [55, 56]. The trend seems promising though very slow.

Hurdles and limitations of research in skeletal muscle tissue engineering

Technical challenges including limited information about the role of environmental cues (such as rigidity and complexity of the matrix [57, 58, 59] and soluble factors [60, 61, 62]) and different kinds of biophysical stimulators (such as mechanical stretch [63, 64, 65, 66], electrical stimulation [67, 68] and motor impulses [10, 69]) in reproduction, growth, differentiation and maturation of myogenic cells have hindered the tissue engineering in the realm of constructing functional skeletal muscle. Much research needs be done on recognition of different kinds of growth factors, cytokine and different genes (or a combination of these molecules), and on spatiotemporal pattern of their release in an organized and controlled manner, in a place adjacent to cells to control myogenesis in a three-dimensional environment. The smaller size of myofibers and their lesser density in engineered muscles, as compared to their natural counterparts, emphasizes as ever before the importance of investigation into the reciprocal interaction between cells and the surrounding matrix in producing force and tension [14].

While previous research has shown that mechanical stretch would have positive effects on growth,

differentiation and production of tension in tissue engineered muscles [65, 66], the subject of electrical stimulation of constructed muscle still needs more attention. It is shown that electrical stimulation of myoblasts in two-dimensional culture media leads to induction of electrical activity and by doing so facilitates the maturation of myofibers and their sarcomere alignments [67, 68]. It is still unknown whether electrical stimulation can, in a three-dimensional environment, enhance differentiation and maturation of engineered skeletal muscle or not.

In the future, improvements in the area of cell-matrix interaction, the combination of bioactive molecules in a fibrin matrix or the use of particular patterns of electrical and mechanical stimulation are required to increase the contracting function of engineered muscles. The construction of such advanced tissues (i.e., cardiac and skeletal muscle) will create a great potential in the realm of developing regenerative medicine. Furthermore, angiogenesis and innervation of the engineered-skeletal muscle fibres are as important. For example, co-culture of C2C12 myoblasts, embryonic fibroblasts and endothelial cells on biodegradable polymeric scaffolds leads to the formation of endothelial networks in engineered muscle *in vitro* and also leads to an increase in angiogenesis, blood profusion and the survival of tissue grafts *in vivo* [70]. Other methods of angiogenesis include the formation of engineered muscle tissue around *in- vitro* perfusion systems such arteriovenous loop or femoral artery [71, 72]. In other words, co-culture of muscle structures with nerve cells [16, 17] or induction of innervation by pieces of sectioned nerves [73], not only increases the differentiation and force production of muscle cells, but also leads to the formation of nerve-muscle connections sensitive to acetylcholine which may facilitate integration of grafted tissue with the host skeletal muscular system after transplanting.

With respect the national vision and achievements out-to-make at 2035, the acquisition of knowledge and skill for constructing skeletal muscle replacement in military forces is of critical importance. To reach predetermined goals, we should know that stem cell activity in intact tissue and body replacements, as three-dimensional environment, is quite different from that in a two-dimensional culture medium. In addition, once the replacement is implanted, it should be able to fuse with the host's body. The realm of stem cells and designing their three-dimensional scaffold with the capability of survival and differentiation in *in-vivo* environment has prominent complexities, due to which no laboratory has yet claimed any productive clinical achievement. Perhaps, the nearest milestone has been introduced by *Bian Weining* to the science arena, but still far away from being ideal [74].

Conclusion

What is a great reward for our military organization is to acquire skills to hire the necessary technology to construct muscle replacements, not only to compensate for the incompetency derived from aging, disease, trauma and injuries, but also to manipulate groups of primary and secondary muscles involved in different military tasks, aiming to boost the muscle force beyond the current maximal physical level. It is critical to better understand the biological properties of various skeletal muscle stem cells, their interaction with the environmental cues and their spatiotemporal behavior in different stages of life. Let us not forget that the concept of being "fit for mission" has infinite breadth and depth.

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