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Trends in Food Science & Technology 21 (2010) 59–66

TRENDS IN FOOD SCIENCE & TECHNOLOGY

Review

Meet the new meat: tissue engineered skeletal muscle

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Contemporary large-scale farming and transportation of livestock brings along a high risk of infectious animal diseases and environmental burden through greenhouse gas emission. A new approach to produce meat and thereby reducing these risks is found in tissue engineering of skeletal muscle. This review discusses the requirements that need to be met to increase the feasibility of meat production *in vitro,* which include finding an appropriate stem cell source and being able to grow them in a three-dimensional environment inside a bioreactor, providing essential cues for proliferation and differentiation.

Introduction

The demand for meat continues to grow worldwide. With this growing demand, the increasing production of meat leads to environmental problems as well as animal suffering. We propose *in vitro* meat production using stem cells as an appealing alternative for general meat production through livestock. Reasons for promoting *in vitro* meat production include animal well fare, process monitoring, environmental considerations as well as efficiency of food production in terms of feedstock. *In vitro* meat production through stem cell technology potentially leads to a dramatic reduction in livestock. In addition, the production process can be monitored in detail in a laboratory, which could result in the elimination of food borne illnesses, such as mad cow disease or salmonella infection. Furthermore, less livestock could lead to a decrease in intense land usage and greenhouse gas emissions (Stamp Dawkins & Bonney, 2008). At the moment, 70% of all agricultural land, corresponding to 30% of the total global surface, is being used for livestock production in terms of grazing and food stock (FAO, 2006). Producing meat in the way we propose could dramatically decrease this percentage, since one could suffice with less livestock and use only limited space to manufacture meat *in vitro*. At the same time, the reduction of greenhouse gas emission could be enormous once livestock numbers have decreased. By way of comparison, 18% of greenhouse gas emission is currently produced by livestock, which is more than the total emission of the transportation sector (FAO, 2006). The animals themselves are mostly responsible for the emission of greenhouse gases (Williams, Audsley, & Sandars, 2006) and therefore a reduction of the number of animals that could be achieved by *in vitro* meat production would result in an appreciable decline of greenhouse gas emission. Although this may be balanced by *in vitro* production processes, novel techniques might be introduced that recycle oxygen by way of concomittant photosynthesis, thus reducing CO_2 emission. The idea of culturing muscle tissue in a lab *ex vivo* already originates from the early nineteen hundreds. In 1912, Alexis Carrel managed to keep a piece of chick heart muscle alive and beating in a Petri dish (Carrel, 1912). This experiment demonstrated that it was possible to keep muscle tissue alive outside the body, provided that it was nourished with suitable nutrients. Among other great thinkers, Winston Churchill predicted that it would be possible to grow chicken breasts and wings more efficiently without having to keep an actual chicken (Churchill, 1932). Although he predicted that it could be achieved within 50 years, his concept was not far off from reality today.

Some efforts have already been put into culturing artificial meat. SymbioticA harvested muscle biopsies from frogs and

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0924-2244/$ - see front matter
doi:10.1016/j.tifs.2009.11.001

kept these tissues alive and growing in culture dishes (Catts & Zurr, 2002). Other research initiatives have also achieved keeping muscle tissue alive in a fungal medium, anticipating on the infection risk associated with serum-based media (Benjaminson, Gilchriest, & Lorentz, 2002).

Obviously, small biopsies will not be practical for large-scale meat production. Therefore, we propose to use tissue engineering to produce *in vitro* cultured meat. Tissue engineering is a powerful technique that is mainly being designated for regenerative medicine in a wide variety of tissues and organs (Bach, Stern-Straeter, Beier, Bannasch, & Stark, 2003; Mol *et al.*, 2005). In particular, tissue engineering of skeletal muscle has many applications, ranging from *in vitro* model systems for drug-screening (Vandenburgh *et al.*, 2008), pressure sores (Gawlitta, Oomens, Bader, Baaijens, & Bouten, 2007) and physiology to *in vivo* transplantation to treat muscular dystrophy and muscular defects (Boldrin *et al.*, 2008) (Fig. 1). Obviously, tissue engineering could also be employed to produce meat (Edelman, McFarland, Mironov, & Matheny, 2005).

For tissue engineering to be used for meat production, a number of demands need to be met. First, a cell source is required that can proliferate indefinitely and also differentiate into functional skeletal muscle tissue. Furthermore, these cells need to be embedded in a three-dimensional matrix that allows for muscle growth, while keeping the delivery of nutrients and release of waste products undisturbed. Last, muscle cells need to be conditioned adequately in a bioreactor to get mature, functional muscle fibers.

Cell sources for tissue engineered meat

Stem cells for muscle tissue engineering

Stem cells are considered the most promising cell source for tissue engineered meat, since in theory these cells can divide indefinitely while retaining the capacity to differentiate into the required phenotype. Different types of stem cells of embryonic and adult origin exist. For now, satellite cells, which are the natural muscle stem cells responsible for regeneration, seem a promising candidate for tissue engineering of skeletal muscle and consequently for *in vitro* meat production. However, their proliferative capacity *in vitro* needs to be improved to match proliferation rates that can be found *in vivo* and which are necessary for the purpose of meat production. Therefore, other sources of stem cells are also still under evaluation.

For instance, embryonic stem cells may also be a potential cell source for *in vitro* meat production. Pluripotent embryonic stem cells show unlimited self-renewal and can differentiate into almost any desired cell type. For embryonic stem cells to become muscle fibers, the cells first need to differentiate into myogenic progenitor cells (MPCs). One of the major challenges when using embryonic stem cells is to direct differentiation into MPCs while avoiding development of other lineages. Interestingly, it seems to be more difficult to induce myogenesis in embryonic stem cells *in vitro* than *in vivo*; myogenic precursor progeny from human embryonic stem cells readily form myofibers when transplanted *in vivo* in mice after muscle damage. *In vitro* formation of myofibers from the same cells, however, has proven challenging (Zheng *et al.*, 2006). Apparently, some important *in vivo* niche components are still missing in the *in vitro* system. Additional concerns with embryonic stem cells for *in vitro* meat production include the risk of uncontrolled proliferation and differentiation, and ethical concerns about the use of this cell source.

Different types of adult muscle stem cells have been isolated from skeletal muscle: muscle derived stem cells

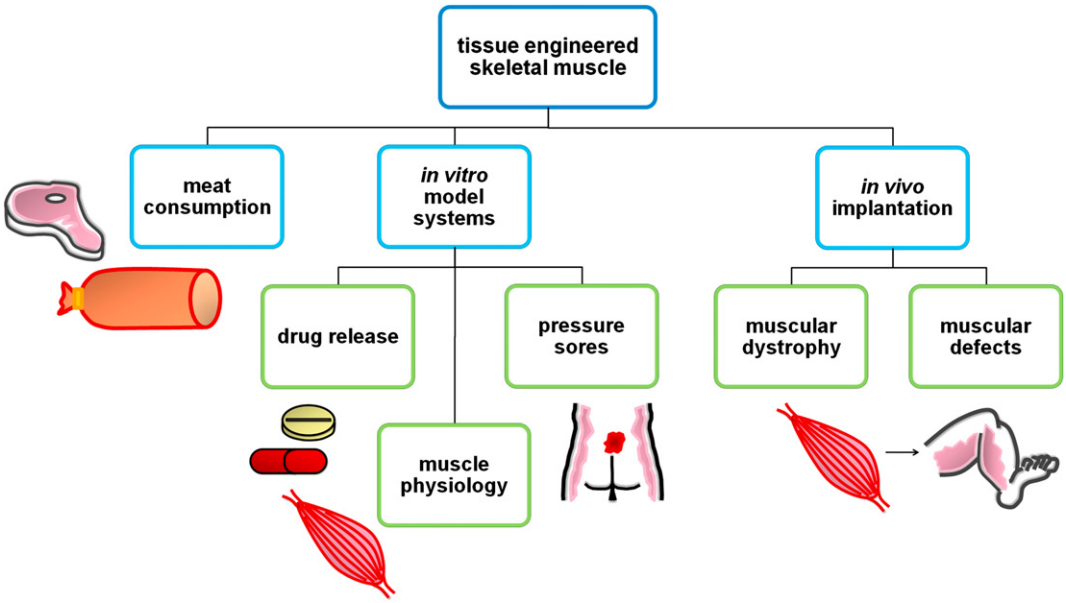

Fig. 1. Applications for tissue engineered skeletal muscle.

(Peng & Huard, 2004), side population cells (Asakura, Seale, Girgis-Gabardo, & Rudnicki, 2002) and satellite cells (Asakura, Komaki, & Rudnicki, 2001). Satellite cells are resident muscle stem cells responsible for regeneration and repair in the adult and are already programmed to differentiate into skeletal muscle. These cells are therefore an appealing source for muscle tissue engineering. Activated satellite cells differentiate to MPCs, which then proliferate and migrate in order to repair defects. Other adult stem cells derived from the muscle or bone marrow, including mesenchymal stem cells, have also appeared to conserve the capacity to differentiate into skeletal muscle and therefore remain potential candidates for muscle regeneration (Gang *et al*., 2004).

Unfortunately, at present, the proliferative capacity of adult stem cells does not match that of embryonic stem cells, mostly because they tend to differentiate spontaneously *in vitro*. It is anticipated that this issue will be tackled by optimizing the culture conditions, for example by mimicking the *in vivo* niche of the cells (Boonen & Post, 2008).

Co-culturing

Once stem cells are differentiated into myoblasts, these cells are specialized to produce contractile proteins but produce only little extracellular matrix. Therefore, other cells likely need to be introduced to engineer muscle. Extracellular matrix is mainly produced by fibroblasts residing in the muscle, which could be beneficial to add to the culture system (Brady, Lewis, & Mudera, 2008). However, co-cultures of fibroblasts and myoblasts involve the risk of fibroblasts overgrowing the myoblasts, due to the difference in growth rate. Next to fibroblasts, regular consumption meat also contains fat and a vasculature. Possibly, co-culture with fat cells should also be considered (Edelman *et al*., 2005). The problem of vascularization is a general issue in tissue engineering. Tissue engineering is currently at the level in which we can only produce thin tissues because of passive diffusion limitations. To overcome the tissue thickness limit of 100–200 μm, a vasculature needs to be created (Jain, Au, Tam, Duda, & Fukumura, 2005). Proof of concept for endothelial networks within engineered tissues has been provided (Levenberg *et al*., 2005) but reproducible and routine incorporation of vascular networks in a co-culture system will pose a special challenge.

Cell matrices

In vivo cell niche

In vivo, stem cells occupy a specific niche, which directs the cellular behavior and comprises soluble factors such as growth factors, insoluble factors including extracellular matrix proteins, physiological factors such as neurological stimulation, and mechanical features such as dynamic stretch and matrix elasticity (reviewed by Boonen & Post, 2008). It was hypothesized that these niche components are essential to mimic the regenerative process *in vitro*, which is necessary to produce mature, functional muscles. For cells to be grown in a 3D structure, for example a scaffold, several of these niche factors should be taken into account.

Extracellular matrix components to which cells attach include fibronectin, collagen and laminin. Myoblasts binding to different matrix molecules leads to induction of different pathways (Grossi, Yadav, & Lawson, 2007; Macfelda, Kapeller, Wilbacher, & Losert, 2007). Another important feature that has to be considered is the overall stiffness of the scaffold material. Engler *et al*. showed that it is possible to direct stem cell lineages by varying matrix stiffness (Engler, Sen, Sweeney, & Discher, 2006). Moreover, they found that the optimal substrate stiffness that gives rise to the characteristic striation of myosin/actin in C2C12 myoblasts is very delicate (Engler *et al*., 2004). In addition, Boonen *et al*. showed that proliferation and differentiation of primary murine satellite cells was affected by the elasticity of the culture matrix. However, they found striations in cells cultured on all elasticities above a certain threshold elasticity (Boonen, Rosaria-Chak, Baaijens, van der Schaft, & Post, 2009). Boontheekul *et al*. also showed that by varying matrix stiffness, gene expression was strongly regulated and the amount of adhesion, proliferation and differentiation of primary myoblasts differed significantly (Boontheekul, Hill, Kong, & Mooney, 2007). However, these results originate from 2D studies and still need translation to a 3D situation. One study using a PLLA/PLGA scaffold in different ratios indicated that the scaffold stiffness can be tailored to direct myoblast differentiation and organization, but these elasticities are of a different order of magnitude compared to the 2D studies (Levy-Mishali, Zoldan, & Levenberg, 2008). Additionally, in a 3D situation not only the stiffness seems important for cell behavior, but also cell forces and deformation of the scaffold will affect cell survival, organization and differentiation (Levy-Mishali *et al*., 2008).

Model systems for 3D tissue engineering

Potential 3D model systems, ideally incorporating the components of the *in vivo* cell niche, need to meet certain requirements. Broadly speaking, the fabrication of dense skeletal muscle tissue necessitates a uniform cell alignment and reproducible architecture. The options that are currently available for 3D muscle tissue engineering are illustrated in Fig. 2. Biocompatible hydrogels are, among others, a promising approach for skeletal muscle tissue engineering, because they allow a spatially uniform and dense cell entrapment (Bian & Bursac, 2008a). In addition, the mechanical properties of a gel system are more comparable to the *in vivo* environment, and the process of myotube alignment is relatively easy by the creation of intrinsic tension by compaction and active force generation by the cells. Gel systems that are currently employed for tissue engineering of skeletal muscle include fibrin gels, and a mixture of collagen and Matrigel (Bian & Bursac, 2008b; Gawlitta, Boonen, Oomens, Baaijens, & Bouten, 2008). However,

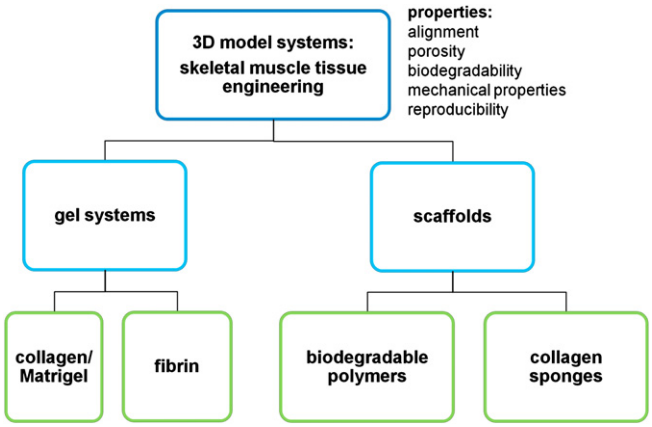

Fig. 2. Examples of 3D model systems for skeletal muscle tissue engineering. Specific properties for optimal muscle development are listed. In addition, examples are given.

one of the largest problems concerning these hydrogels is their stability (Beier *et al*., 2009). A possible solution would be to introduce a co-culture with cells that extensively produce extracellular matrix and could take over stability while the hydrogel degrades. Still, the hydrogel would be required during the initial phase of the culture process to hold the cells together.

Scaffolds produced of synthetic biodegradable polymers are also a potential 3D model system that is suitable for *in vitro* cultured meat. Reproducibility and uniformity of scaffolds can be achieved by producing them with electrospinning techniques. However, this generally results in very dense structures, which are difficult for cells to enter and will preclude homogeneous cell distribution (Beier *et al*., 2009). A new approach to create scaffolds with an open structure is the use of low temperature electrospinning (Simonet, Schneider, Neuenschwander, & Stark, 2007). These scaffolds can be produced from various polymers, such as poly-caprolactone and poly-lactic-acid, dependent on the desired mechanical and degradation properties as well as cell attachment demands. Parameters such as fiber thickness and orientation can be adjusted and optimized in the electrospinning process in order to influence the architecture and mechanical properties (Ayres *et al*., 2007). Also, orienting the fibers in one direction could be accomplished, which is beneficial to muscle development since it resembles the *in vivo* structure of muscle (Riboldi *et al*., 2008).

Conditioning

The creation of a native-like tissue architecture with the capacity of active force generation is crucial in the process towards tissue engineered muscle, and consequently also important for *in vitro* meat production. However, the advances made in culturing of engineered muscle constructs have not yet resulted in satisfactory products. An important hurdle that still has to be overcome is the inability of muscle cells to fully mature within these engineered muscle constructs. Although biochemical stimuli may be more important in the initial differentiation process, biophysical stimuli have proven to be crucial in the maturation towards functional tissue with native-like properties (Kosnik, Dennis, & Vandenburgh, 2003). Therefore, we hypothesize that for successful tissue engineering of skeletal muscle, the design of a bioreactor should also incorporate the ability to apply biophysical stimulation regimes that resemble the native *in vivo* environment regarding muscle regeneration. The effects of both biochemical and biophysical stimuli on muscle differentiation and maturation are summarized in Fig. 3.

Biochemical conditioning

Conventionally, application of a biochemical stimulus can induce the differentiation of muscle precursor cells. Growth factors have been identified that influence myoblast proliferation and differentiation to a great extent. Different members of the Transforming Growth Factor-β (TGF-β) superfamily, Fibroblast Growth Factors (FGFs) and Insulin-like Growth Factors (IGF) are crucial in this respect. TGF-β reduces myoblast recruitment and differentiation (Goetsch, Hawke, Gallardo, Richardson, & Garry, 2003). FGFs are more stimulatory in their actions than TGF-β family members; FGFs increase myoblast proliferation *in vitro* and thereby inhibit differentiation (Hannon, Kudla, McAvoy, Clase, & Olwin, 1996). Comparable to FGFs, a splice variant of IGF-1, called mechano growth factor, increases proliferation of myoblasts (Ates *et al*., 2007). IGF-1 itself is more involved in accelerating differentiation

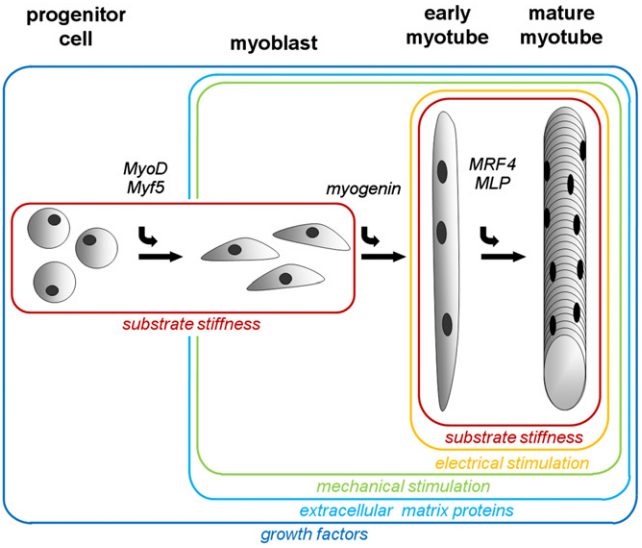

Fig. 3. Factors affecting muscle cell proliferation, differentiation and maturation. *Substrate stiffness* is involved in both the proliferation of progenitor cells and the maturation of myotubes. *Electrical stimulation* results in enhanced maturation of myotubes, whereas *mechanical stimulation* is important for the alignment of myoblast and the maturation of myotubes. *Extracellular matrix proteins* and *growth factors* are involved in the overall process of differentiation and maturation of muscle progenitor cells towards mature myotubes.

in C2C12 myoblasts (Florini, Ewton, & Coolican, 1996) and in inducing hypertrophy *in vitro* (Gawlitta *et al*., 2008).

Biophysical conditioning

Regarding the relatively poor development of sarcomeres *in vitro* (Engler *et al*., 2004), indicated by a lack or limited level of maturation specific cross striations, biochemical stimulation alone may not be sufficient in the maturation process towards fully functional engineered muscle constructs. It appears that in addition to biochemical stimuli, biophysical stimulation is required for full muscle maturation and function.

Electrical stimulation

Neuronal activity has proven to be pivotal in the development of mature muscle fibers (Wilson & Harris, 1993) and can be mimicked by applying appropriate electrical stimuli in *in vitro* cultures (Bach, Beier, Stern-Staeter, & Horch, 2004). In this respect, it has been shown that induction of contractile activity promoted the differentiation of myotubes in culture by myosin heavy chain expression of different isoforms and sarcomere development (Fujita, Nedachi, & Kanzaki, 2007; Naumann & Pette, 1994). We also showed that early electrical stimulation accelerated maturation of myotubes with respect to sarcomere development in the C2C12 murine myoblast cell line (personal observations). Within a relatively short differentiation period of 5 days, mature cross striations had developed in the electrically stimulated cultures, whereas non-stimulated control cultures did not show these cross striations. This effect was accompanied by upregulated expression levels of the muscle maturation inducer muscle LIM protein, and the sarcomere components perinatal myosin heavy chain, actin and α-actinin.

Alternatively, electrical stimulation can provide a non-invasive, accurate tool to assess the functionality of engineered muscle constructs (Dennis, Smith, Philp, Donnelly, & Baar, 2009). By generating a homogeneous electrical field inside the bioreactor, functional muscle constructs will exert a force due to active contractions of the muscle cells. So far, these forces generated by engineered muscle constructs only reach 2–8% of those generated by skeletal muscles of adult rodents (Dennis, Kosnik, Gilbert, & Faulkner, 2001). Therefore, at this moment, functional properties of tissue engineered muscle constructs are still unsatisfactory.

Mechanical stimulation

Another important biophysical stimulus in myogenesis is mechanical stimulation (Vandenburgh & Karlisch, 1989). Mechanotransduction, the process through which cells react to mechanical stimuli, is a complex but increasingly understood mechanism (Burkholder, 2007; Hinz, 2006). Cells attach to the insoluble meshwork of extracellular matrix proteins mainly by means of the family of integrin receptors (Juliano & Haskill, 1993), transmitting the applied force to the cytoskeleton. The resulting series of events shows parallels to growth factor receptor signaling pathways, which ultimately lead to changes in cell behavior, such as proliferation and differentiation (Burkholder, 2007).

Different mechanical stimulation regimes affect muscle growth and maturation. The application of static mechanical stretch to myoblasts *in vitro* resulted in a facilitated alignment and fusion of myotubes, and also resulted in hypertrophy of the myotubes (Vandenburgh & Karlisch, 1989). In addition, cyclic strain activates quiescent satellite cells (Tatsumi, Sheehan, Iwasaki, Hattori, & Allen, 2001) and increases proliferation of myoblasts (Kook *et al*., 2008). These results indicate that mechanical stimulation protocols affect both proliferation and differentiation of muscle cells. The applied stimulation should be tuned very precisely to reach the desired effect. Percentage of applied stretch, frequency of the stimulus and timing in the differentiation process are all parameters that presumably influence the outcome of the given stimulus.

Discussion

Challenges in tissue engineering of meat

This review has dealt with the challenges of *in vitro* meat production. By taking the appropriate stem cells, proliferating them under the right conditions to reach sufficient numbers and providing them with the right stimulatory signals in a 3D environment, industrial meat production seems feasible (Fig. 4). We described three major issues in skeletal muscle tissue engineering, being the proper cell source, the optimal 3D environment for cells to be cultured and differentiated in, and adequate conditioning protocols. Adult stem cells, i.e. satellite cells, seem a promising cell source. However, there still is room for improvement of the proliferative capacity as well as the differentiation protocol of these cells. Unfortunately, culturing of only muscle cells in a construct will not result in a tissue structure comparable to an *in vivo* muscle. Co-culturing with other cells, such as fibroblasts or adipocytes, is probably the solution to this problem.

Examples originating from the field of regenerative medicine show that culturing of meat could be technologically feasible. Transplantations of tissue engineered muscle have been undertaken in several model systems. Human muscle precursor cells (Boldrin *et al*., 2008) or mouse satellite cells (Boldrin & Morgan, 2007) seeded in a polymeric scaffold were implanted into muscular defects in mice. These constructs survived and contributed to the regeneration of the host muscle. These implantation experiments show that it is possible to engineer skeletal muscle tissue that is compatible with authentic muscle tissue, although we are still quite far from producing large volume muscle constructs that can generate forces within the physiological range of skeletal muscle.

The farm animal derived stem cells that are, in our view, required for the production of artificial meat as mentioned before, will be available over time. Adult stem cells,

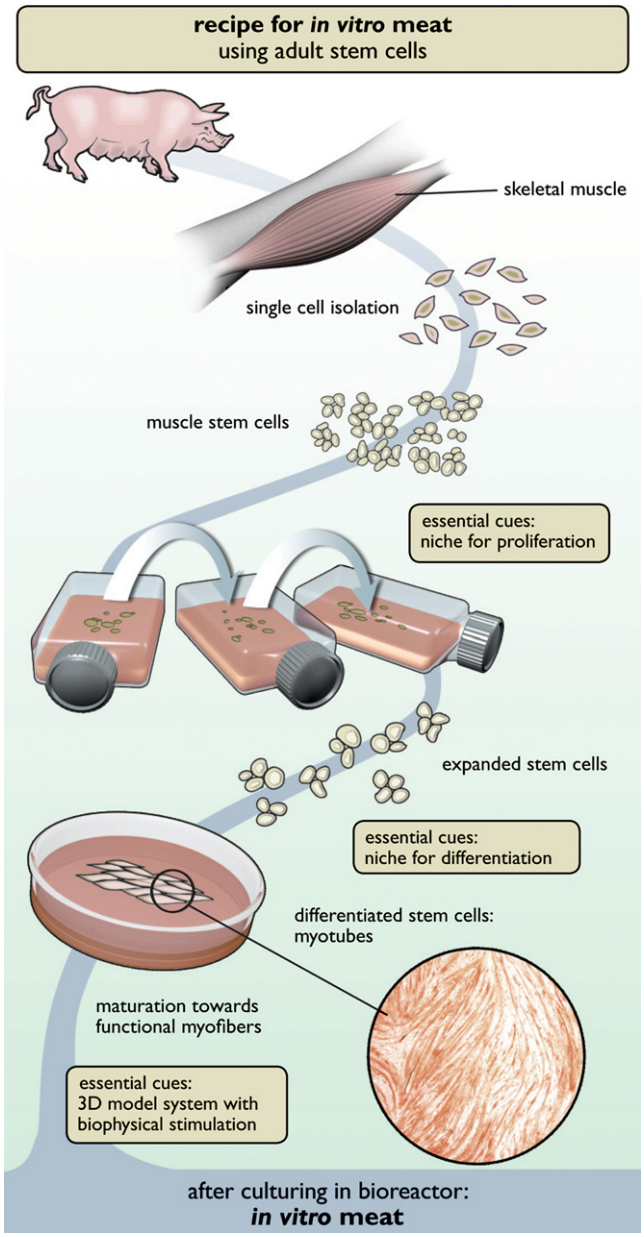

Fig. 4. Recipe for *in vitro* meat using adult stem cells. The essential cues indicate the challenges that have to be met in the distinctive processes (illustration Sebastiaan Donders).

derived from skeletal muscle, have already been isolated from pigs (Wilschut, Jaksani, Van Den Dolder, Haagsman, & Roelen, 2008). Until now, only embryonic stem cell lines originating from several model species and humans have been isolated and cultured successfully. Deriving a new embryonic stem cell line from livestock animals is a matter of time and continuous effort. For practical reasons, most research on skeletal muscle regeneration has been performed in mice (Beauchamp, Morgan, Pagel, & Partridge, 1999). Indeed, we study the scientific foundation of *in vitro* meat production also with murine satellite cells; remaining aware that mouse meat will not appeal to projected consumers.

Future considerations

The next hurdle that has to be overcome in time is the size of the cultured meat. At the moment, skeletal muscle constructs of approximately 1.5 cm in length and 0.5 cm in width can be cultured (Gawlitta *et al*., 2008). These sizes of artificial meat can already be used as a supplement in sauces or pizzas, but the production of a steak, for example, demands for larger tissue sizes. Up-scaling of the cell and tissue culturing processes is therefore necessary.

Since no other animal sources are wanted in the process of *in vitro* cultured meat, conventional culture medium, which is commonly supplemented with fetal bovine serum, has to be adjusted. For example, a cocktail of growth factors and other essential additives can be produced by bacteria or yeast cells (Halasz & Lasztity, 1990) resulting in a defined culture medium.

Obviously, for artificial meat to compete with its livestock counterpart, it should approximate the authentic color, taste and structure. Myoglobin is responsible for the red color of meat (Miller, 1994), and is also expressed by skeletal muscle cells in culture (Ordway & Garry, 2004). In addition, artificial food coloring is a generally accepted and approved process. The same holds for taste; artificial meat flavors do exist and are currently being used in meat replacements. Artificially adapting the taste of engineered meat would even be more practical in the process of *in vitro* meat production, since it still remains undetermined which components of meat are responsible for the flavor (Toldrá & Flores, 2004). We believe that texture is the most important aspect for tissue engineered meat. Myofibrils, fat, and connective tissue are responsible for this texture (Toldrá & Flores, 2004) and it therefore seems important to create functional muscle tissue containing these myofibrils. The connective tissue and fat content should be realized by co-culture with different types of cells. As far as the nutritional value of meat is concerned, we aim at reproducing actual skeletal muscle tissue and therefore we believe that important nutritional components, such as the essential amino acids that make meat an important part of the human diet (Reig & Toldrá, 1998), will also be present in *in vitro* cultured meat. In addition, by tuning the substrates used for cultured cell metabolism, for instance using polyunsaturated fatty acids, we theoretically can affect the biochemical composition of muscle cells to make the product healthier (Jiménez-Colmenero, 2007).

When all technological challenges regarding artificial meat production are overcome, the next step towards a successful substitute for authentic meat is product marketing. Introduction of artificially cultured meat is undoubtedly challenging, but potential negative connotations may be off-set by the impact of such a product on animal suffering, environment and world food supply. Therefore, the idea that people would eat meat originating from the lab does not seem so farfetched.

Acknowledgements

This work was financially supported by SenterNovem, grant ISO 42022.

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