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Review

Cultured meat from stem cells: Challenges and prospects

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ABSTRACT

As one of the alternatives for livestock meat production, in vitro culturing of meat is currently studied. The generation of bio-artificial muscles from satellite cells has been ongoing for about 15 years, but has never been used for generation of meat, while it already is a great source of animal protein.
In order to serve as a credible alternative to livestock meat, lab or factory grown meat should be efficiently produced and should mimic meat in all of its physical sensations, such as visual appearance, smell, texture and of course, taste. This is a formidable challenge even though all the technologies to create skeletal muscle and fat tissue have been developed and tested. The efficient culture of meat will primarily depend on culture conditions such as the source of medium and its composition. Protein synthesis by cultured skeletal muscle cells should further be maximized by finding the optimal combination of biochemical and physical conditions for the cells. Many of these variables are known, but their interactions are numerous and need to be mapped. This involves a systematic, if not systems, approach. Given the urgency of the problems that the meat industry is facing, this endeavor is worth undertaking. As an additional benefit, culturing meat may provide opportunities for production of novel and healthier products.

Contents

1. Introduction

In recent years the notion has been growing that alternatives may be needed for conventional meat production through livestock. This is generally based on concerns about sustainability, environmental burden and animal welfare. These concerns have grown due to further intensification of livestock herding and slaughtering, and on the other hand a predicted rapid increase in global meat consumption (FAO, 2006).

In this review the state of the art of meat alternatives is discussed, with a particular emphasis on cultured meat. The urgency of the problem is apparent. The focus will be on tissue-engineering methods rather than bio-printing or expanding existing pieces of tissue through culturing.

2. Why do we need meat alternatives?

There are at least three motivations to intensify the exploration of production alternatives to livestock meat production. First, with the

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predicted substantial increase in meat demand, we will quickly run out of production capacity as already a large portion of arable land is dedicated to livestock feeding and management. Second, there is growing concern about the environmental impact of livestock breeding and management. Last, high volume herding and slaughtering has sparked societal concerns about animal welfare and public health.

Due to an expanding world population and to increasing meat consumption in developing economies, it is predicted that meat consumption will double in the coming forty years (FAO, 2006). Although these predictions are associated with considerable uncertainty, the sheer magnitude of this alleged increase supports the assumption that demand will increase appreciably. At the same time it appears – also with margins of error – that the capacity of conventional meat production is close to its maximum (FAO, 2011). As a result, meat will become scarce, therefore more expensive and eventually a luxury food. This may then serve to aggravate the already unequal global distribution of food. Alternatively, many other techniques are being investigated to improve the efficiency of the entire supply chain of foods, such as decreasing post-harvest losses (wasting of food). In addition to these, efficient production of food and meat in particular will have a great impact.

Livestock meat production accounts for a considerable portion of greenhouse gas (GHG) emission, land usage, water and energy consumption. Of the three major greenhouse gases specifically carbon dioxide, methane and nitrous oxide, the contribution of livestock to their total emission is 9%, 39% and 65% respectively (FAO, 2006). It has been noted that these numbers vary greatly per country and continent, depending on many factors, including the presence or absence of collateral damage by deforestation (Capper, 2011; Cederberg, Persson, Neovius, Molander, & Clift, 2011; FAO, 2006; Peters et al., 2010; Steinfeld, Mooney, & Schneider, 2010). It is clear however, that major improvements can be made in the environmental impact of meat production, either through conventional (Capper, 2011) or other technologies. In a preliminary life cycle analysis Tuomisto and de Mattos (2011), calculated for instance that in vitro production of meat when using for instance cyanobacteria-produced biomass as a nutrient source might reduce energy consumption and land usage by 99%, water usage by 90% and energy consumption by 40%. If realized, these reductions lead to a large reduction in GHG emission.

Another motivation for livestock alternatives is the concern about animal welfare. Public debate on animal welfare surfaces on a regular basis. As shown by Tonsor and Olynk (2011), non-vegetarians decrease consumption of meat proportional with exposure to awareness campaigns of animal welfare through public media. The effects were rather small and pertained mostly to poultry and pork, not to beef, but at the same time the number of publications on animal welfare issues in livestock meat production rose gradually during the 1982–2008 observation period. Thus, public concern about animal welfare may affect consumer behavior thereby forcing the meat industry to continuously evaluate its practices in view of that concern.

Lastly, there are public health problems surrounding livestock production. Cardiovascular disease, diabetes and colorectal cancer are associated with the consumption of red meat (Larsson & Wolk, 2006; Song, Manson, Buring, & Liu, 2004). Over-consumption of meat may be responsible for a quarter of all ischemic heart disease, or 1.8 million deaths, annually (Key, Davey, & Appleby, 1999). Specifically, the meta-analysis of Larsson and Wolk (2006) suggests that as little as 120 g red meat/day or 30 g processed meat/day would significantly raise the relative risk of colorectal cancer. It remains to be established which nutrients in meat are causing this risk, which makes it very difficult to specifically develop alternatives aiming to reduce this risk.

In addition to these adverse health effects, foodborne pathogens found in meats, such as *Salmonella*, *Campylobacter* and *E. coli*, are responsible for millions of episodes of illness each year (CDC, 2012). From an epidemiological point of view it is evident that these pathogens and emerging diseases, such as avian and swine influenza, are associated with the intensity of livestock farming and other anthropogenic developments in the bio-industry (Greger, 2007; Slingenbergh, Gilbert, de Balogh, & Wint, 2004).

In summary, there are numerous and pressing reasons to explore alternatives to relieve the burden and pressure of livestock meat production.

3. Requirements for a meat alternative

Mimicry and efficiency are the two key requisites for a meat alternative to be accepted and industrialized. For a new meat substitute to be widely adopted, it needs to exactly mimic or even better, recreate conventional meat in all of its physical sensations, such as visual appearance, smell, texture and of course, taste (Bredahl, Grunert, & Fertin, 1998; Verbeke et al., 2010). If such a product can be created, it will deserve the name “meat”, without any pejorative adjectives. Of these challenges, taste is arguably the most difficult, especially since the more than 1000 water soluble and fat derived components may make up the species and perhaps strain specific taste of meat (Claeys, De Smet, Balcaen, Raes, & Demeyer, 2004; Mottram, 1998).

A high efficiency, bioconversion rate, is the basis for a sustainable product that will be able to improve on the carbon footprint of livestock meat production and as a consequence will require less water, land and energy input per kg of meat. The low bioconversion rate of pigs and cattle of approximately 15% (Egbert & Borders, 2006; Pimentel & Pimentel, 2003) offers a wide margin for improvement. Nevertheless, the challenge to design an in vitro production process that is much more efficient will be formidable.

Opportunities on the other hand are also numerous. In the production phase, recycling mechanisms and combining culture with nutrient supplying systems through for instance photo-synthesis would create substantial benefit and value (Tuomisto & de Mattos, 2011). In vitro culturing of meat would also facilitate the design and production of novel products. For instance, stem cells from probably every mammalian source or blends of cell sources can be used as a basis for hitherto unimaginable meats. In addition, the biochemical composition of meat might be changed to make it a healthier or specialized diet product, for instance by increasing the content of poly-unsaturated fatty acids through changes in culture conditions.

4. Meat alternatives

Several meat substitutes have been developed and are being developed. Meat substitutes entirely made of vegetable components have gained a small market share, which is gradually, but slowly, increasing (Egbert & Borders, 2006). In the US, in 2010, the total sales of frozen meat substitutes reached 267 million USD (Salvage, 2012) as opposed to 74 billion USD in beef sales alone (Mathews & McConnell, 2011). Most products are based on soy (Tofu, Tempeh, “TVP: textured vegetable protein”), milk proteins, wheat proteins (“Seitan”) or mycoprotein (“Quorn”), which all fit the criterion of efficient protein production and a beneficial carbon footprint (Hoek, Luning, Stafleu, & de Graaf, 2004). Although the technology of texturization to improve the feel and taste of these products is continuously improving it appears difficult to closely mimic meat with proteins, sugars and fats from vegetable origin (Elzerman, 2006). The vegetable origin meat substitutes are therefore mainly being used in processed meats such as burgers, sausages or other types of minced products.

Insects are another source of natural proteins. In addition to sufficient minerals, insects have a high protein content (Defoliart, 1992) and can therefore be considered nutritious. For a general overview of the possibilities of insects for food production see the FAO publication from 2010 (Durst, Johnson, Leslie, & Shono, 2010). Their high bioconversion rate is favorable for large-scale protein production

although processing for insects with an exoskeleton is generally required to get rid of non-digestible chitin (Verkerk, Tramper, van Trijp, & Martens, 2007). Crickets for instance have an estimated five-fold higher efficiency than cattle (Defoliart, 1992). The taste for insects is subject to strong cultural influence, with little rational foundation. While populations in East Asia and South America accept insects as common food (Defoliart, 1992; Schabel, 2010) or even a delicacy, it generates aversion in Europe and North America. Part of this aversion may disappear if insect cells, instead of whole organisms are cultured and used as an unrecognizable protein source.

It remains to be shown if changes in livestock meat production such as vertical farming, recycling, selective breeding, feeding (Durham, 2011) and perhaps scaling up, will increase the edible protein yield per unit of input (land surface, water volume, energy, feed). Only then will these strategies allow either more production with the same resources or stationary production with less demand on natural resources (FAO, 2011). This will require substantial improvement of the bioconversion rate of pigs and cattle.

All the above alternatives are being investigated and implemented but as of yet, no particular strategy has proven a perfect or practical solution.

5. Cultured meat

One of the many alternatives under investigation is culturing meat based on stem cell technology. The idea of growing meat without livestock is not new. For instance, Winston Churchill in his book "Thoughts and adventures" (Churchill, 1932) wrote "…..Fifty years hence, we shall escape the absurdity of growing a whole chicken in order to eat the breast or wing, by growing these parts separately under a suitable medium…". Three technologies that have emerged over the last six decades make it possible to generate skeletal muscle and other mesenchymal tissues such as bone, cartilage, fat and fibrous tissue: stem cell isolation and identification, ex vivo cell culture, and tissue engineering. In fact, bio-artificial muscles (BAMs) produced from the skeletal muscle resident stem cells, a.k.a. satellite cell, have been generated for the last 15 years, mainly to serve as research tools or potential medical implants (Dennis & Kosnik, 2000; Vandenburgh, Shansky, Del Tatto, & Chromiak, 1999). These BAMs can already be regarded as a valuable source of animal proteins, but they are still far removed from an efficient and convincing meat mimic.

In vitro production of meat would also enable novel products. For instance, stem cells from pretty much every source or blends of cell sources can be used as a basis for hitherto unimaginable meats. In addition, the biochemical composition of meat might be changed to make it a healthier or specialized diet product, for instance by increasing the content of poly-unsaturated fatty acids.

5.1. Stem cells

During the last two decades the identification, selection and modification of stem cells have greatly advanced. For culturing of meat, several stem cell types are of interest. The first and foremost is the myoblast or satellite cells, described by Mauro (1961). This adult, tissue derived stem cells is the bona fide cell responsible for muscle regeneration after injury. However, it has proven difficult to maintain its replicative state in cell culture. On the other hand, once cultured to sufficient numbers, the satellite cell easily differentiates into myotubes and more mature myofibrils and was therefore selected as the preferred cell source for tissue engineering of skeletal muscle. Recent data of aging populations of satellite cells suggests that there may be a subset of satellite cells with even better regenerative capacity (Collins, Zammit, Ruiz, Morgan, & Partridge, 2007). Currently however, direct methods to select these subsets are lacking.

Embryonic stem cells are a theoretical alternative at this stage, as the search for the porcine and bovine embryonic stem cells is still ongoing. Although it is likely just a matter of time and continuous effort to keep cells taken from the inner cell mass of porcine or bovine epiblasts (Telugu, Ezashi, & Roberts, 2010) in an undifferentiated state, so far the attempts have not been completely successful. Quite recently, induced pluripotent porcine stem cells (iPSC) have been generated (Ezashi et al., 2009) and these might be an alternative source for in vitro meat generation. iPSCs are differentiated cells, e.g. fibroblasts, that have been rendered pluripotent by stable transfection with a set of four specific transcription factors (Oct4, Sox2, KLF4 and c-Myc) driving embryonic gene expression programs in the cell (Takahashi & Yamanaka, 2006). To date, no bio-artificial muscles with myotubes derived from iPSCs have been made, although they are capable of myogenic differentiation and in vivo repair of muscle injury (Mizuno et al., 2010). For the production of other components of meat such as fat tissue, again several cell sources may be selected. For this, we have selected another adult tissue resident stem cell; the adipose tissue derived stem cell (ADC), which has shown a propensity to form pre-adipocytes and further differentiate into mature adipocytes. Tissue engineering of adipose tissue with these cells has been described elsewhere (Frerich, Winter, Scheller, & Braumann, 2011; Verseijden et al., 2012).

5.2. Cell culture

Long before stem cells were identified, in the twenties, but really catching on in the fifties, large-scale cell culture of mammalian cells had become available due to advances in cell media, incubators and serum production. In a skeletal muscle cell culture for meat production, thousands of variables can be – and have to be – controlled to make the process reliable and efficient. This creates challenges as well as opportunities. The biggest challenge is to define the level of each variable (e.g. feed item, biochemical and biophysical culture conditions), but also the possible interactions between these variables. Current culture protocols have largely developed through trial and error, leading to a gradual optimization. As a result, a theoretical basis for a systematic approach is still lacking. For simpler, prokaryotic organism like bacteria and simple eukaryotic organisms like yeast such a systems biology approach is just starting to be developed (Brul, Mensonides, Hellingwerf, & Teixeira de Mattos, 2008; Gutteridge et al., 2010). Either a biological systems strategy should be developed for more complex mammalian cells as well or a large-scale, high-throughput analysis should be set up to optimize culture media. This is true for the synthetic part of the culture medium as well as the serum part — together, a considerable task. Eventually, culture media should be completely synthetic and devoid of serum products. A limited number of such products have been developed for medical purposes and it is to be expected that more of these will become available (van der Valk et al., 2010). In our hands, serum-based media are still superior to synthetic ones (unpublished data). Opportunities to increase the efficiency of skeletal muscle cell culture on the other hand are also numerous. In the production phase, recycling mechanisms and combining culture with nutrient supplying systems through for instance photosynthesis would create substantial benefit and value (Tuomisto & de Mattos, 2011).

Culturing of skeletal muscle cells from satellite cells can be separated into two phases with distinct goals: the proliferation phase and the differentiation phase. The challenges in optimizing culture conditions for large-scale skeletal muscle growth are therefore also different for these two phases.

In the proliferation phase the goal is to obtain the maximum number of cells from the starting batch of cells, i.e. to maximize the number of doublings. As a result of the theoretical power of 2 relationship between number of doublings and number of cells, moving from 20 to 30 doublings makes a tremendous difference when, in fact 30

doublings give a thousand-fold higher yield. With the current isolation and culturing methods for satellite cells 20 doublings can be achieved. Higher doubling numbers have generally been obtained by delaying differentiation, therefore much attention has focused on the mechanisms that determine differentiation. A major improvement in maintaining the replicative capacity of satellite cells indeed resulted from a combination of mild enzymatic treatment and trituration of remaining skeletal muscle fibrils during harvesting according to Collins et al. (2005). Once harvested, the concept is to recreate the stem cell niche as closely as possible to retain the stem cell behavior of the satellite cells (Boonen & Post, 2008). For instance, success has been achieved with changing the elasticity of the substrate on which the satellite cells are cultured (Gilbert et al., 2010). In our hands however, the effect of physiologic substrate stiffness on myoblast cell proliferation was mild, higher than on very flaccid substrates (3 kPa range) but not significantly different from stiff plastic surfaces (Boonen, Rosaria-Chak, Baaijens, van der Schaft, & Post, 2009). Likewise, coating the culture surface with proteins that mimic the basal membrane, such as laminin and collagen IV, has some impact on the proliferation rate of satellite cells (Wilschut, Haagsman, & Roelen, 2010). Finally, a large variety of biochemical mechanisms stimulate differentiation, such as TGFβ1, Pax7, Notch and Wnt (Zammit, 2008). For most of these mechanisms, biological modulators have been designed and may be used to optimize proliferation by delaying differentiation.

After having produced sufficient cells, the next goal is to differentiate them into skeletal muscle cells and coerce them into maximum protein production i.e. to undergo hypertrophy. For satellite cells, this process occurs almost naturally with very little adjustment to culture conditions. The cells will merge, form myotubes, and will start to express early stage skeletal muscle markers such as MyoD, myogenin and embryonic isoforms of muscle myosin heavy chain. The cues for subsequent hypertrophy are a mix of metabolic, biochemical and mechanical stimuli. It appears that mechanical stimuli are extremely important in triggering protein synthesis and protein organization into contractile units. The latter gives the muscle its typical striated microscopic morphology. Usually, these cells are cast in a collagen-like gel or onto a temporary biodegradable scaffold. In both conditions the cell constructs or bio-artificial muscles (BAMs) are anchored (e.g. Velcro™ or silk wires) to the culture dish to simulate tendons. Differentiation therefore takes places in a tissue engineering construct.

5.3. Tissue engineering

Most mesenchymal cells, including skeletal muscle cells, will organize a collagen or collagen/Matrigel gel in a tight fiber in between the anchors and will develop tension within that fiber (Grinnell, 2000). This apparently static tension boosts protein production by the so-called bio-artificial muscle tremendously (Vandenburgh et al., 1999). Interestingly, imposition of cyclic stretch did not further improve protein synthesis but had in fact a slight negative effect (Boonen et al., 2010; Kook et al., 2008). This result is somewhat controversial as others have observed positive effects of cyclic stretch on muscle maturation (Powell, Smiley, Mills, & Vandenburgh, 2002). In addition to passive stretch and tension, we and others have investigated the effect of electrical stimulation to further stimulate protein production and force generation (Boonen, van der Schaft, Baaijens, & Post, 2011). In combination with specific coatings, electrical stimulation did lead to earlier maturation of the skeletal muscle fibers, but it remains to be shown if for large scale production the rather inefficient use of energy warrants the improvement in protein synthesis. For a more extensive discussion on mechanical stimulation of muscle differentiation please see (Langelaan et al., 2010).

With the above described techniques it is feasible to generate BAMs of small dimensions, limited by the dependence on an adequate nutrient and oxygen supply through diffusion. No attempts have been made yet to create large BAMs with a built-in blood vessel or channel system conducting a continuous flow of oxygenized, nutrient-rich medium. However, printing and biomaterial technologies have been described that would make this possible and certainly testable (Skardal, Zhang, & Prestwich, 2010; Visconti et al., 2010).

Although contractile proteins comprise the bulk of protein content and quality of muscle tissue, there are other proteins that are important for texture, color and taste of the BAM tissue. One particularly important protein is myoglobin. As a heme-carrying protein it is in part responsible for the pink color of meat and since it is a major carrier of iron, its presence will likely determine taste as well. The transcriptional regulation of myoglobin is reasonably well understood and involves the transcriptional activators MEF2 and NFAT/calcineurin (Kanatous & Mammen, 2010) and co-activator PGC-1α. It appears that contractile activation of muscle in the setting of hypoxia will stimulate myoglobin maximally. It seems therefore feasible to increase the myoglobin content using stimuli that are compatible with tissue engineering of products that eventually should be consumed.

In summary, the effective culture of skeletal muscle is possible with current technology. There are numerous options for refinement and extension suggesting that it will take time and effort to optimize the product, but current successes indicate that an acceptable mimic of meat tissue will likely be generated.

6. What after in vitro meat can be grown effectively?

Efficiency and mimicry are the keys to success and acceptance of any meat alternative and in vitro meat is no exception. It is clear that we have a long way to go until we have reached that goal and once that is achieved there may still be obstacles. Important issues to consider are scalability of the production process, quality control of mammalian cell/tissue cultures, maintaining sterility in the culture, prevention of contamination or disease and the controlled breeding of stem cell donor animals. It is likely that these technical issues can at some point be solved.

If so, it is possible that culturing of meat in laboratories and eventually in factories will transform the meat industry. This will require time, a great deal of research and development and a gradual transition in our thinking about meat.

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