POSSIBILITIES FOR AN *IN VITRO* MEAT PRODUCTION SYSTEM

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Abstract

Meat produced *in vitro* has been proposed as a humane, safe and environmentally beneficial alternative to slaughtered animal flesh as a source of nutritional muscle tissue. The basic methodology of an *in vitro* meat production system (IMPS) involves culturing muscle tissue in a liquid medium on a large scale. Each component of the system offers an array of options which are described taking into account recent advances in relevant research. A major advantage of an IMPS is that the conditions are controlled and manipulatable. Limitations discussed include meeting nutritional requirements and large scale operation. It will be speculated the direction of further research and prospects regarding the future of *in vitro* meat production.

Keywords: *in vitro* meat; myocyte culturing; meat substitutes

1. Introduction

In light of the sizable negative effects of livestock production, establishment of an *in vitro* meat production system (IMPS) is becoming increasingly justifiable. Current meat production methods are a major source of pollution and a significant consumer of fossil fuels, land and water resources. World meat production at present is contributing between 15% and 24% of total current greenhouse gas emissions; a great proportion of this percentage is due to deforestation to create grazing land. (Steinfeld, Gerber, Wassenaar, Castel, Rosales & De Haan, 2006). The production of beef requires 15 500 m^3/ton of

water, while chicken requires 3918 m^3/ton (Hoekstra & Chapagain, 2007) and with a growing population and great proportion of which facing starvation, it no longer makes sense to contribute staple crops toward inefficient meat production, where 1 kg poultry, pork and beef requires 2kg, 4kg and 7 kg of grain, respectively (Rosegrant, Leach & Gerpacio,1999). Satisfying the demand for meat in the future will be a challenge if we intend on maximizing the use of agricultural resources and reducing greenhouse gas production, as Fiala (2008) calculates the amount of total meat consumed worldwide in 2030 to be 72% higher than that consumed in 2000 following current consumption patterns.

It is suspected that myocyte culturing would have a reduced water, energy and land requirement because a) solely muscle tissue is cultivated, bypassing the development of by-products and non-skeletal muscle tissues; b) for the same mass of meat, tissue cultivation is anticipated to be faster than growth to a slaughter-ready age and c) *in vitro* meat production systems are capable of increasing in volume vertically, making deforestation to create pasture unnecessary. The controlled conditions would theoretically eliminate product losses from infected and diseased animals. As a method with little to no waste products or byproducts and a minimized land and resource requirement, myocyte culturing could possibly alleviate the environmental burden exhibited by today's meat harvesting techniques.

To provide meat globally, modern industrial meat production has become a complex landscape of trade where feed production, animal husbandry, processing and consumption may all take place in different countries (Burke,

Oleson, McCullough and Gaskell, 2009). The comparatively minimal land requirement of an in vitro meat production system allows meat production and processing to take place domestically in countries which would normally rely on imported meats. By bringing the stages of the meat production process closer together spatially and temporally, meat supply can be better determined by demand.

Humans are taxonomically omnivorous and meat provides several essential nutrients unavailable in plant sources. Meat is specifically valuable as a source of omega-3 fatty acids, vitamin B_{12}, and highly bioavailable iron (Bender, 1992). The health benefits of meat are countered by its association with cancer and cardiovascular disease (Demeyer, Honikel & De Smet, 2008), though these are the result of overconsumption and high saturated fat content, not the muscle tissue.

While it is possible that a cultured meat product could consist of a variety of animal cell types, meat is being defined here as primarily skeletal muscle tissue. An *in vitro* meat production system involves culturing muscle-like tissue in a liquid medium, therefore bypassing animal husbandry and slaughter. The controlled conditions of the IMPS are impossible to achieve by traditional livestock methods and therefore allow for a safer, healthier product. Myocyte culturing prevents the spread of animal-borne disease which may or may not affect meat products. Moreover, by reducing the amount of close quarter human-animal interaction, the incidence of epidemic zoonoses developing will decline. The employment of aseptic technique throughout the

culturing process ensures the meat product is free from contamination. Controlled conditions also offer the capacity for manipulation to create meat products with different nutritional, textural and taste profiles. This can be accomplished by co-culturing with different cell types, medium supplementation or genetic engineering.

Considering the benefits of an IMPS, is not surprising that a number of parties have proposed (and patented) the methodology for actualizing this idea (Vein, 2004), (Van Eelen, van Kooten & Westerhof, 1999), (Edelman, McFarland, Mironov & Matheny, 2005). This paper introduces the techniques so far proposed. As of yet none of these processes, though detailed, have been tested. This is partially because livestock animal cell lines have not been well-established *in vitro* (Talbot & Blomberg, 2008) and because growing muscle cells *ex vivo* on a large scale it is certainly a vast and unexplored undertaking. The technical demands of large scale production are unseen in the world of medical research, where most efforts in growing tissue *ex vivo* have been directed. The nutritional composition of *ex vivo* engineered muscle tissue has not yet been paid much attention. As a result, establishment of an IMPS is faced with many unique challenges so far unexplored in the field of tissue engineering.

Breakthroughs in relevant research since the publishing of these patents and proposals have widely altered the scope of designing an IMPS. Because the proposed systems at present approximate to a schematic with modifiable elements, the discrete elements will be attended to individually, taking into

account recent relevant scientific developments. This will be followed by a discussion of some drawbacks and limitations of the methodology, concluded with a conjecture of the future of *in vitro* meat production, and suggestions for further research.

2. Basic methodology

The methods so far proposed have a common set of elements. Each proposal employs the growth of myoblasts or myosatellite cells on a scaffold in a suspension culture medium within a bioreactor. Neither of these cell types need to be stimulated to muscle cell lineages but both have limited regenerative capacity. Van Eelen et al. (1999) proposed growth of myocytes on a collagen meshwork, while Edelman et al. (2005) suggest collagen beads as the scaffold. The scaffold-based method can only produce a thin myocyte layer of 100 - 200 μm thick on the scaffold in static culture due to diffusional limitations (Carrier et al., 1999). As a result, the products of these methods lack the structure of native muscle tissue, and therefore could only be put towards processed meat products (Edelman et al., 2005). To create a three-dimensional meat product, van Eelen et al. (1999) suggest layering several confluent sheets of myocyte culture.

An alternative method of creating a three-dimensional product is the expansion in volume of an explant of muscle tissue of animal origin. Benjaminson, Gilchriest and Lorenz (2002) were able to expand the surface area of an explant of fish by growth in a medium containing crude cell mixture;

the resulting product was prepared and well-rated by a food panel. This method also faces diffusional limitations and is unlikely to translate well into a large scale operation.

3. Cells

It is worth mentioning that non-skeletal muscle cell types may have relevance as a cultured meat product, but in vitro meat is being defined here as a culture of primarily skeletal muscle tissue therefore cell types destined to create this desired tissue type are discussed below.

3.1. Native muscle tissue formation

During embryological development, committed muscle tissue formation as seen in Fig. 1a) begins with mononucleated myoblasts of limited proliferation capacity (Benjaminson et al., 2002). Myoblasts fuse into a multinucleated myotube, which matures into a non-proliferative myofiber (Campion, 1984). Postnatally, increases in number of myofibers and number of nuclei per myofiber is kept minimal, except in instances requiring repair or regeneration. In these cases, myosatellite cells are responsible for generating new myofibers or contributing additional myonuclei to existing ones (Fig. 1b), (Le Grand & Rudnicki, 2007). Located between the basal lamina and sarcolemma of an associated myofiber, mononucleated myosatellite cells are normally in a quiescent, non-dividing state (Hill, Wernig & Goldspink, 2003). When activated *in vivo* by weight-bearing stress or injury, myosatellite cells

asymmetrically divide into self-renewing myoblasts and committed myofibers (Benjaminson et al, 2002, Le Grand et al., 2007).

Myosatellite cells are a very small proportion (1-5%) of the cell population of muscle tissue, and this percentage is dependent on muscle fiber composition and organism age (Allen, Temm-Grove, Sheehan & Rice, 1997). As an organism ages, the regenerative potential of its myosatellite cell population also decreases rapidly. As a result, the cells that offer optimal regenerative potential and myofiber morphology *in vitro* (longer myofibers and greater myofiber density) must be harvested from neonates (Delo, Eberlie, Williams, Anderrson, Atala & Soker, 2008).

3.2. Possible cell types

Proposed cell types: At the time when van Eelen et al. (1999) and Edelman et al. (2005) made their IMPS proposals, the two viable cell options were embryonic stem (ES) cells or myosatellite cells. In theory, after the ES cell line is established, its unlimited regenerative potential eliminates the need to harvest more cells from embryos, however, the slow accumulation of genetic mutations over time may determine a maximum proliferation period for a useful long-term ES culture (Amit et al., 2000). While ES cells are an attractive option for their unlimited proliferative capacity, these cells must be specifically stimulated to differentiate into myoblasts and may inaccurately recapitulate myogenesis (Bach, Stem-Straeter, Beier, Bannasch & Stark, 2003). In addition to this, there are so far no “proven” bovine, porcine, caprine nor ovine ES cell lines that have been established to the degree of a biological reagent like that of human,

monkey or mouse ES cells (Talbot et al., 2008). Efforts invested into establishing ungulate stem cell lines over the past two decades have been generally unsuccessful with difficulties arising in the recognition, isolation and differentiation of these cells (Keefer et al., 2007).

Although myosatellite cells have the disadvantage of being a rare muscle tissue cell type with limited regenerative potential, Bach et al. (2003) indicate that they are the preferred source of primary myoblasts because they recapitulate myogenesis more closely than immortal myogenic cell lines. Myosatellite cells have been isolated and characterized from the skeletal muscle tissue of cattle (Dodson, Martin, Brannon, Mathison & McFarland, 1987), chicken (Yablonka-Reuveni, Quinn & Nameroff, 1986), fish (Powell, Dodson & Cloud, 1989), lambs (Dodson, McFarland, Martin & Brannon, 1986), pigs (Blanton, Grant, McFarland, Robinson & Bidwell, 1999 and Wilschut, Jaksani, Van Den Dolder, Haagsman & Roelen, 2008), and turkeys (McFarland, Doumit & Minshall, 1988). Each animal species has its own benefits and limitations as a cell source, and myosatellite cells isolated from different muscles have different capabilities to proliferate, differentiate, or be regulated by growth modifiers (Burton, Vierck, Krabbenhoft, Byrne & Dodson, 2000). Wilschut, Jaksani, Van Den Dolder, Haagsman and Roelen (2008) have shown that porcine muscle progenitor cells have the potential for multilineage differentiation into adipogenic, osteogenic and chondrogenic lineages, which may play a role in the development of co-cultures.

Recent advances in tissue engineering and cell biology some offer alternate cell options which may have practical applications with multilineage potential allowing for co-culture development and with suitability for large-scale operations.

Adult stem cells: Myosatellite cells are one example of an adult stem cell type with multilineage potential (Asakura, Komaki & Rudnicki, 2001). Adult stem cells have been isolated from several different adult tissues (Wagers & Weissman, 2004), but another cell type relevant to in vitro meat production are adipose tissue-derived adult stem cells (ADSCs), a rare population of multipotent cells found in adipose tissue (Gimble, Katz & Bunnell, 2007). Kim et al. (2006) have noted that these highly expandable cells can be obtained relatively non-invasively from subcutaneous fat and subsequently transdifferentiated to myogenic, osteogenic, chondrogenic or adipogenic cell lineages.

The greatest concern and matter of debate regarding adult stem cells is their proneness to malignant transformation in long-term culture (Lazennec & Jorgensen, 2008). Rubio et al. (2005) have found that adipose tissue-derived adult stem cells immortalize at high frequency and undergo spontaneous transformation in long term (4-5 months) culturing, while evidence of adult stem cells remaining untransformed have also been reported (Bernardo et al., 2007). In an in vitro meat production system, re-harvesting of adult stem cells to minimize the risk of spontaneous transformation may be necessary. With this in mind, harvesting ADSCs from subcutaneous fat is far less invasive than

collection of myosatellite cells from muscle tissue and samples can be taken from certain organisms without causing substantial harm.

Dedifferentiated cells: Dedifferentiation is the reversion of a terminally differentiated cell into a multipotent cell type. It has been reported by Matsumoto et al. (2007) that mature adipocytes can be dedifferentiated *in vitro* into a multipotent preadipocyte cell line termed dedifferentiated fat (DFAT) cells. Following this, DFAT cells are capable of being transdifferentiated into skeletal myocytes (Kazama, Fujie, Endo & Kano, 2008). By floating a piece of glass on top of a suspension of mature adipocytes, buoyant mononucleated adipocytes attach to the upper surface and either a) release their fat droplet and assume the fibroblast-like shape of a DFAT cell or b) asymmetrically divide to produce one fibroblast-shaped DFAT daughter cell (Matsumoto et al., 2007). Termed the "ceiling culture" method, the process certainly seems achievable on an industrial scale. Because mature adipocytes are the most common cell type in adipose tissue, tissue samples will have high cell yields compared to other cell options.

Cell dedifferentiation appears to be an attractive alternative to the use of stem cells but Rizzino (2007) has put forth the argument that many of the claims of transdifferentiation, dedifferention and multipotency of once terminally differentiated cells may be due to abnormal processes resulting in cellular look-alikes.

3.3. Replicative ability

An IMPS requires many cell divisions to mass culture muscle tissue, but most cells have a finite number of divisions in culture before natural cell death; this number is termed the Hayflick limit. Edelman et al. (2005) put forth the three possible means for overcoming this limitation in an IMPS: a) regularly replenishing the culture, b) using an immortal cell line or c) immortalizing a cell line. Most cell types will require the first method though embryonic stem cells fall into the second category. Use of the third strategy is controversial because immortalization of cell lines requires genetic manipulation. It is also important to draw parallels between the genetic modifications that would facilitate large scale production and the mutations exhibited by cancerous cells.

Increasing the regenerative potential of cells without immortalizing them is one additional possibility. The Hayflick limit is determined by telomere length. Telomeres are the guanine-rich repeats found at the ends of linear chromosomes. Due to the linearity of the chromosome and the mechanism by which replication occurs, telomeres are shortened with every round of DNA replication (and cell division). Telomere length therefore corresponds to the number of divisions a cell type is capable of. This explains why a neonatal source is best for harvesting myosatellite cells; very early in development the cells still have long telomeres. Telomerase is a ribozyme capable of lengthening telomeres, naturally found in immortal cell lines. Many different cell types have been immortalized with ectopic telomerase expression and have showed no signs of the growth deregulation associated with cancer cells (Harley, 2002).

Unfortunately, this helpful genetic alteration will likely be subject to consumer rejection.

4. Scaffold

Scaffolding mechanisms differ in shape, composition and characteristics to optimize muscle cell and tissue morphology. Myoblasts are anchorage-dependent cells, capable of spontaneous contraction. An ideal scaffold would have a large surface area for growth and attachment, be flexible to allow for contraction, maximize medium diffusion and be easily dissociated from the meat culture. A scaffold that closely mimics the in vivo situation is best; myotubes differentiate optimally on scaffold with a tissue-like stiffness (Engler, Griffin, Sen, Bönnemann, Sweeney & Discher, 2004). The best materials would be natural and edible, though inedible scaffold materials cannot be disregarded. Development of new biomaterials offer additional characteristics that may be beneficial for *in vitro* muscle tissue growth, such as fulfilling the requirement of contraction for proliferation and differentiation (De Deyne, 2000)

4.1. Shape

As mentioned before, Edelman et al. (2005) proposed beads made of edible collagen as a substrate while Van Eelen et al. (1999) proposed a collagen meshwork described as a “collagen sponge” of bovine origin. The tribeculate structure of the sponge allows for increased surface area and diffusion, but may impede harvesting of the tissue culture. Other possible scaffold forms include

large elastic sheets or an array of long, thin filaments. Conformation choice is based primarily on maximizing surface area, which increases diffusion and the amount of anchorage-dependent tissue that can be grown.

4.2. Texture and microstructure

Texturized surfaces can attend to specific requirements of muscle cells, one of which is myofiber alignment. To mimic native muscle architecture, Lam, Sim, Zhu and Takayama (2006) cultured myoblasts on a substrate with a wavy micropatterned surface and found that the wave pattern aligned differentiated muscle cells while promoting myoblast fusion to produce aligned myotubes. Myofiber organization is important for the functional characteristics of muscle and the textural characteristics of meat. Micropatterned surfaces could allow muscle tissue cultured by the scaffold-based technique to assume a two-dimensional structure more similar to that of meat of native origin.

Electrospinning is the process of using electrical charge to extract very fine fibers from liquids. Riboldi, Sampaolesi, Neuenschwander, Cossu and Mantero (2005) have suggested electrospun microfibrous meshwork membranes as a scaffold for skeletal myocytes, as the membranes offer high surface area to volume ratio and some elastic properties. Electrospinning creates very smooth fibers, which may not translate well into a good adhesive surface; Riboldi et al. (2005) have shown that coating electrospun polymer fibres with extracellular matrix proteins, such as collagen or fibronectin, promotes attachment by ligand-receptor binding interactions. Electrospinning shows promise for scaffold formation because the process is simple,

controllable, reproducible and capable of producing polymers with special properties by co-spinning (Riboldi et al., 2005). Microfibre organization can also affect myofiber morphology. Electrofibers can be spun with nanometer to micrometer width; this corresponds to the wave periodicity (6μm was found to be optimal by Lam et al. (2006)) found to be beneficial for myocyte alignment on a micropatterned surface. Knowing the effects of micropatterning, introducing electrospun fibers aligned at the correct periodicity could theoretically align myofibers.

Beyond facilitating attachment and alignment, a scaffold capable of increasing nutrient diffusion and medium circulation would prove invaluable to the process. Noting that tissue thickness cannot exceed 100-200 μm without experiencing cell death, Borenstein et al. (2002) microfabricated a synthetic vascular network. Edelman et al. (2005) acknowledges that a cast of an existing vascularization network, such as that in native muscle tissue, can be used to create a collagen network mimicking native vessel architecture. Taking this a step further, Borenstein et al. (2002) created artificial networks of channels in sheets of biodegradable, biocompatible polymer, then seeded the network with endothelial cells. Following dissolution of the polymer mold, successful proliferation could theoretically leave behind a network of endothelial tissue onto which one could grow myocytes. A synthetic vascular system would then require a circulation pumping system and a soluble oxygen carrier in the medium to be fully functional. Unfortunately, at this moment creation of these

artificial vascular networks does not translate well into mass production due to the microfabrication processes required.

4.3. Composition

Several different polymers could suffice as the scaffold material for an *in vitro* meat production system. Examples of edible, naturally derived polymers are collagen, cellulose, alginate or chitosan. These polymers would be safe to leave in the meat product and could add a textural quality. Edelman et al. (2005) have suggested that porous beads made of these polymers capable of undergoing surface area changes under different pH and temperature conditions could fulfill the contraction requirement of myoblast cells.

By contrast, inedible polymers confer some interesting qualities that can aid muscle tissue formation. The aforementioned micropatterned surfaces which can aid in myofiber alignment are inedible, as are the thermoresponsive coatings described below. Jun, Jeong and Shin (2009) have found that growing myoblasts on electrically conductive fibers induces their differentiation, forming more myotubes of greater length without the addition of electrical stimulation. Use of inedible scaffolding systems necessitates simple and non-destructive techniques for removal of the culture from the scaffold.

4.4. Scaffold removal

A technical challenge of growing by the scaffold-based technique is removal of the scaffolding system. Removal of confluent cultured cell sheets is conventionally done enzymatically or mechanically, but these two methods damage the cells and the extracellular matrix they may be producing (Canavan,

Cheng, Graham, Ratner & Castner, 2005). Da Silva, Mano and Reis (2007) note that thermoresponsive coatings which change from hydrophobic to hydrophilic at lowered temperatures can release cultured cells and extracellular matrix as an intact sheet upon cooling. Termed “thermal liftoff,” this method results in undamaged sheets that maintain the ability to adhere if transferred onto another substrate (da Silva et al., 2007). This opens the possibility of stacking sheets to create a three dimensional product.

Lam et al. (2009) have presented a method for detaching culture as a confluent sheet from a non-adhesive micropatterned surface using the biodegradation of selective attachment protein laminin.

It should be noted that culturing on a scaffold may not result in a two-dimensional confluent “sheet” of culture. After scaffold removal, the contractile forces exerted by the cytoskeleton of the myocyte are no longer balanced by adhesion to a surface, causing the cell lawn to contract and aggregate, forming a detached multicellular spheroid (da Silva et al., 2007). To remove the culture as a sheet, a hydrophilic membrane or gel placed on the apical surface of the culture before detachment can provide physical support. Lam, Huang, Birla and Takayama (2009) found that use of a fibrin hydrogel was ideal for skeletal muscle tissue because cells can migrate, proliferate and produce their own extracellular matrix within it while degrading excess fibrin. These two-dimensional sheets could be stacked as suggested by Van Eelen et al. (1999), to create a three-dimensional product.

5. Culture conditions

Perhaps the most difficult task in designing an *in vitro* meat production system is determining the best culture medium formulation. The medium should support and promote growth while being made of affordable, edible components available in large quantities. Medium composition will be a substantial cost determinant if not solely for the fact that large quantities will be required.

5.1. Growth media

Myoblast culturing usually takes place in animal sera, a costly media that does not lend itself well to consumer acceptance or large scale use. Animal sera are from adult, newborn or fetal source, with fetal bovine serum being the standard supplement for cell culture media (Coecke et al., 2005). Because of its in vivo source, it can have a large number of constituents in highly variable composition and potentially introduce pathogenic agents (Shah, 1999). The harvest of fetal bovine serum also raises ethical concern. Commercially available serum replacements and serum-free culture media offer some more realistic options for culturing mammalian cells in vitro. Serum-free media reduce operating costs and process variability while lessening the potential source of infectious agents (Froud, 1999).

Serum-free media have been developed to support in vitro myosatellite cell cultures from the turkey (McFarland, Pesall, Norberg & Dvoracek,1991), sheep (Dodson & Mathison, 1988) and pig (Doumit, Cook & Merkel, 1993). Variations among different serum-free media outline the fact that satellite cells from different species have different requirements and respond differentially to

certain additives (Dodson et al., 1996). Ultroser G is an example of a commercially available serum substitute specially designed to replace fetal bovine serum for growth of anchorage-dependent cells in vitro. It has a consistent composition containing growth factors, binding proteins, adhesin factors, vitamins, hormones and mineral trace elements, all necessary for eukaryotic cell growth (Duque, Gómez, Diaz, Facal, Hidalgo & Diez, 2003). It has one-fifth the protein content of serum (Pope, Harrison, Wilson, Breen & Cummins, 1987), yet growth of mammalian skeletal myocytes with serum substitute Ultroser G showed more maturation over the same period of time, longer lasting viability and longer myotubes with more localized nuclei (Benders, van Kuppevelt, Oosterhof and Veerkamp, 1991). While Ultroser G has many beneficial effects on the growth and maturation of muscle tissue in vitro, its costliness may make this an unlikely candidate for scale up. In addition, while the Ultroser G and other commercially available serum replacements may have advantageous effects on the growth of tissues, their exact formulations are protected by commercial copyright and an evaluation of their suitability on a large scale can only be determined by cost and effects. In most cases, serum-free media are supplemented with purified proteins of animal origin (Merten, 1999).

Benjaminson et al. (2002), in their investigations with fish explants, found that mushroom extracts were comparable to serum as a growth medium in promoting explant surface area expansion. A cheap, rich serum is necessary for an *in vitro* meat production system; it is possible that amino acid-rich

mushroom extracts could be applied here. The development of an appropriate serum-free media completely free of any animal derived components appears ideal, but the potential for allergens from plant-derived proteins are a risk factor to be mindful of.

5.2. Regulatory factors

Creating an optimal cocktail of hormones, growth factors is a complex undertaking, one which requires an extensive amount of investigation. Extrinsic regulatory factor selection must be specific to the chosen cell type and species, as myosatellite cells for instance, of different species respond differentially to the same regulatory factors (Burton et al., 2000). It is also likely that the formulation may be required to change over the course of the culturing process. For instance, the proliferation period may require one certain combination of growth factors and hormones while the differentiation and maturation period may require a different set.

A multitude of regulatory factors have been identified as being capable of inducing myosatellite cell proliferation (Cheng et al., 2006), and the regulation of meat animal-derived myosatellite cells by hormones, polypeptide growth factors and extracellular matrix proteins has also been investigated (Dodson, McFarland, Grant, Doumit & Velleman, 1996; Doumit, Cook & Merkel, 1993). Purified growth factors or hormones may be supplemented into the media from an external source such as transgenic bacterial, plant or animal species which produce recombinant proteins (Houdebine, 2009). Alternatively,

a sort of synthetic paracrine signalling system can be arranged so that co-cultured cell types (a feeder layer) can secrete growth factors which can promote cell growth and proliferation in neighbouring cells. Co-cultured hepatocytes for instance could provide insulin-like growth factors which stimulate myoblast proliferation and differentiation (Cen, Zhang, Huang, Yang & Xie, 2008) as well as myosatellite cell proliferation in several meat-animal species in vitro (Dodson et al., 1993) Human growth hormone, routine produced from a transgenic bacterial source can be supplemented into the medium to stimulate production of insulin-like growth factors by hepatocytes.

Alternatively, autocrine growth factor signaling can play a role, as certain muscle cell-secreted growth factors such as insulin-like growth factor II stimulate myocyte maturation (Wilson, Hsieh & Rotwein, 2003) Similarly, growth factor production can occur in genetically engineered muscle cells.

5.3. Contraction

Regular contraction is a necessity for skeletal muscle. It promotes differentiation and healthy myofiber morphology while preventing atrophy. Muscle *in vivo* is innervated, allowing for regular, controlled contraction. An *in vitro* system would necessarily culture denervated muscle tissue, so contraction must be stimulated by alternate means. Cha et al. (2006) have found that administration of cyclic mechanical strain to a highly porous scaffold sheet promotes differentiation and alignment of smooth muscle cells. Edelman et al. (2005) and van Eelen et al. (1999) proposed mechanical stretching of scaffolds and expandable scaffold beads to fulfill the requirement of providing

contraction. This in mind, de Deyne (2000) noted that external mechanical contraction is less effective than electrical stimulation in promoting muscle development. Electrical stimulation, feasible on a large scale, induces contraction internally as opposed to passively and aids in differentiation and sarcomere formation. As mentioned before, even growth on electrically conductive fibers without application of electrical stimulation sufficed in reaping the benefits of induced contraction (Jun et al., 2009).

6. Bioreactor

Achieving adequate perfusion of the cultured tissue is key to producing large culture quantities. To construct viable tissue greater than 100-200 μm in thickness it is necessary to have adequate oxygen perfusion during cell seeding and cultivation on the scaffold (Radisic et al., 2008). Adequate oxygen perfusion is mediated by a) bioreactors which increase mass transport between culture medium and cells, along with b) the use of oxygen carriers to mimic hemoglobin-provided oxygen supply.

6.1. Medium perfusion

The design of a bioreactor is intended to promote the growth of tissue cultures which accurately resemble native tissue architecture while providing an environment which allows for increased culture volumes.

The cylindrical wall of rotating wall vessel bioreactors rotate at a speed that balances centrifugal force, drag force and gravitational force, leaving the three-dimensional culture submerged in medium in a perpetual free fall state (Carrier et al., 1999). This creates a laminar flow of medium which improves

diffusion with high mass transfer rates at minimal levels of shear stress, producing three-dimensional tissues with structures very similar to those *in vivo* (Martin et al., 2004).

More appropriate for scaffold-based myocyte cultivation, direct perfusion bioreactors flow medium through a porous scaffold with gas exchange taking place in an external fluid loop (Carrier et al., 2002). They offer high mass transfer but also significant shear stress, so determining an appropriate flow rate is essential (Martin et al., 2004). Direct perfusion bioreactors are also used for high-density, uniform myocyte cell seeding (Radisic, Euloth, Yang, Langer, Freed & Vunjak-Novakovic, 2003).

Another method of increasing medium perfusion is by vascularizing the tissue being grown. Levenberg et al. (2005) had induced endothelial vessel networks in skeletal muscle tissue constructs by using a co-culture of myoblasts, embryonic fibroblasts and endothelial cells co-seeded onto a highly porous biodegradable scaffold. This is certainly a method that can increase the diffusional limitation of tissue thickness beyond 100-200 μm in vitro.

6.2. Oxygen carriers

Cell viability and density positively correlate with the oxygen gradient in statically grown tissue cultures (Radisic et al., 2008). Oxygen carriers can be supplemented to medium to maintain high oxygen concentrations in solution, similar to that of blood. There are two distinct varieties of oxygen carrier: those which are modified versions of hemoglobin and those which are chemically inert, artificially produced perfluorochemicals (PFCs) (Lowe, 2006a).

Though many chemically modified hemoglobins have been developed, their bovine or human source makes them an unlikely candidate for scale up. Alternatively, human hemoglobin has been produced by genetically modified plants (Dieryck et al., 2007) and microorganisms (Zuckerman, Doyle, Gorczynski & Rosenthal, 1998). Efforts to produce heme proteins and blood subsitute components using the microorganisms *Escherichia coli, Pichia pastis,* and *Aspergillus niger* are underway as these organisms are already used to commercially produce human pharmaceuticals and food additives (Lowe, 2006b).

Perfluorochemicals (PFCs) dissolve large volumes of oxygen and therefore can perform the same function as hemoglobin. To be miscible in aqueous conditions PFC's must be emulsified and emulsifications have been available for use *in vitro* and *in vivo* (Lowe, 2006a). Shine et al. (2005) note that medical PFCs are extremely potent greenhouse gases on a per molecule basis and though they presently make a trivial contribution to climate change, increasing their use requires careful consideration. Development of an artificial blood is an active area of research and many applicable options are likely to arise with time.

7. Control and manipulation

An IMPS offers a level of control unattainable by traditional livestock methods of producing meat. Myocyte culturing prevents the uncontrollable, unpredictable complications present in livestock production including the spread of disease among animals and the development of zoonoses. The

diseases of concern in industrial agriculture which have become increasingly more difficult to contain with expanding international trade - avian flu, swine flu, foot-and-mouth disease, and bovine spongiform encephalopathy - will not threaten an IMPS. In addition, the problems caused by pre-slaughter environment: pale, soft, exudative (PSE) and dark, firm, dry meat (DFD), would not exist in the products of myocyte culturing.

While the controlled conditions *in vitro* allow the likelihood of unpredictable complications to be minimized, they can be manipulated to intentionally create products with differing qualities. With the advent of functional and enriched foods, consumers are more willing to try products that have been altered to have particular nutritional characteristics (Korhonen, 2002; Burdock, Carabin & Griffiths, 2006). By co-culture, medium formulation or genetic engineering, it is theoretically possible to create products with different tastes, textures and nutrient profiles.

Fat content is an example of a nutritional quality that can be manipulated. The greatest criticism against meat is the high saturated fat content which contributes to cardiovascular disease. *In vitro*, saturated fat content is determined by the amount of adipocyte co-culture present; without co-culture, pure myocyte culture would produce a product rich in nutritionally beneficial fatty acids from phospholipids. Furthermore, genetic manipulation of myocytes could allow the fatty acid composition to be altered to enrich for particularly beneficial fatty acids such as those which are omega-3 polyunsaturated.

While in theory nutrient content can be altered, one major obstacle likely to postpone the development of an IMPS is ensuring that the product has the full complement of nutrients available in meat harvested *in vivo*.

8. Limitations to the methodology

The scientific development of an IMPS is most hindered by the facts that a) a major tissue will be cultured in the absence of *in vivo* homeostatic regulation and b) that the process needs to be carried out on a large scale. The lack of homeostasis affects the nutritional value of the meat product, as often other organ systems are involved in nutrient absorption and distribution in the live organism. Muscle tissue is also a highly metabolic tissue and the products of metabolism need to be removed or recycled at a pace matching the provision of reactants. Large scale tissue culturing is a concept crucial to the development of an IMPS but is as of yet unexplored in the field of tissue engineering.

8.1. Nutritional value

In addition to having high protein content with the full complement of amino acids, meat is an exclusive source of several essential nutrients. It is necessary that an *in vitro* grown meat product meets if not exceeds the nutritional value of traditional meat products to be competitive on the market. Nutrients found in meat *in vivo* which are not synthesized by muscle cells must be supplemented.

For instance, the essential vitamin B12 is synthesized exclusively by certain species of gut-colonizing bacteria and is therefore found solely in food

products of animal origin. Supplementation of crystalline vitamin B_{12} produced commercially by biosynthetic microbial fermentation would be necessary in an *in vitro* meat product grown in an aseptic environment.

Iron in meat is present as the Fe(II) ion in the highly bioavailable form of heme, the prosthetic group found in myoglobin. To provide iron to growing myocytes in a bioavailable form, Fe(III) ions bound to the plasma binding protein transferrin will have to be supplemented to the culture medium. By transferrin-mediated iron transport, iron can enter the myocyte mitochondria and be incorporated into heme synthesis and subsequent myoglobin synthesis (Aisen, Enns & Wessling-Resnick, 2001). It must be noted that levels of transferrin must be closely monitored, so as not to allow free ferric or ferrous ions to be present in the medium, as they can readily catalyze the production of damaging reactive oxygen species in aerobic environments (Papanikolaou & Pantopoulos, 2005). In addition, Graber and Woodworth (1986) found that myoglobin levels are not significant in culture until a stable population of myotubes has formed, a fact which can help determine the optimal growth time necessary before harvest.

Vitamin B_{12} and heme iron are two especially important and exclusively meat-source nutrients of several. A great challenge in producing a competitive *in vitro* grown meat product is ensuring that all necessary nutrients are present. Dietary minerals and vitamins not synthesized by myocytes will often require binding proteins in medium and effective transport mechanisms for entry into the cells. Knowledge of the complex metabolism of each crucial vitamin and

mineral is necessary to develop a nutritionally valuable meat product. While determining the proper nutrient profile will be a major hurdle to overcome, it comes with the knowledge of how to manipulate the culturing system to make nutritionally tailored products.

8.2. Metabolism

An IMPS lacks the organ systems that maintain homeostasis in an organism and therefore metabolism needs to be carefully and strictly monitored. Myocytes must be metabolizing aerobically to prevent acidification of the culture medium with lactic acid. Oxygen must be readily available to myocyte culture to prevent hypoxia and the acidification of medium, two situations which are damaging to the cells. As mentioned above, adequate oxygenation is dependent on the ability of the bioreactor to enhance perfusion and the availability of oxygen in the medium. Adequately providing the chemical and physical requirements for metabolism while removing damaging waste products can be a difficult task, with the possibility of unforeseen shortcomings. For instance, co-culturing hepatocytes to convert lactate back to the glucose via the Cori cycle can be one method of preventing lactic acidification of medium in the event of anaerobic conditions. This can be complicated, however, by the individual metabolic needs of hepatocytes, which may not coincide well with those of myocytes.

Also important to consider is that muscle tissue and meat are biochemically (and therefore qualitatively) dissimilar. The metabolic reactions that proceed post-slaughter: anaerobic glycolysis, lactic acid accumulation,

protein denaturation and enzymatic proteolysis, are responsible for producing the textural quality, taste and appearance of meat. One uncertainty concerning metabolism is whether these typical post-slaughter reactions will occur in cultured meat after harvest in like manner to properly convert cultured muscle tissue to meat as it is traditionally defined.

8.3. Large scale operation

The development of a large scale IMPS facility with the capability to mass produce meat at a rate comparable to traditional slaughterhouses is greatly hindered by the lack of investigation into large scale culturing. Presently, "large" pieces of cultured tissue are measured on a centimetre scale with most relevant tissue engineering efforts have been put towards the medical application of *in vivo* tissue repair. Vladimir Mironov (Medical University of South Carolina, personal communication, 19 April 2009) has stated that an industrial scale bioreactor would need to be at least three to five storeys to produce industrially relevant amounts of cultured meat. The best source of inspiration for mass production at this time would be to look towards the pharmaceutical industry and microbial biotechnology where living organisms in bioreactors "manufacture" purified chemicals. Though the mass production of purified molecules is quite different from the mass production of cultured tissue, some of the technology and methodology may prove relevant.

While stem cell lines are defined by ability to be propagated in culture indefinitely while maintaining broad plasticity, a great criticism of stem cells in long-term culturing is spontaneous transformation.

9. Conclusion and future prospects

Developments in regenerative medicine, stem-cell research and biomaterial engineering in the past decade have yielded highly applicable, viable options which can aid in the scientific development of an IMPS. One possible in vitro meat production scheme is shown in Fig. 2. With the scaffold-technique being a practicable method for scale up, the materials and techniques now available make the IMPS seem more achievable, however, there remain several aspects of the concept yet to be investigated.

Because nearly all research applicable to the development of an IMPS is intended for biomedical application, many of the investigations into multipotent and myogenic cell lineages have been done with human, mouse or rat cell lines, with relatively few studies describing cell lines of agriculturally relevant animals. Adult stem cells and dedifferentiated cells are recently innovated cell types which require further exploration into their safety and suitability for an in vitro meat production system. The biological aspects of an IMPS need to be further investigated to ensure that the resulting tissue closely mimics native muscle tissue morphologically and nutritionally. In addition, relevant studies have so far focused on small-scale applications, with pertinent biological research focused towards tissue replacement *in vivo*. Undoubtedly, a major hindrance in development of the science of an IMPS is based around application on a mass production scale, where little research has been done. Many of the cell lines, scaffold materials and medium components described

are unreasonable for scale up economically, and are often complicated by other issues such as environmental impact and safety. It absolutely necessary that an in vitro meat production system be developed according to the rules and regulations of good cell culture practice (GCCP) (Coecke et al., 2005) as well as current good manufacturing practice (cGMP) as pertaining to food and drug production.

The greatest stumbling block comes with the commercial implementation of an IMPS, where cost-effectiveness and consumer acceptance determine if cultured myocyte tissue will become a significant meat alternative on the market. A preliminary economics study reviewing the financial viability of *in vitro* grown meat estimated the cost of manufacturing to be Euro 3500/tonne, but notes that because such technology has not yet been developed, this estimation could be largely inaccurate (eXmoor Pharma Concepts, 2008). *In vitro* meat production on an industrial scale is feasible only when a relatively cost-effective process creating a product qualitatively competitive with existing meat products is established and provided with governmental subsidization like that provided to other agribusinesses.

Cultured meat can certainly have an application in ground, processed foods such as hamburgers or hotdogs as a main component or as an additive. In this form, the textural shortcomings of the *in vitro* grown product will not compromise the final processed product and one would expect greater consumer acceptance. The second goal of cultured meat is to create

three-dimensional products resembling traditional cuts with proper textural characteristics.

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Fig 1: a) Prenatal Myogenesis: Stem cells give rise to proliferative muscle precursor cells, (myoblasts), which lose proliferative capability upon fusing into multinucleate myotubes. Myotubes undergo morphological changes to become mature nonproliferative myofibers.
b) Postnatal/posthatch myogenesis is for repair and regeneration of existing muscle tissue. Myofiber-associated myosatellite cells respond to weight bearing stress or injury by asymmetrically dividing into a self-renewing daughter cell and a nonprolierative myofiber-committed cell. Committed cells can fuse with other committed cells to produce new myofibers or add nuclei to existing myofibers.

Fig 2: A possible in vitro meat production scheme: (1) Media is formulated and kept in a feed tank before being deposited (2) into the bioreactor containing the scaffolding system. Flow of media allows cells previously isolated and expanded to be seeded (3) onto the scaffold within the bioreactor. During the culturing process, media is being constantly oxygenated in an external fluid loop (4). When culturing is complete, media can be recycled (5) and reformulated, while the scaffold and cultured tissue are removed from the bioreactor (6). Harvest of the tissue involves separation of tissue from the scaffold for further processing (7).