Universiteit Utrecht

FACULTEIT DIERGENEESKUNDE / FACULTY OF VETERINARY MEDICINE

PRODUCTION OF ANIMAL PROTEINS BY CELL SYSTEMS

DESK STUDY ON CULTURED MEAT ("KWEEKVLEES")

H.P. HAAGSMAN, K.J. HELLINGWERF, B.A.J. ROELEN

October 2009
Faculty of Veterinary Medicine
http://www.uu.nl
Utrecht

Production of animal proteins by cell systems

Desk study on cultured meat ("kweekvlees")

H.P. Haagsman
K.J. Hellingwerf
B.A.J. Roelen

Contents

Boxes:

Preface

This report was commissioned by the Ministry of Agriculture, Nature and Food Quality as part of a survey on meat alternatives. It presents the current state of research and development of cultured meat ('kweekvlees"), a completely new idea to produce edible skeletal muscle (i.e. meat) by culturing and differentiating stem cells of farm animal species to skeletal muscle cells. This hypothetical method of producing 'meat' has been patented by the Dutchman Willem van Eelen in 1999, and research on its feasibility has been conducted by as part of a SenterNovem research project between 2005-2009.
The current study is performed by three researchers of two Dutch universities who also took part in the SenterNovem research project. Since these authors were involved in the 'in vitro meat' research project they had already acquired a vast amount of knowledge about the scientific aspects of cultured meat. Also in the past few years they have build up an extensive global network of those working on or interested in this subject. These contacts range from colleague scientists to process technologists, food specialists, and representatives of vegetarian organizations to psychologists and sociologists. The authors have used these contacts and did an extensive literature research for this report.
The study is therefore broad; not only scientific aspects are taken into account but also societal and economic factors. This was not an easy task since research and development of cultured meat is still in its infancy.
In this report, it is first described why there is a current dire need for meat alternatives. Subsequently, the theoretical background and short history of cultured meat are described and the types of culture media that can be used or developed. This is followed by a section on bioengineering and process technology. Ethical and societal issues are discussed, as well as challenges, strengths and weaknesses of the current approach. Besides literature studies on technological and societal aspects of cultured meat, the opinions of many interviewed experts were also an important source of information. The opinions of the experts can be read throughout the report in the various boxes. We would like to thank all experts that have contributed to this report.

The authors

Introduction

The use of livestock for the production of food has always been an essential part of man's existence on earth, and its impact has until recent years been primarily positive, both economically and socially. However, current production methods are rather demanding. Their impact may be direct, by ruminant methane production for example, or indirect, such as expansion of soybean production for feed in South America, replacing rain forests. About 70% of the fresh water use, 35% of land use and 20% of the energy consumption of mankind is directly or indirectly used for food production, of which a considerable proportion is used for the production of meat. The total area of ice-free land in use for grazing is about 26% of the earth's total, and an additional 33% of arable land is in use for feed crop production. In total, livestock is responsible for 70% of agricultural land exploitation, and without change of policies this percentage will only increase in the near future.

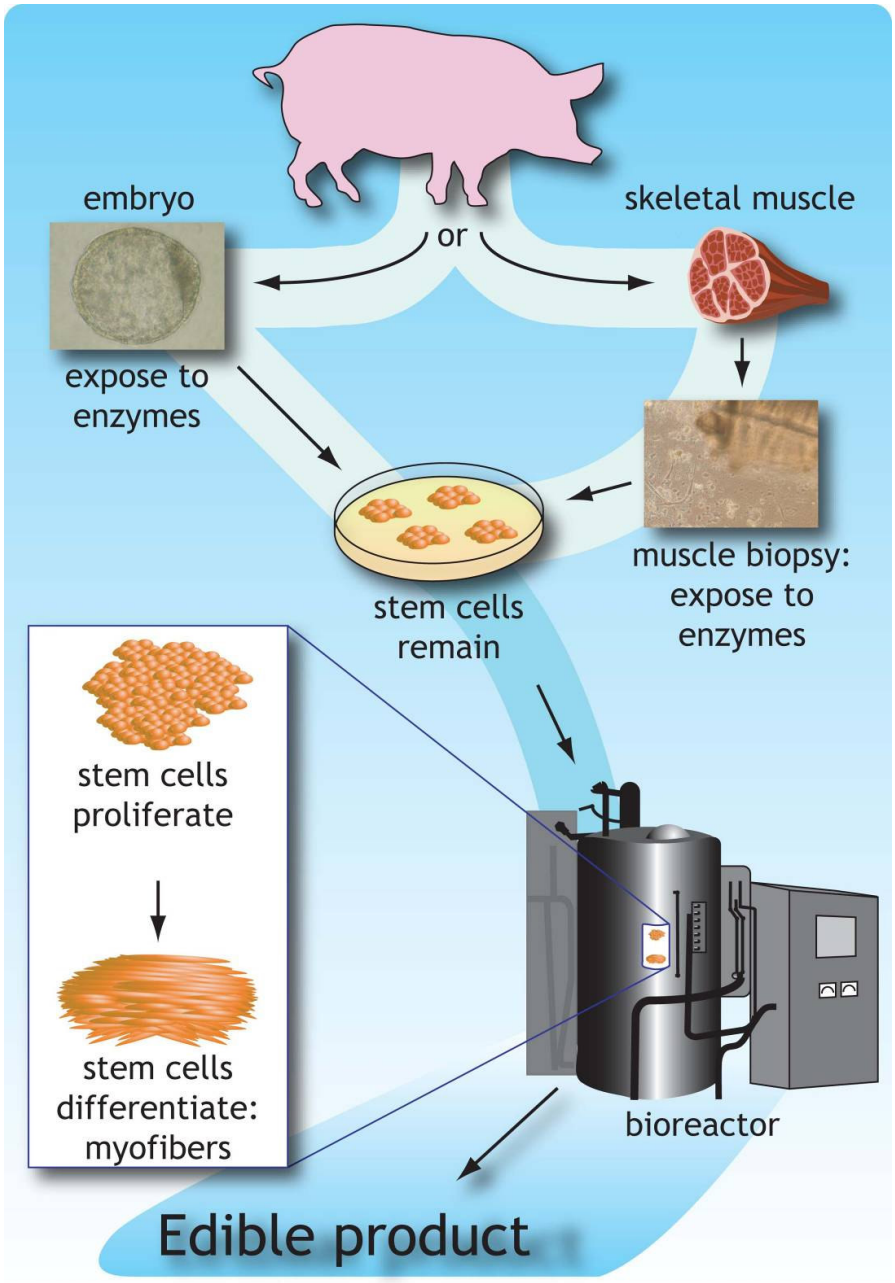
It has been estimated that the global population will increase from 6 billion people in 2000 to 9 billion people in the year 2050. This population increase will be accompanied by a rise in annual greenhouse gas emissions from 11.2 to 19.7 gigatonne of carbon dioxide, carbon equivalent. It is anticipated that in the same period annual global meat production will rise from 228 to 465 million tonnes due to rising incomes, urbanization and growing populations (FAO 2006. Livestock's long shadow, environmental issues and options, Rome.
http://www.virtualcentre.org/en/library/key_pub/longshad/A0701E00.pdf).
The amount of additional land available for the required increase in production capacity, however, is limited. This has serious consequences at various levels and makes it a major policy focus. It is now evident that we are experiencing a climate change, and that anthropogenic influences seem to be (at least partly) responsible for this. This includes the current level of meat production. Feed crop production demands high levels of energy, which in itself, leads to increased CO_2 emission. Livestock species, particularly ruminants, are responsible for greenhouse gas emissions, including methane from alimentary tract fermentation and nitrous oxide that may be emitted from decomposing manure and fertilizer. Indeed ruminants constitute the biggest anthropogenic source of methane emissions.
If the climate change with increasing global temperatures that we experience today will continue at the current pace this will have enormous consequences for plant and animal life, including the human population. A significant rise in the sea-water level is expected due to melting glaciers and ice caps, combined with an increased frequency in extreme weather events. The water use for livestock and accompanying feed crop production also has a dramatic effect on the environment such as a decrease in the fresh water supply, erosion and subsequent habitat and biodiversity loss. In order to limit temperature increase to an acceptable level it has been calculated that in 2050 greenhouse gas emissions need to be between 40 and 80% reduced compared with the levels of 2000.

Land use, including that for the livestock sector, has increased dramatically in the past decades, leading to loss and fragmentation of habitats. As a result the total area of habitats important for biodiversity such as rain forests and wetlands has decreased dramatically. Since the total land area of planet Earth is finite, the land surface that can be used for the livestock sector either for cropping or grazing is limited. The future increase in agricultural production will therefore have to stem from intensified agriculture on land already used, and/or from a more efficient conversion of plant material to edible meat products.

An additional complication is that, particularly in developed countries, animal welfare has become a societal issue and keeping animals for consumption is a matter of debate for a significant part of the population.

Based on the above, several scenarios can be sketched for the future of meat production. Continuation of 'business as usual' will lead to further environmental degradation and destruction of habitats. Solutions are however within reach, many of which are at political levels, such as subsidy programs for environmental services and regulation of land rights. Solutions can also come from the scientific sector, however, although these will not be immediate. These solutions will need investment in the form of time and money, and possibly changes in consumer's habits.

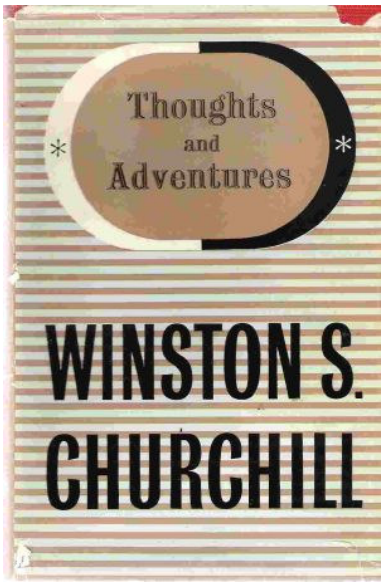
Scientific innovations can and should come from all sectors involved, such as transport, machinery, fertilizers etc. An important contribution can be made via the generation of meat alternatives. Many of these innovations will be improvements of already existing concepts and products. Great leaps can be made by radically new concepts that require 'out of the box' thinking.



An example of such a concept is to make edible products from skeletal muscle cells, cultured from stem cells, outside the animal in a bioreactor. Although this technology is still at a very early stage, and requires many fundamental problems and questions to be solved, this technique holds great promise as a solution to reduce livestock's impact on the environment. A schematic overview of how this technology could look like can be seen in the figure left.

Cultured meat: a short history

The idea of culturing animal parts in vitro for human consumption is not new. In fact, already in the 1920s it was Winston Churchill who predicted that within 50 years from then, animal parts would be 'cultured separately under a suitable medium' (Churchill, essay 'Fifty Years Hence' later published in 'Thoughts and adventures', 1932) [1]. In 2002 a study was published in which the possibilities of culturing animal muscle protein for long-term space flights or habituation of space stations were explored. For this, muscle tissue from the common goldfish (*Carassius auratus*) ranging from 3-10 cm in length was cultured in Petri dishes. The results from the experiments were rather promising and a limited increase in cell mass was observed when the muscle tissue was cultured with crude cell extracts. In addition, the cultured explants were washed, dipped in olive oil with spices, covered in breadcrumbs and fried. A test-panel judged these processed explants and, although actual tasting was not performed according to Food and Drug Administration rules, agreed that the product was acceptable as food [2]. This promising study was not continued because of a lack of further funding.



In the Netherlands, it was Willem van Eelen in the early 1950s who independently had the idea of using tissue culture for the generation of meat products. Since at that time the concept of stem cells and the in vitro culture of cells still had to emerge, it took until 1999 before van Eelen's theoretical idea was patented. Van Eelen, as part of the company VitroMeat BV, sought collaboration with academic partners, and in 2004 a consortium consisting of the Faculty of Biomedical Technology (Technical University Eindhoven), the Swammerdam Institute of Life Sciences (University of Amsterdam) and the Faculty of Veterinary Medicine (Utrecht University) together with an industrial partner, the meat concern Meester Stegeman (at that time part of Sara Lee Foods Europe) was founded. This consortium was awarded a SenterNovem research grant for 4 years to study the proof of principle of culturing skeletal muscle cells from farm animal stem cells.

Experts' opinions (1): Need for protein sources other than meat

- There is definitely a market for meat substitutes. Examples are legume-based and mycoprotein-based meat substitutes. Pros: the production is efficient, and the technology is already mature. Cons: allergies, meat texture difficult to replicate, aftertaste, psychological resistance to "substitute"
- First of all, the demand for meat is at present increasing. It will not be possible to produce all that meat in an environmental and animal friendly way. So there is a rather conventional meat market for in vitro meat. Another smaller market comprises the vegetarians that do not eat meat for ethical reasons.
- If with conventional meat we mean the production of meat via 'factory farming than there certainly is a market for alternative protein sources. These alternatives will include biological production and extensive farming. These has as advantages that they will cause less animal suffering, but as disadvantage that it still is a very inefficient way of protein production. Additionally, 'meat protein' can be produced using plants, such as for instance Quorn, soy etc. These have as advantages that they are animal friendly and sustainable. Such products are however no solution for the craving for meat.
- On a society level there is definitely a need and a market for cultured meat, but there is no need for a different new 'taste' or something like that. I think that in the future cultured meat will be safer than conventional meat. The production will also be more sustainable, but I do not think that it will have a wider range of applications. I also do not think that it can ever be tastier than conventional meat.
- There is a need for other protein sources but plant proteins will suffice if we returm to the consumption patterns of the 1960s-1970s.
- There is a need for protein sources other than meat but the development of cultured meat will only be a success if there will be an impulse with public money. Animal proteins could also be produced by transgenic plants.
- Other protein sources are available like fungi and plants (soy, peas) that have been used to make a variety of good products that are not expensive. The disadvantage of these products is the lack of a good texture and a taste that does not approach the original 'benchmark'.
- Due to the non-sustainability of traditional meat production I think there is a huge market for this. However, unless people change their habits dramatically I do not think other products than something that looks like, and tastes like, ordinary meat will have a great success. If the processing technology become very advanced one may think that the actual protein sources can be from bacteria, algae, plants, yeast as well as well as from tissue culture. However, given that a muscle tissue product was offered in the same price range as other products, I would think that it would be preferred by the consumers.
- There is a need for protein sources other than conventional meat. Cultured meat may be the preferred alternative because it is, unlike the other products, animal-derived and with respect to composition most like meat as we know it.

Cell culture

Prokaryotic cells. Research into the use of unicellular (micro)-organisms as a source for human consumption has gained momentum in parallel with the first oil crisis. In that period, from the mid seventies onwards, a lot of research has been conducted to get to the use of single-cell protein as animal feed or human food. In the same period there was a growing awareness of the world-hunger problem, and this also stimulated research on alternative sources of food.

The fact that the microbial biomass indeed is suited as a human food is clear for a long time. For example, local African tribes have centuries-old traditions of eating prokaryotic cells. Some of these tribes made the biomass of the cyanobacterium *Spirulina* a main component of their daily meal. In addition, many fermented food products contain large numbers of living bacteria, especially from the group of the lactic acid bacteria. However, continued research on single cell proteins has made clear that a prokaryotic biomass (i.e. bacterial cells) has a specific disadvantage when these cells are used as the main component of the diet of a mammal [3] [4]. Because of the high nucleic acid content of this particular type of biomass, and the uric acid produced from nucleic acids, the consumption of this type of biomass increases the risk of kidney stone formation. Nevertheless, prokaryotic biomass can, provided that the required precautions of concerning application, etc. are taken, become very useful as a source of nutrients of a culture medium for mammalian cells.

Research on single cell protein has provided a lot of information regarding the theoretical conditions, as dictated by the laws of Nature, on the large-scale culturing of unicellular organisms. In particular, knowledge has been gained about energy requirement (driven by the question whether influx or efflux of heat is required), supplementation of oxygen, optimization of the composition of the growth medium, and efficiency of the conversion of catabolic substrates (i.e. nutrients) towards biomass [5]. Regarding the latter it has become clear that after optimization many cell types can be cultured with a conversion efficiency of sugars to biomass of up to 50% (based on weight). This is, however, only possible provided that the cells can be cultured under optimal conditions, that means with saturating levels of oxygen. Without oxygen, metabolism can only take place by fermentation, a form of catabolism that provides only about 10% of the yield of aerobic metabolism.

This particular type of research on single cell protein has been almost completely ended at the end of the seventies when, because of, among others, the oil crisis, the prices for raw materials like methanol and petroleum for the production of proteins increased dramatically. Other factors also contributed to the termination of these projects, including the discovery of traceable amounts of petroleum in the protein products. Because of this, the knowledge transfer to those working outside the microbiology field has not been optimal.

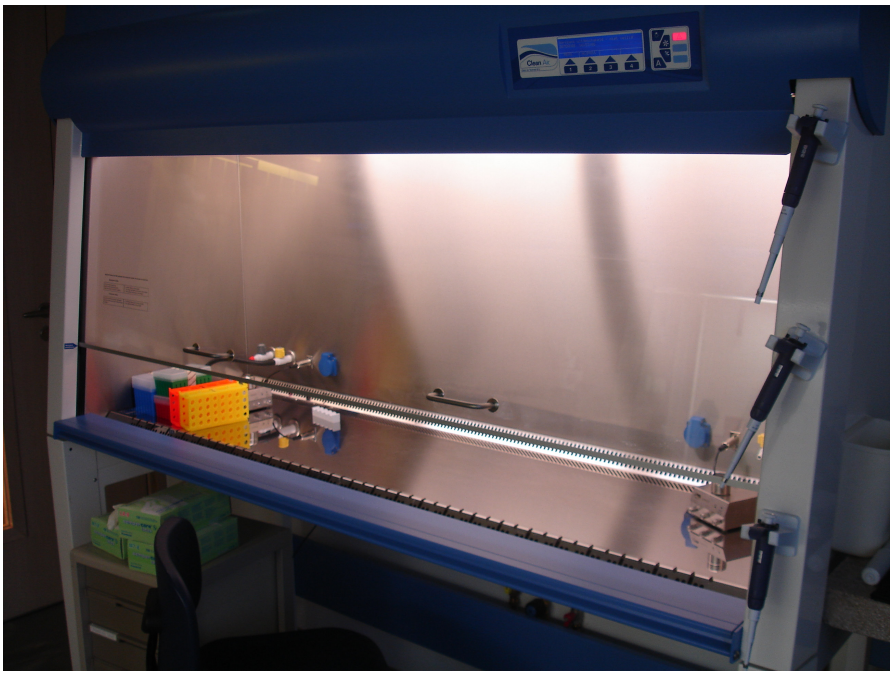
Experts' opinions (2): The most important reasons to produce cultured meat

- Potential impact on reducing cardiovascular disease and greenhouse gas emissions
- To discontinue the use of animals as bioreactors to grow food and other products
- Animal ethics. This can be "meat without suffering". Also environmental.
- There are a number of advantages of replacing conventional meat with cultured meat: 1) Prevention of animal suffering; 2) Prevention of food scarcity that can be expected with an increasing world population; 3) Liberation of land for nature (including wild animals); 4) Cultured meat will be more sustainable and better for the climate.
- For me the most important reason to produce in vitro meat would be consumer demand. More and more people are interested in cultured meat, and it can be a very successful product.
- Sustainability of the meat supply to an increasing population society, that is gaining buying power and demanding for more protein. Environmental concerns as even more fertile land would be used to grow meat (cows).
- It could be sustainability but that has not been proven yet.
- Avoid the use of animals in factory farming.
- Allegedly the safety and sustainability of cultured meat production are the most important reasons to produce cultured meat but I have my doubts.
- Personally, the major reasons are the environmental impact and the effect it will have on animal welfare.
- Nutrition of a growing world population with reduced ecological impact.

Eukaryotic cells. At the end of the 1900s it was discovered that is possible to keep tissue alive outside the animal from which it was derived for several days in a warmed physiological salt solution. With this method, however, the cells will eventually become necrotic and the tissue will disintegrate because of lack of a bloodstream and therefore insufficient supply of oxygen and nutrients to the cells. Growth of such tissue is not possible, and culturing is merely maintenance. Alternatively, single cells can be liberated from tissues by enzymatic digestion, and these cells can be kept in culture as a suspension or attached to a suitable surface such as that of flasks or dishes. Equivalent to culturing of whole tissue, single cells derived from most specialized tissues have largely lost the capacity to divide. Indeed, tissues of adult animals also have ceased to grow; intrinsic cellular mechanisms take care of cellular senescence after differentiation. In vitro culture of cells derived from healthy tissue therefore results in cells that stop dividing after a few population doublings.

Cells derived from tumors have escaped the mechanism of limited cell division and are therefore immortal; in other words they can divide indefinitely. Indeed, the first human cells that have been cultured for a prolonged time were derived from a patient with an cervical tumor in 1951, and since then these cells, referred to as HeLa cells, are still in culture and have become the most widely used cell-type in biomedical research. Alternatively, cells can actively be immortalized by deliberate genetic modification such as through irradiation, chemical mutagenesis or targeted introduction of specific genes. When such cells can be sub-cultured for a prolonged period of time they are referred to as a cell line.

In warm-blooded animals, such as mammals, cells are kept at a temperature of around 37°C. For proper in vitro culturing of such cells it is important that the cells are kept at the appropriate temperature and under an appropriate gas atmosphere. Cells are therefore cultured in insulated incubators with a humidified atmosphere of 36-39°C and 5% CO_2 in air (depending on the species from which the cells were derived). Nowadays mammalian cells are routinely cultured in vitro for research purposes in complex liquid growth media containing salts, glucose, amino acids and other nutrients. Since not only mammalian cells, but also bacteria, fungi and yeast thrive well in these rich media it is essential that all reagents and equipment used for culturing are made and kept sterile. Cells are cultured in sterile disposable culture flasks or dishes, and passaging is done with sterile disposable pipettes. To create a sterile environment when handling the cells (*e.g.* for passaging) these procedures are performed in laminar flow cabinets (also called tissue culture hoods), by which a sterile environment is created by air that is drawn through a filter, and thereby sterilized, and flowing towards the user (crossflow) or from top to bottom (downflow).



Since cells of a cell line continue to proliferate, the bottom of the tissue culture flask or dish in which they are growing will eventually become completely covered with cells. Most animal cells show the phenomenon of 'contact inhibition', *i.e.* they cannot grow over each other. When the surface is fully covered by a monolayer of cells, they will gradually loose viability and die. To avoid this, cells are routinely passaged to new flasks or dishes before they have reached the maximal surface density. For this passaging (or splitting), cells are exposed to low concentrations of enzymes which will hydrolyze the extracellular matrix between the cells (and the plate) so that a suspension of single cells is formed. Part of this suspension is subsequently transferred to a new flask.

Culture media

The possibilities of culturing mammalian cells in vitro has evolved primarily because of biomedical interest (particularly cancer research), much less so because of a more fundamental cell biological interest. A much richer and more complex culture medium is needed for culturing mammalian cells than the medium needed for culturing prokaryotic cells. Dependent on the cell type, many specific growth factors, vitamins, lipids, amino acids, etc., are needed to maintain the viability of the cells and allow them to replicate. In addition, in contrast to prokaryotic cells, many mammalian cell types prefer to be attached to a solid surface.

To encourage attachment mammalian cells are usually cultured in flat plastic (for instance polyethylene) culture flasks that have a large surface/volume ratio. If needed

the flasks can be coated with proteins such as, for instance, laminin. More recently, several mammalian cell lines have become available that can be cultured in suspension. In comparison to bacteria these mammalian cells are extremely sensitive to mechanical stress, for instance caused by the forces that occur by stirring. This poses severe limitations to the type of bioreactors that can be used for such cells [6].

Historically, mammalian cells have been cultured in liquid media containing blood plasma and serum, of which the exact content was a) unknown and b) variable. Particularly, the addition of fetal calf serum, usually 5-20% of the final concentration, has been very successful. Even today, the majority of mammalian cells is cultured in medium containing a substantial percentage of fetal calf serum. Apparently, the fetal calf serum contains the growth factors that are required for mammalian cell proliferation. A disadvantage of the ample availability of fetal calf serum (commercially available) for biomedical researchers, is the fact that it has delayed the development of alternative serum-free culture media [7].

Research on mammalian cell cultures that is aimed at the basic understanding of cell physiology has been less successful because of the complex, largely unknown and highly variable composition of fetal calf serum. Furthermore, research on the effects of individual protein growth factors in cell culture media has been limited, because of the high costs of purified growth factors.

Chemically defined culture media. In the past 10-15 years there has been a considerable improvement in the development of growth media that support large scale culture of mammalian cells. This has been largely made possible by the increased use (and the added value) of particularly antibodies, growth factors, other recombinant proteins, etc. Examples of mammalian cells that are cultured in media with bovine serum include PER.C6 cells (a human cell line developed by Crucell) and Chinese hamster ovary (CHO) cells [8]. Importantly, several mammalian cell lines can now also be cultured in suspension, without adherence of the cells to a solid surface. Using this suspension method, culture systems can be used with a much better productivity per volume [9].

Culture media that contain fetal bovine serum are being described as 'complex' media, to stress the fact that the media are composed of many different factors of which the exact nature and concentration is unknown. This makes it practically impossible to accurately define the chemical composition of such media. In the past few years it has however become clear that it is also possible to compose growth media for mammalian cell using combinations of limited numbers of purified chemical compounds. Since the exact composition of these chemically defined media is known, these media can be produced with the guarantee that they do not contain animal products. The risk of contaminating mammalian cells with animal components that create a health hazard is thus eliminated. Importantly, production of cells and cell-derived products is much better standardized with these chemically defined media.

For the past 10 years, much research has been conducted on the growth of cells like the CHO and PER.C6 cell lines in chemically defined media. The composition of various such 'chemically defined media' for specific cell type purposes has been published in the past few years. The price of these media, however, is still so high that these media can only be used on a commercial basis for the generation of products with a very high added value (such as monoclonal antibodies or therapeutics).

Origin of components of culture media. For some products, for instance those used in human therapies, it is extremely important that the culture medium does not contain animal derivatives. In those cases it has been demonstrated that complex amino acid or protein mixtures can - instead of being harvested from animals - also be derived from plants. Most of the specific animal proteins essential for mammalian cell culture will not be naturally occurring in plants. But by using recombinant-DNA technology it has become possible to let plant cells produce such animal proteins [10]. One or several genes that

encode for animal growth factors can be introduced into plant cells. The plant cells will produce the proteins that can subsequently be isolated by fractionation. Using these techniques it is nowadays possible to efficiently produce culture media which are completely free of animal-derived products [11].

The elemental composition of all living cells, including bacteria, plants cells, and animal cells is carbon (C), hydrogen (H), oxygen (O), nitrogen (N), sulfur (S) and phosphorus (P) (in order of numerical contribution) and the minerals potassium (K) and magnesium (Mg). Other minerals are also needed but only in minute amounts, and these are sufficiently available in for instance normal tap water. The composition of living cells dictates that all culture media have to contain these elements, and the cells have to be able to extract them from the medium, preferentially in balanced proportions.

On the basis of our textbook knowledge of cellular physiology and more recently acquired knowledge of the genome, it is possible to define and compose culture media for simple cell types in which these elements are present as molecules in such a way that they can almost quantitatively be transformed into cell material. Vice versa, it is possible to analyze the efficiency of conversion of a specific compound (like glucose) into cellular material, provided that the compound is present in sufficient amounts. For the latter type of analyses a chemostat is used, while for less complex cell culture experiments batch cultures are mostly used.

The most extreme example of a simple efficient culture medium is a medium that can be used for growth of cyanobacteria. These types of bacteria can grow efficiently by only using carbon dioxide (CO_2), phosphate, nitrogen gas and rain water. By using energy from sunlight they can produce cell material (i.e. grow) from these compounds. There are also non-photosynthesizing cells that can use CO_2 as a carbon source, but that quality is relatively rare. Conversely there are various examples of chemotrophic organisms for which one single carbon-containing compound suffices to synthesize all the complex molecules necessary for the formation of new cells. Commonly these properties are specific for bacteria, although certain lower eukaryotic cells, such as yeast, can also exhibit this type of metabolism.

For the in vitro culture of cells from more complex organisms, such as mammalian cells, the composition of the culture medium is much more critical and therefore more demanding. Mammalian cells are dependent on the supply of specific molecules that are normally produced elsewhere in the body (for instance growth factors) and on compounds that are directly taken up from the food. In addition, these cells need to burn or metabolize part of their nutrients to produce energy in the form of adenosine triphosphate (ATP). Energy is required for cell maintenance but also for various synthesis processes (also termed: anabolism).

When sugars (carbohydrates) represent the main component in nutrients, two alternative metabolic routes can be exploited: aerobic catabolism and anaerobic fermentation, which leads to lactic acid production. Both processes will take place via glycolytic degradation of the sugar. The use of either the aerobic pathway or the anaerobic pathway greatly affects the energy yield per gram of sugar but much is still unknown about how these catabolic pathways are regulated (see below).

The elementary building blocks needed by mammalian cells as a carbon source can be divided into three classes: sugars, fatty acids and amino acids. Each of these classes is made up of numerous representatives. Mammalian cells can synthesize most of these compounds from one class to another, except for a group of essential amino acids that have to be taken up via the bloodstream, or, in case of in vitro culture, via the culture medium. This implicates that an almost unlimited variability in culture media is possible. Historically, the sugar glucose has been the most important source of carbon in tissue culture media. A possible disadvantage of using this sugar as carbon source is that it will

steer the catabolic process of the cells for the generation of metabolic energy towards fermentation, which is a rather inefficient process. This can be somewhat compensated by the use of an alternative monosaccharide that is easier to catabolize, for instance because the monosaccharide is less efficiently taken up by the cells. Examples of such alternative monosaccharides are galactose and fructose. Alternatively, the supply of carbon and nitrogen can be combined using specific amino acids. Specific fatty acids can also be used.

If it is not essential that the culture medium is chemically defined, it can be decided to provide most of the nutrients for cell growth via a complex mixture of 'undefined' components. Such mixtures can be derived from for instance hydrolysates of yeast cells (i.e. yeast extract). Alternatively, hydrolysates from plant cells can be used (see above). A disadvantage of these complex media is that it is much more difficult to determine beforehand whether all necessary elements are present at the correct balanced ratios. The possibility exist therefore that large amounts of non-metabolized compounds remain present in the medium after culturing of the mammalian cells as a waste.

Despite this disadvantage, it is to be expected that the use of complex mixtures of components, such as extracts from plant cells, in combination with partly purified growth factors is the most straightforward method to develop culture media that can be used for the generation of cultured meat on a large scale. The required growth factors can be synthesized by the same plant cells from which the extracts are derived. This will be extremely cost-effective, and can significantly reduce the price of culture media. As an extra advantage, this production of culture medium offers the possibility of creating a 2-step process for the generation of in vitro meat, relatively similar to the in vivo situation: plant cells grow and photosynthesize using light, and these same cells can produce the main ingredients for the culture medium used for the culture of the mammalian cells (see also box on photosynthesis).

Photosynthesis

In photosynthesis, plants and cyanobacteria use carbon dioxide, water and minerals, to produce biomass. The most efficient photosynthesizers are the photosynthetic microorganisms: cyanobacteria and (green) algae. This is due to the absence of non-productive parts in the latter organisms, like stems and roots, to their more complete surface coverage and because of their lower maintenance energy requirement. Overall this leads to an approximately 10-fold higher photosynthetic biomass yield for the microorganisms as compared to plants (10 versus 100 tonnes per hectare per year under optimal conditions).

If this photosynthetic biomass would be hydrolysed for subsequent use as a food supply for muscle cells, and one would assume a conversion yield into muscle cell biomass of 0.25 (g/g), for a production facility of 10 tonnes of meat product annually, a surface area of 80 by 80 meters of an algal mass culture would be required to provide the main medium ingredient. This calculation shows that it should be feasible to integrate the algal mass culturing facility and the muscle cell production facility into one operational unit.

Skeletal muscle from stem cells

Traditional meat market

Although meat can be referred to as edible animal tissue, with meat we commonly mean the flesh part of farm animals, in other words the skeletal muscle tissue of these animals. Skeletal muscle is composed of bundles of muscle fibers. When muscle tissue is formed, single muscle cells (myoblasts) fuse with each other and form multinucleated myotubes, which assemble to form muscle fibers. In vivo, skeletal muscle tissue specific types of stem cells (satellite cells), that reside in the existing muscle, can become activated in response to specific local factors that are generated for instance in case of trauma.

With tissue engineering it is attempted to mimic neo-organogenesis outside the animal (ex vivo). For medical purposes, tissue engineering of muscle tissue from human cells holds promise for the treatment of various diseases such as muscular dystrophy and spinal muscular atrophy. Additionally, engineered muscle tissue can be used for surgical reconstruction that may be needed after traumatic injury or tumor ablation. The scientific and technological know-how for the engineering of skeletal muscle tissue for regeneration purposes is in essence identical to the knowledge needed for the in vitro production of skeletal muscle tissue from farm animals for consumption purposes. In order to be effective the latter purpose requires much larger numbers of cells that with current technologies only can be achieved with bioreactors. These bioreactors need to be developed. In addition, a change in consumer's mind set might be needed. Nevertheless, many alternative methods are available to grow muscle cells. For example muscle-derived stem cells can be grown on the surface of micro-carriers suspended in growth medium and proliferate almost indefinitely (> 100 doublings). Such systems may also be used for the large-scale production of muscle cells, which then could be processed to a meat-related product, after differentiation of these cells into myoblasts. One could even envisage an edible nature of the micro-carriers. This approach will allow the use of much simpler bioreactors than in the approach to produce tissue cells. These simpler bioreactors will briefly be addressed below.

Stem cells. Production of tissue in vitro necessitates the use of large quantities of cells, but differentiated cells exhibit a limited proliferative capacity. In contrast, cells exist that maintain or regain the capacity to self-renew, which means that these cells continue to proliferate. Stem cells are unique in their capacity to remain in a rather undifferentiated state for a substantial amount of population doublings while retaining the ability to differentiate into at least one specific cell type [12]. Stem cells have a tremendous potential for human medicine as these cells may be used to repair damaged or diseased tissues in our body [13]. Indeed, it has been hypothesized to amplify stem cells and subsequently introduce these into patients, such as is currently performed in bone marrow transplantations. Alternatively, stem cells can be used for so-called tissue engineering techniques by which complete tissues or organs are constructed outside the body (in vitro). Non-stem cell based tissue engineering already has a diversity of applications

ranging from formation of cardiac valve substitutes, construction of cartilage or construction of a urinary bladder [14]. The list of tissues that can potentially be engineered with stem cells is even more extensive and includes blood vessels, bone, cartilage, skin, liver, cardiac muscle and skeletal muscle.

Different types of stem cells have been identified and cultured in vitro. The classification of stem cells is largely dependent on the tissues or cell population from which they were derived. Stem cells derived from pre-implantation (blastocyst stage) embryos are known as embryonic stem cells, whereas stem cells derived form postnatal tissue are generally called adult stem cells. The variety in the types of adult stem cells is obviously much larger than the variety of embryonic stem cells.

Embryonic stem cells. The fertilized egg is a totipotent (from the Latin 'totus' meaning entire) cell that, in mammals, will give rise to all the structures of the conceptus, both embryonic (the fetus) and extra-embryonic (the yolk sac and umbilical cord). This cell is not a stem cell since it cannot self-replicate without differentiation. As the embryo develops by rapid cleavage divisions, simultaneously the first differentiation process takes place by which lineages are being segregated: the trophectoderm that gives rise to extra-embryonic structures and the inner cell mass that will give rise to the developing fetus. The cells of the inner cell mass of several animal species can be taken from the embryo and grown in the laboratory. When these cells are cultured under appropriate conditions the cells will not differentiate as they would do in the conceptus but will duplicate while maintaining the capacity to differentiate into all cells of the fetus. These cells are called pluripotent (from the Latin 'plures' meaning many) embryonic stem (ES) cells. There is an understandable academic interest in embryonic stem cells. Firstly, these cells can provide information about early differentiation processes since they can recapitulate the sequence of processes that take place in embryos after implantation into the uterus which still are poorly understood. Secondly, when differentiated to specific tissue types the cells can be used for screening of drugs or toxic compounds. Thirdly, it has been proposed that, after differentiation, these cells can be used in human medicine for cell replacement. The first embryonic stem cell lines were derived from mouse blastocysts in 1981 [15] [16] but from then it took another 14 years before well-characterized embryonic stem cells were derived from another species (rhesus monkey) [17]. Human ES cells were first derived in 1998 from surplus embryos that had been generated in fertility clinics [18]. Remarkably, well-characterized embryonic stem cell lines from other animal species, including farm animals, have not been described, although many attempts have been made to generate such cell lines [19, 20]. Only recently the establishment of true embryonic stem cell lines from rat embryos has been described, but derivation of these cell lines required the addition of various inhibitors to the cells [21] [22].

Adult stem cells. Adult stem cells comprise a more heterogeneous and in certain aspects less well understood population of cells. The bodies of animals (and humans) contain different groups of cells that sustain a certain level of self-renewal and it is generally thought that these cells are necessary for regeneration and repair of tissues in which for instance the cells have a short life span or when the tissue is damaged by disease or trauma. To what extent these cells are true stem cells, i.e. capable of unlimited self-renewal, or that this would be a more transient capacity, so that they would eventually become a differentiated cell type, is in many cases not completely clear. Similar to embryonic stem cells it has been hypothesized that pluripotent adult stem cells do not exist in vivo but arise during in vitro culture [23].

Independent of their possible in vivo occurrence, adult stem cells or progenitor cells can be excellent sources for the generation of cultured meat. In contrast to embryonic stem cells, adult progenitor cells have been derived from farm animal species such as pig [24] [25] [26] and cattle [27, 28]; these cells have, at least to a certain extent, the capacity to differentiate into skeletal muscle cells. A disadvantage of adult stem cells could be their limited differentiation potential, meaning that these cells can only differentiate into a

limited number of cell types. This is a serious drawback if stem cells are to be used for biomedical purposes, but for the generation of cultured meat this would hardly be a disadvantage since cells do not need to differentiate to other cells than myoblasts. For cultured meat, it is however important that the cells have a minimal self-renewal capacity since most adult stem cell types cannot be cultured in vitro indefinitely.

iPS cells. Differentiated cells can also be reprogrammed into an embryonic-like state by introducing four (or less) genetic factors [29]. These cells, called induced pluripotent stem (iPS) cells, behave exactly like embryonic stem cells in that they self-renew with conservation of their truly pluripotent character if cultured under the right conditions. With this technique both mouse and human cells have been reprogrammed [30, 31]. This new technology has sparked the attention of many biomedical researchers, most importantly because it opens up a possibility of creating human, even patient-derived, pluripotent cells without the ethical difficulties that accompany pluripotent cells derived from human embryos.

For the production of cultured meat, bovine or porcine iPS cells could be useful cells, as these cells can also differentiate into muscle tissue. There are, however, some difficulties in this approach. For the first iPS cells that were derived, the DNA that codes for four transcription factors was delivered to the cells by retroviral infection. As a result, the viral DNA integrated into the genome of the targeted cells at (multiple) random locations may lead to uncontrolled behavior of the cells, and makes them non-suitable for large scale production of food. For the production of an edible product, cells that have been infected with a retrovirus also cannot be used, because of potential safety hazards. Very recently however, human iPS cells have been generated that were made free of vector and transgene sequences and it is anticipated that this technology will advance rapidly[32].

The iPS technology proceeds rapidly and many labs are working on these cells, and indeed the first iPS cells from farm animal species have recently been described. In 2009 two articles were published that describe the generation of porcine iPS cells [33 34]. Although the use of these cells for the generation of an edible product is debatable, it is clear that these results are very promising.

Bioengineering and bioreactors for tissue cultures

Adult skeletal muscle tissue is characterized by elongated, multinucleated cells with a highly organized network of cytoskeletal proteins. Skeletal muscle cells can reach considerable lengths and adult cells have a large myonuclear domain. Culturing skeletal

muscle cells in vitro, however, results in cells with a relatively high nuclear density when compared to the in vivo situation. Also, the characteristic highly organized architecture of the cytoskeleton often is lacking under in vitro circumstances. This indicates that the cells are immature, which makes them at that moment an inefficient protein source. Such immature myotubes are referred to as primary myotubes. After fusion of the myoblasts, primary myotubes need a secondary differentiation step in order to mature. Only then, they will produce sufficient cell mass to be an efficient protein source.

It would be ideal to refrain from the addition of growth stimulating factors such as hormones in the production of cultured meat although the use of growth factors (produced in plants or lactic acid bacteria) may increase the efficiency of myotube formation (see below).

In order to produce sufficient biomass in vitro, the primary myotubes need to be directed towards secondary differentiation. The approach may be threefold:

Co-culturing. Commonly, cell cultures are expanded and differentiated in monoculture, which creates a well-controlled environment without the interaction of different cell types. The in vivo situation is distinctly different from this. In skeletal muscle, nerve cells, cells forming blood vessels and fibroblasts that form the basal membrane are ubiquitously present. It has been shown that in vitro, the presence of fibroblasts improves the efficiency of myotube formation. Accruing evidence suggests that the basal membrane is paramount in directing regeneration and controlled growth in many tissues, including skeletal muscle. It regulates the activity of locally active growth factors such as fibroblast growth factor (FGF), transforming growth factor beta (TFG-β) and hepatocyte growth factor (HGF). Since the presence of extracellular matrix (ECM) gives meat its texture and 'bite', co-culturing primary myotubes with fibroblasts may be a good way to improve the maturation and to produce a meat-like texture at the same time. Co-culturing has been shown to be very effective in the in vitro engineering of skeletal muscle when the highly proliferative fibroblasts are seeded as a low percentage of the total cell population.

Mechanical stimulation. In the body, regular activity of the skeletal muscle is a potent stimulus for increase of muscle mass. Upon contraction, myotobes produce the IGF-1 splice variant mechano growth factor (MGF) which increases protein synthesis in the myotubes. Furthermore, internal forces that occur inside the myotubes during contraction are potent regulators of the cytoskeletal arrangement. In culture, it takes some time before myotubes have matured enough to contract in such a way that they exert significant intracellular forces. Stretch can be applied to the culture to yield a similar effect on the immature myotubes. This procedure has the added advantage that the myotubes will align in the direction of the applied stretch, which will make electrical stimulation more effective, and it does stimulate the production of ECM proteins by the fibroblasts in the culture.

Electrical stimulation. In vivo, nerve activity controls muscle contraction. Contraction of developing myotubes upon electrical stimulation will increase the maturation of their cytoskeletal structure. Moreover, contraction increases the total amount of the contractile proteins actin and myosin. Since the contractile apparatus makes up by far the largest part of the mature myotube, increased expression of those proteins is necessary to achieve the myonuclear domain similar to that in the in vivo situation. If the electric stimulus of the culture, and thus its contraction, is correctly tuned with the activity of the stretch device, it will be possible to submit the cultured myotubes and surrounding ECM to eccentric contractions. In vivo, such contractions have been shown to be a potent stimulus for increase in muscle mass.

Scale-up of muscle culture bioreactors. If, in an experimental small-scale bioreactor, the optimal myotube maturation protocol is established, the production of engineered meat can be scaled up in specifically designed bioreactors. It is possible to culture cells

on flexible membranes. Mounting the culture over a post and applying a slight vacuum under that post will suck down the membrane and stretch the cells in culture. The amount of stretch must be tightly regulated as too much stress will damage the cells and lead to cell death.

Electric stimulation can be applied by putting two electrodes into the culture medium, in close proximity to the cells. Preferentially, such electrodes are of an inert material and span a significant area. Carbon plate electrodes would suit these requirements. Polarity should be changed during stimulation to avoid hydrolysis of the medium and separation of positively and negatively charged ions. Stimulation over larger areas should be avoided since it requires high voltages, which will increase the temperature of the culture with detrimental effects to the cells, and an increased evaporation that will affect the osmolarity of the medium.

Whereas the proliferation of stem cells may ultimately be possible in suspension cultures in large fermentors, the production by mechanostimulation of mature secondary myotubes, that contain high amounts of the nutritious proteins actin and myosin, may always require a solid support. Since diffusion of gases, nutrients and metabolic products is limited, the muscle tissue only grows in two dimensions. This necessitates the harvest of the product as large sheets and subsequent processing into consumer products. Alternatively, deformable micro-carrier beads of edible (non-animal) material may be developed that enable production of secondary myotubes in suspension. Myotubes may be used as an animal protein ingredient in a wide variety of products. Alternatively, products with a meat-like appearance and texture can be made by using, for example, cell printing techniques. Addition of fibroblasts (for firmness) and fat cells (for taste) to the myotubes might result in meat completely of animal origin.

Bioreactors for suspension cultures. The possibility to grow cells in suspension simplifies their proliferation considerably. The most simple and straightforward way is to do this in a batch culture, well-stirred to facilitate oxygen transfer into the growth medium. During the past few years a large variety of simple, often disposable, stirred-tank type of reactors have been described, often for use at an industrial scale. Proper reactor design can add a lot to the rate of oxygen transfer. For detailed studies of physiology it is most ideal to grow cells in a chemostat: By continuous supply of fresh medium and removal of cells plus medium, a constant, time-independent, environment can be created which gives optimal opportunity to characterize metabolic processes. This system also allows continuous supply of cells. Nevertheless, chemostats are challenging devices for use of slow-growing mammalian cells. Generally, batch cultures and chemostats do not result in very high cell densities. This parameter can be further increased by running so-called fed-batch cultures, in which – after an initial phase of exponential growth – the limiting nutrient is added in a steady supply. Accordingly, cell densities of e.g. CHO-cells used for recombinant antibody production can increase up to 100 grams of cells per liter [35].

As explained above, the degree to which metabolism of the mammalian cells proceeds in the presence of saturating amounts of oxygen determines the overall efficiency of growth of the cells. For this reason it is important to be able to measure oxygen concentrations in situ. The development of fluorescence lifetime probes has recently simplified these measurements considerably, to the extent that this is a very straightforward measurement in most large-scale culturing facilities.

With the use of bioreactors for suspension cells many scale-up problems in the production of muscle-derived cells can be solved by the lessons learned in the single cell protein producing industries (in for instance the BioProtein process operated by Norferm). Therefore, is will be possible to operate reactors with a productivity of > kg scale.

Life cycle assessment of cultured meat production (cradle to cradle)

Livestock production in total currently occupies about 30 % of the ice-free terrestrial surface of our planet and amounts to 18 % of the global warming effect (FAO, 2006). The consumption of meat has been predicted to double between 1999 and 2050, which will further increase its negative impact on the environment. Cultured meat will be produced in a reactor by growing only muscle cells/tissues, instead of growing the whole animals. Its development has started mainly from attempts of producing space food for astronauts, but it could potentially offer many environmental, heath, and animal welfare benefits in the future. To assess the overall environmental impact of cultured meat production, it would be advisable to carry out a formal Life Cycle Assessment (LCA), to estimate the energy-, water-, and land-use and the greenhouse gas (GHG) emissions. Here we present some preliminary results that are based on an LCA study by Hanna Tuomisto (University of Oxford, UK) and Joost Teixiera de Mattos (University of Amsterdam) that is still in progress.

The basic production unit considered is one ton (i.e. 1000 kg, of which 30% is dry matter en 20 % protein) of cultured meat. With due scientific development this cultured meat product should be producible from hydrolyzed algal biomass (for sugars and amino acids) and recombinant growth factors produced via lactic acid bacteria. The most relevant input factors are the production of the input materials and fuels, and production of the feedstock (presumably ~ 1.5 ton biomass with 50 % (w/w) protein), and the fermentation of muscle cells. The cost of nutrients (mainly: K^+, Na^+ and inorganic phosphate are negligible, because of the low amounts required (~ 1 kg potassium and 0.1 kg phosphate).
Taking proper literature data one can estimate that 500 m^2 algal mass culture will be required (for cost estimate: see (Chisti, 2008)). The resulting extract will be fed into a 1000 l stainless steel fermenter, which will have to run for two months (for costs: see Akiyama *et al.*, 2003). It was estimated that the average energy use is 45-60% lower; greenhouse gas emissions are 80-95% lower; land use is 98% lower and water use is 90-98% lower. Only poultry production has a lower energy use compared to cultured meat.

References

Akiyama, M., Tsuge, T., Doi, Y., 2003. Environmental life cycle comparison of polyhydroxyalkanoates produced from renewable carbon resources by bacterial fermentation. Polymer Degradation and Stability 80, 183-194.

Chisti, Y., 2008. Response to Reijnders: Do biofuels from microalgae beat biofuels from terrestrial plants? Trends in Biotechnology 26, 351-352.

FAO, 2006. Livestock's long shadow –environmental issues and options. Food and Agricultural Organization of the United Nations, Rome, p. 390.

Experts' opinions (3): Technological challenges

- I assume that the technology would involve standard mammalian cell culture techniques (anchorage-dependent cells on microcarriers cultured to high density in large-scale bioreactors). This can be challenging. In addition to the standard engineering challenges (sufficient mass transfer, mixing, etc. while minimizing shear profiles) the costs of goods will need to be extremely low to be competitive.
- There are many challenges for the production of in vitro meat, but I would say the most important is the achievement of adult phenotype muscle in vitro, which has never been reported in the scientific literature. This is important because it is adult phenotype muscle that has the protein density, texture, and other important features that make meat an important dietary component. Failing this, what you will have will be more like loose connective tissue, not "meat" in any real sense of the word.
- There are quite a number of challenges that have to be overcome: (a) Generation of stem cell lines from farm animal species; b) Proliferation of these stem cells without differentiation; c) Efficient differentiation into muscle cells; d) Large-scale production of myofibers; e) Large-scale production of cheap growth media.
- Here are some hurdles to be overcome. What are the best (stem) cells? What is the best design of the bioreactor? And not least in the early phase (if the bioreactor can produce just cells on some surfaces etc): What is the best way to process the product? In vitro meat involves some basic biology but there are no fundamentally basic problems.
- The technological hurdles that need to be overcome are: (a) efficient differentiation of the cells; (b) engineering of tissue that is larger than only a few cell layers; (c) processing of the cells to something tasty and visually attractive
- To make a product with the required structure that is safe.
- I would suggest research is done to move the muscle cells into a suspension type culture; this would greatly simplify the manufacturing process and eliminate potentially harmful agents. Consideration should be made as to the culture vessels, capacity, disposable or permanent stainless steel.
- Large-scale production, reduction of costs and producing the right texture (bite) are important hurdles.
- The most critical steps are to be able to produce a serum free cell culture medium at a low enough price, to be able to produce 3d muscle tissue either from stem cells, satellite cells or mesenchymal cells having a phenotype very close to normal tissue, and then to be able to scale up this process to an industrial level.
- An important challenge is to increase the mass of the tissue from a few cell layers with natural diffusion to many cell layers that still are able to take up nutrients efficiently. Another hurdle is consumer acceptance. The consumer may not like 'high-tech' food.
- In vitro meat may be made (a) through suspension culture of single cells or small cells clumps that are not differentiated or (b) by tissue culture of differentiated cells. Single cell or cell clump culture is an established technology that is used for the production of BioPharmaceuticals with (very) high added value, such as monoclonal antibodies. The major challenges for meat production based on singe cell technology are in my opinion: (a) The very high cost of goods of the current processes (must be performed under sterile conditions to prevent microbial contaminations, use of expensive culture media, etc.) (b) The lack of resemblance to meat (texture, appearance, etc.). Tissue culture for the generation of muscle tissue, potentially including blood vessels etc., may result in something more resembling meat. The costs however may be even higher and the technology and especially the scale-up still need significant further development.

Ethical and societal issues

The concept of cultured meat appeals to many people. Since the pioneering work of Benjaminson and Mironov, and particularly since the start of the Dutch Vitro Meat Consortium in 2005, many articles appeared in newspapers and magazines about cultured meat. These articles, and also programs on radio and television, helped to start a discussion about a future technology that is not even in its infancy. It may seem somewhat premature to start a societal discussion at such an early stage. However, food is a subject that evokes many emotions: it is, if we recall the turmoil associated with the introduction of genetically modified foods, a good idea to educate citizens about all aspects of producing animal proteins for human consumption by cultured cells.

Does food in general evoke strong feelings; meat is a commodity that sometimes is even controversial. Indeed, it can be stated that controversy exists in the minds of people: the different opinions about meat between citizens and consumers are striking. One may argue that the disconnection between animals and meat that exists in consumer's minds would make it more difficult to lure people and convince them to eat cultured meat. On the other hand, if a product is available that has all the characteristics of meat (like good sensory properties), but is not produced from animals, citizens would probably buy it for a variety of reasons. These could range from worries about the environment or animal welfare to the fear of zoonotic diseases.

Unfortunately, no scientific studies have been conducted yet on society acceptance of cultured meat production. It must be said, however, that the authors experienced a positive attitude by the audience in national and international debates. Also web-surveys and internet-discussions indicated that many people are in favor of the production of 'victimless' meat (see box). Other people, however, have expressed feelings of disgust because they consider cultured meat as another step away from 'natural' food production. In addition, the cultural background probably also plays an important role. Interesting in this respect is that among the many requests for interviews none came from journalists of Mediterranean countries. In contrast, dozens of requests came from the Nordic countries (and many more from English- and German-speaking countries). Social-cultural aspects of acceptance of cultured meat are probably important and should be subjects of investigation. In addition, it will be interesting to compare the views of people on current intensive farming with organic farming and the production of animal proteins by cell systems. Replacement of (part of) the conventional meat production by cultured meat would entail social, technological and economic changes. However, it is to be expected that these transitions occur relatively slowly and farmers do not need to be worried.

Best of both worlds

In their paper 'Vegetarian Meat: Could Technology save Animals and Satisfy Meat Eaters' Hopkins and Dacey argue that cultured meat would be the best of both worlds: eat meat and not harm animals. They specifically analyze the most important and most morally complex objections to cultured meat and conclude:

"Cultured meat has the potential to make eating animals unnecessary, even while satisfying all the nutritional and hedonic requirements of meat eaters. It also has the potential to greatly reduce animal suffering. As such, the development of cultured meat would seem to have a moral claim on us – whether moral vegetarians (for whom a greater opportunity exists to reduce animal suffering) or conflicted meat eaters (for whom practice could now cohere with beliefs) or even for recreational hunters (for whom ancillary arguments about providing food would fall by the wayside and require defenses of getting pleasure from animal death per se). The development of cultured meat, then, is not merely an interesting technological phenomenon, but something that we may be morally required to support. In doing so, we recognize that morality is not something that must simply respond to new technologies as they arrive, throwing us into confusion, but rather that morality may champion and assist in the development of new technologies, as a step toward the production of a world that in fact, and not merely in ideal, mirrors the moral vision we possess for it."

Holmes, P.D. and Dacey, A., 2008. J. Agric. Environ. Ethics 21, 579-596.

Challenges

Cultured meat technology is still in its infancy. Important challenges for the production of animal proteins by cell systems are:

1) Generation of suitable stem cell lines from farm animal species

2) Safe media for culturing of stem cells

3) Safe differentiation media to produce muscle cells

4) Tissue engineering of muscle fibers

5) Industrial bioreactors

6) Food processing technology

7) Customer preferences and adapted marketing strategies

1. Generation of suitable stem cell lines from farm animal species. For the generation of cultured meat it is important that cells from farm animal species (like pig, cattle or chicken) can be cultured in large quantities starting from a relatively small number. Stem cells would fit this criterion. Embryonic stem cells have been cultured for many generations. Since under normal conditions each cell will duplicate roughly every day, if one would start with 10 cells within 2 months these 10 cells would have proliferated to 1.2 E19 cells. If we assume that 0.5 gram of meat is approximately equivalent to 116 million cells, this would amount to about 50,000,000 kg of meat (assuming that these cells have also differentiated to skeletal muscle cells).

Culturing embryonic stem cells would be ideal for this purpose since these cells have an (almost) infinite self-renewal capacity. In theory, one such cell line would be sufficient to literally feed the world. Despite attempts from many research groups including our own [36] it has so far not been possible to culture cell lines with unlimited self-renewal potential

from pre-implantation embryos of farm animal species. Many attempts have been made, however, to generate permanent cell lines from embryos of animals including hamster [37], mink [38], rabbit [39], pig [40], cattle [41], sheep [42]and goat [43]. Until now, true embryonic stem cell lines have only been generated from mouse, rhesus monkey, human and rat embryos. It is to be anticipated that the social resistance to cultured meat obtained from mouse, rat or rhesus monkey will be considerable and will not result in a marketable product. The reasons why it has not been possible to culture embryonic cells from farm animal species without differentiation of these cells, and thereby a loss of proliferative capacity, is unknown. Apparently, the culture conditions as have been derived to keep mouse and human embryonic cells undifferentiated are different from the conditions that will be required for embryonic cells of farm animal species. Most likely, fundamental research on the early development of embryos of these species can shed light on this issue and thereby provide clues on how to maintain cells undifferentiated.

Another strategy would involve the use of adult stem cells from farm animal species. Adult stem cells have been derived from, for instance, pig and cattle. A disadvantage of these cells is that their in vitro proliferation capacity is not unlimited. However, it has been demonstrated that these cell can proliferate in vitro for several months, and that these cells also have the capacity to differentiate into skeletal muscle cells, albeit not very efficiently. Nevertheless, for now, adult stem cells are the most promising cell type for use in the production of cultured meat.

2. Safe media for culturing of stem cells. Mammalian adult and embryonic stem cells are routinely cultured in a rich broth that contains salts, sugars, amino acids and other supplements. For proper culturing, serum from fetal calves (aptly called fetal calf serum) is added to the medium. Exactly which factors in the serum are important for proper cell growth is unknown, but it is well-accepted that some batches of serum are better suited for the culture of stem cells than others. Therefore, laboratories usually test different batches of fetal calf serum for their suitability for stem cell culture. For the generation of an animal-free protein product, the addition of fetal calf serum to the cells would not be an option and it is therefore essential to develop a serum-free culture medium. Indeed such media have already been generated and are available from various companies for biomedical purposes; however, their price is incompatible with the generation of an affordable edible product. A cell culture medium therefore has to be developed that enables culturing of cells for an affordable price but that does not contain products of animal origin.

Mouse and human embryonic stem cells are cultured on a feeder layer of embryonic fibroblasts for the maintenance of stem cell characteristics, but recently culture media and methods have been developed that do not require culturing on layers of feeder cells. The medium that will be developed for the culturing of farm animal stem cells should also be compatible with feeder free culture. Adult stem cells are less dependent on such a layer of feeder cells for their proliferation.

3. Safe differentiation media to produce muscle cells. For stem cell culturing it is important that these cells remain undifferentiated and maintain their capacity to proliferate. For the production of cultured meat these cells have to be differentiated to skeletal muscle cells. This differentiation process needs to be specific and efficient: Specific in the sense that no other cell types must be formed and efficient in the sense that the majority of the cells will differentiate into muscle cells. Differentiation of the cells will have to be initiated with a specific (set of) growth factor(s). Currently, the most efficient method to let (mouse) stem cells differentiate into skeletal muscle cells is to culture them in a medium that contains 2% horse serum instead of 10 or 20% fetal calf serum. For the generation of cultured meat, however, it is essential that the cells are cultured and differentiated without animal products, so without horse serum. A chemically defined culture medium therefore has to be developed that (efficiently) enables the differentiation of stem cells to skeletal muscle cells.

4. Tissue engineering of muscle fibers. In the absence of blood flow that provides oxygen and nutrients to the cells and removes metabolic end products, the possibility to form a 3-dimensional structure of cells is restricted. The in vitro culturing of cells is limited to only a few layers of cells, which would represent tissue of a thickness of less than a millimeter at maximum because limitations in nutrient diffusion. This problem also has to be addressed by those who pursue tissue engineering for biomedical purposes. Culturing of cells on biological or synthetic scaffolds may provide a solution to this problem. In this way the scaffold would provide shape and structure to the engineered tissue. In the case of cultured meat, the scaffold should be either edible or biodegradable. A more straightforward solution would be the processing of thin layers of cells into a (meat-like) product.

5. Industrial bioreactors. Production of sufficient numbers of muscle cells for the generation of edible products will require large-scale culturing. Since stem cells and skeletal muscle cells require a solid surface for culturing (in contrast to, for instance, blood cells that can be cultured in suspension) a large surface area is needed. Culturing should be performed in large bioreactors containing many sheets of printed cells, cells grown on scaffolds, or cells cultured on microspheres that can be kept in suspension.

In a model for mammalian muscle cells in a 3-D matrix, cells are supported and supplied within the bioreactors such that the natural tissue builds 'self-organizing constructs', where the 3D self-organization of tissues allows the provision of the nutrient supply, aeration, waste removal etc. (one of the models from the in vitro meat economics study; see supplementary materials). This subsequently allows cell, and consequently, tissue growth. It was concluded from this study that several areas require further development: (1) the mechanism for cell support and growth within the bioreactor; (2) the mechanism for harvesting; (3) the need for pharmaceutical grade cleanliness and ability to sterilize; (4) instrumentation and process control.

6. Food processing technology. Depending on the starting material (suspensions of small myotubes, myofibers on scaffolds, microspheres, etc.) new technologies need to be developed to make attractive products. It is expected that at first small pieces of cultured muscle fiber will be produced that serve as raw materials for making a wide variety of products ('cultured meat inside').

7. Consumer preferences and adapted marketing strategies. Why would a consumer prefer cultured meat if meat from animals is available? If it is because of sustainability or animal welfare issues, why not eat less meat and instead, more plant proteins? Many questions can be asked and many factors determine consumer preferences. Studies are required to determine the preferences and, consequently, the marketing strategies. Interesting in this respect is the summary from a workshop on cultured meat held on December 3rd, 2008 as part of an NWO application (see boxes). This workshop was organized by scientists from Wageningen University (Dr. Cor vd Weele and Dr. Hilde Tobi) and Utrecht University (Prof. dr. Henk Haagsman and Dr. Bernard Roelen).

Participants of the valorisation workshop

Name	Affiliation	Field of expertise
Ing. C.J.G. Wever	Ministry Agriculture, Nature & Food Quality (LNV)	Food Quality
Dr. J.C. Dagevos	LEI (WageningenUR)	Sociology of consumption
Dr. B.J. van 't Hooft,	Stegeman	Meat industry
Drs. Ing. J.A.C. Peters	Voedingscentrum	Food quality & safety
Ir. C. van Dooren	Voedingscentrum	Food quality & sustainability
Mr. H.P. Voormolen	Albron	Catering & sustainability
V. Helder	Vegetariërsbond	Vegetarian concerns
Ir. C.P.G. Driessen	Applied Pilosophy Group (WageningenUR)	Technology & animal ethics
K. Gruijters	Katja Gruijters Food Design	Food design

The workshop was moderated by Dr. Nicolien Wieringa of Groningen University. Several important conclusions could be drawn from this workshop, in particular with respect to consumer acceptance.
First of all it was acknowledged that various different consumer groups can be identified in relation to cultured meat:

- Those that would readily eat cultured meat.
- Those that would accept cultured meat based on religious or fundamental principles (for instance vegetarians).
- Those that do not immediately reject cultured meat, but will only accept when the sustainability is demonstrated.
- Those that reject cultured meat.

During the workshop, three main areas of valorisation were recognised:
1) Sustainability
2) Societal embedding: reception and demand
3) Commercial production

Ad 1) Sustainability.
It was agreed between participants of the workshop that gains of sustainability are of central importance for the success of a cultured meat product, and a basic requirement for success. These gains in sustainability need to be calculated and indeed properly demonstrated for credibility. It was also recognized that the sustainability of cultured meat is not static but will be a topic of continuous development and improvement, most likely in combination with other activities/discoveries.

Ad 2) Societal embedding: reception and demand.
On the one hand cultured meat is an alternative for 'traditional' meat from animals. For many people meat is an important aspect of their daily food, because of the taste and nutritional value and it is therefore important that an alternative should have a similar taste and nutritional value. On the other hand it is important for a product to have its own image and can be considered as something totally new and different. From this perspective, it is not essential for the product to resemble and should in fact be clearly distinctive from traditional meat.
From a perspective of social acceptance, the technological character of cultured meat can have a negative value, and associations with Frankenstein, cloning, transgenesis and unknown risks are close at hand. The name of the product can be of importance in this aspect. In vitro meat and cultured meat are likely not to be names of consumer products that will appeal to the average consumer. Marketing strategies designed by experts can become very important in this respect. Transparency, that is a clear picture of the whole production process could help in gaining public acceptance, whereby one can imagine production in open agro-parks where in vitro meat cultures are being 'fed' with the

products of cultured algae. Technology would thus not increase but rather decrease the distance between producers and consumers. It has to be mentioned that in the western society, the distance between producers and consumers is already quite big, as one thinks about (commercially successful) consumer products such as beer, soda drinks, different types of candy, instant meals. This distance is therefore not necessarily negative for commercial success, and could in fact also be beneficial (consumers simply do not want to know). Proper consumer feedback and validation studies are therefore essential for the introduction of a product like cultured meat.

Ad 3) Commercial production.
A requirement for commercial production is societal demand. Industry is clearly not interested in a product that will not be profitable. On the other hand, there is a serious commercial interest provided that technology has advanced more. In comparison with animals, a product from a bioreactor could be attractive as it does not come with all the vicissitudes of animals such as uneven growth, disease, consequences of stress, animal killing etc.

During the workshop, marketing strategies for cultured meat were also discussed. Because of the technology involved, it as suggested that the focus should be more on the characteristics of the product rather than on the production process. Independent of this, it was generally agreed that a broad acceptance is necessary for the product to become commercially successful. The product should not only appeal to vegetarians or an elite group of consumers. It was suggested that well known media cooks might be called in for marketing help. Also, availability and price of traditional meat can be important decision factors. The question arose whether a demand for cultured meat will have to wait for a serious crisis in the availability of traditional meat. Finally, it is likely that first products will not be end products but ingredients, which will most likely be less offensive for many consumers.

Traditional product

Conclusions from the valorisation workshop (1)

Which factors determine success or failure of artificial meat?

Sustainability: Participants overwhelmingly emphasized sustainability as the central success factor. They also stressed the need to quantify the gain in sustainability of cultured meat in comparison with normal meat.

Name: The name of the product (in vitro meat; kweekvlees) was seen as a big risk factor. For some, this had to do with Frankenstein-like associations, while others thought cultured meat should not be presented as meat (see below).

Meat or no-meat? On the one hand, cultured meat is explicitly introduced as an alternative to the problems of normal meat. Because people like meat, cultured meat should be as meat-like as possible in order to be a real alternative. On the other hand: a new product needs a profile of its own, otherwise it will not be able to compete. Some participants thought it should therefore not be meat-like at all.

Process or product? On the one hand, food production as a process should be transparent, and this is especially true for technologically produced food: "the mistakes of the GM debate should not be repeated". In order to expose big strengths of the product, such as sustainability, transparency is also needed. On the other hand: embryonic stem cells, bioreactors and high tech production trigger associations with cloning, Frankenstein etc. (Cultured meat therefore amounts to a "worst case challenge for a marketing campaign", as one participant of the valorization workshop put it). Would it therefore be better to focus on the characteristics of the product rather than the production process?

Place-independence. Cultured meat is not bound to soil or place, which opens up possibilities for new places of production and for alternative land use. Given the fact that 80 % of agricultural soil is directly or indirectly used for husbandry, this is not a trivial perspective.

Synergy. Cultured meat could be combined with other sustainable innovations. Algae cultures, for example, might provide meat cultures with nutrients and energy.

Reliability. From a commercial perspective, animals are notoriously unreliable as a raw material, due to illness, stress and uneven growth. Cultured meat is potentially a much more reliable alternative.

Conclusions from the valorisation workshop (2)

What would an introduction campaign focus on?

Broad acceptance needed. In order to make a sustainability-difference, cultured meat should not just appeal to vegetarians or other small elites; broad acceptance is needed. Wellknown media cooks (etc.) might be called upon for marketing help.

Demand: crisis needed? At the moment, protein is not in short supply in the Western world. In countries where protein supply is a problem, consumers might insist on "real" meat as soon as increasing wealth allows this. The question arises whether substantial demand for cultured meat will have to wait for a serious crisis in the availability of protein.

What kind of product? Participants thought it most likely that the (first) products will not be end products, but ingredients. It is not easy to think of a campaign for a "mere" ingredient. Completely new products gave more inspiration, even though their production may not be realistic, at least in the short term.

Current developments in cultured meat research

The Dutch 'In vitro meat' project funded by SenterNovem. After having obtained a patent an international patent on the 'industrial production of meat from *in vitro* cell cultures' in 1999 (international application number PCT/NL98/00721), Willem van Eelen sought collaboration with academia and industry to obtain funding in order to realize his dream. In 2004, Utrecht University (Faculty of Veterinary Medicine, prof. dr. Henk Haagsman and dr. Bernard Roelen), Eindhoven University of Technology (Department of Biomedical Engineering, dr. Carlijn Bouten), University of Amsterdam (Swammerdam Institute for Life Sciences, prof. dr. Klaas Hellingwerf), Meester Stegeman BV (at that time part of Sara Lee foods, Peter Verstrate) and Vitro Meat BV (Willem van Eelen) submitted a grant proposal to SenterNovem that was honored, resulting in the start of the Dutch In Vitro Meat project in May 2005 that lasted until April 2009.
The project was subdivided into 3 different areas: a) stem cell biology, conducted at Utrecht University; b) tissue engineering, conducted at Eindhoven Technical University; and c) culture media, conducted at the University of Amsterdam.
To conduct this research, at Utrecht University 2 PhD students were appointed for a period of 4 years, and 1 post-doctoral fellow for a period of 2 years. Also at Eindhoven Technical University 2 PhD students were appointed for 4 years, and 1 post-doctoral fellow for a 2 year period. At the University of Amsterdam, 1 technician was appointed on this project for a period of 4 years.

Experts' opinions (4): Presumed qualities of cultured meat

- There is no "objective" reason why in vitro meat should not have the same properties as conventional meat. Safety and sustainability are two major issues that favor cultured meat.
- Production may be less safe because of risks of contamination.
- Cultured meat will be safer and more sustainable than conventional meat.
- Cultured meat may have a completely different risk profile than conventional meat. Much attention should be paid to the safety of added substrates and other compounds of the culture medium. So, less risks with respect to microbial contamination but more risk of contamination of substrates.

Stem cell biology. To be able to generate sufficient skeletal muscle cells, these cells have to be derived from stem cells. Stem cells have the capacity to self-renew, i.e. the capacity to replicate without losing their characteristics. Also, for the generation of cultured meat it is essential that the cultured stem cells can specialize into skeletal muscle cells. Before the start of this project, no well-characterized stem cell lines of farm animal species were available that fulfilled these criteria. At Utrecht University the focus has therefore been on the generation of stem cell lines of pig origin, both of embryonic and of adult origin. For the stem cells of embryonic origin, embryos of blastocyst stages and inner cell masses of these embryos have been cultured at a variety of culture conditions. Both in vitro produced and in vivo produced embryos have been used, but the in vivo produced embryos gave far better results than the in vitro embryos, which is an important finding. The main difficulty of propagation of these cells was to keep the cells undifferentiated. Indeed the cells could be cultured for few passages, after which they had a tendency to spontaneously differentiate, primarily to a neural lineage.

Cells are cultured in special incubators

Cells have also been collected from skeletal muscle of newborn piglets. It is to be expected that skeletal muscle tissue contains a small fraction of stem cells, and these cells can potentially be used for propagation in vitro and differentiation to skeletal muscle cells. Indeed, cells were sorted from the population of skeletal muscle cells from newborn piglets, and these cells have been cultured in vitro for several months, while maintaining the capacity to specialize to skeletal muscle tissue, and can therefore be referred to as adult stem cells (despite the fact that the original cells were isolated form newborn piglets they are still, confusingly, referred to as *adult* stem cells). The differentiation procedures that are used until now are not very effective and other cells or differentiation conditions need to be developed for the generation of large quantities of skeletal muscle cells.

Tissue engineering. The differentiation of naïve cells to skeletal muscle cells is rather complicated and is dependent on many factors. By creating conditions that resemble the environment, or niche, that stem cells undergo in vivo when stimulated to differentiate to muscle cells, the group at Eindhoven Technical University has investigated which factors are important for efficient differentiation. In particular, the influence of substrate elasticity and protein coating has been investigated. A difficulty in these studies has been lack of properly characterized stem cells of farm animal origin. For this reason, stem cells of mouse origin were used. It is anticipated that results from these studies can be directly used for studies with porcine cells.
When stem cells are freshly isolated from muscle tissue, their differentiation capacity has been reported to be limited. One of the reasons for this limitation could be a lack of pivotal niche factors, for instance substrate elasticity and extracellular matrix proteins.

The proliferative and differentiative response of primary (ie freshly isolated) mouse muscle cells was investigated to different substrate stiffness and protein coatings in vitro. It was discovered that the capacity to divide was primarily influenced by substrate elasticity, with a substrate elasticity of 21 kPa, similar to the physiological elasticity of skeletal muscle, being optimal. Differentiation capacity on the other hand was less dependent on surface elasticity, but very much dependent on the type of surface protein, with laminin and poly-D-lysine being the best stimuli for differentiation of myotubes.
In addition to these findings, researchers at Eindhoven Technical University also studied the final maturation of skeletal muscle cells by electrical and mechanical stimulation. It has been identified that, using the mouse C2C12 cell line as experimental model cells, electrical stimulation leads to further maturation of differentiating myotubes.
Currently experiments are ongoing that analyze the influence of physical loads on cellular differentiation. For this a computer-regulated bioreactor is used that uses vacuum pressure to apply strains to cells (cyclic or static) that are cultured on flexible-bottom culture plates. This can provide information on the behavior of muscle cells in a mechanically-active 3 dimensional culture environment.

Culture media. At Amsterdam University studies have been conducted to optimize culture media for in vitro cultured meat. Importantly, culture media to produce animal-friendly meat should not contain animal derived factors such as fetal bovine serum, but should contain enough nutrient including sugars and amino acids. Photosynthetic algae can use energy from sunlight to produce nutrients for stem and muscle cell cultures. Progress has so far mostly been on theoretical and small-scale experiments, and a suitable culture medium is not yet available. A start was made with the production of required growth factors by bacteria.

Experts' opinions (5): Organization of research on cultured meat

- First and foremost, I would start with some focus groups to see what kind of reception this conceptual product would receive in the market place. If there is a strong market, then compile a team of large scale mammalian cell culture professionals.
- An ideal approach is one that attracts significantly more funding to accelerate research. It is important to think strategically about which research milestones will attract further funding. I believe the most important of these milestones is the production and human consumption of a small amount of ground meat, in a form that resembles an existing product. Most of the public and potential funders will remain skeptical about the technology until this milestone is achieved. Even if it is a "$10,000 chicken nugget," the case can be made that the costs will decrease with improvements in the technology, but it must be shown that a product is possible.
- Other analytic work can help support the case for cultured meat, including economic feasibility analysis, environmental life cycle analysis, regulatory analysis, and consumer surveys. There is opportunity for international collaboration, as there are many different proficiencies required for success. Partners in East Asia should be especially valuable, as this technology could have the greatest impact in that region. Other important things moving forward are transparency about the research, in order to build trust with public and potential funders; and publication of results in peer-reviewed literature, in order to build trust with scientists.
- I believe there should be a large effort to systematically investigate various animal stem cell types, nutrition and bioreactor design.
- The most ideal approach would be when there is a collaboration between technological, cell biological and ethical-social sciences.
- Most importantly, research on in vitro meat should be done in an international project, perhaps even beyond EU only. Aside from technical aspects there should be research directed on consumer acceptation.
- This project should be developed by a multidisciplinary group involving experts from different areas going from cell culture to bioprocess engineering and polymer and food technologists. An international consortium should also be created.
- First, public money is required. Stakeholders (consumers, producers, scientists) should act together to start research on a large scale. It is pointless to only fund a small project.
- The market should decide if and how the technology must be developed. I am doubtful that companies are interested at this point.
- The challenges involved are daunting and they cannot be met a small group of academics in collaboration with a company or two. Like the thermonuclear fusion project, in vitro meat technology development will need international concerted efforts from a whole range of academic and engineering disciplines for many years before it becomes mature enough to be taken over by commercial interests. An international body or society is needed to guide the technology development and fuel it by attracting money from various sources.

- In my opinion, it is extremely important that there is sufficient attention for the societal aspects of cultured meat, and that grant money is also used to study these aspects. For this reason, I would plead for an interdisciplinary research centre.
- First step should be to develop the technology conceptually, including consumer acceptance. Second step should be to mathematically model these concepts and to define under which conditions they can become economically viable, so to define the development focus. Then technology development programs could be initiated.
- First phase: make a good team consisting of cell biologists, microbiologists, engineers, etc. Determine the best approach. Next, start a biotech company to test process technology. Sell technology worldwide.

Scientific publications from the 'In vitro meat' project

- Roelen BA, Lopes SM. Of stem cells and gametes: similarities and differences. Curr Med Chem, 2008; 15(13):1249-56.
- Wilschut KJ, Jaksani S, Van Den Dolder J, Haagsman HP, Roelen BA. Isolation and characterization of porcine adult muscle-derived progenitor cells. J Cell Biochem, 2008; 105(5):1228-39.
- Boonen KJ, Post MJ. The muscle stem cell niche: regulation of satellite cells during regeneration. Tissue Eng Part B Rev, 2008; 14(4):419-31.
- du Puy L, Chuva de Sousa Lopes S, Haagsman H, Roelen B. Differentiation of porcine ICM cells into proliferating neural cells. Stem Cells Dev, 2009; doi: 10.1089/scd.2009.0075.
- Boonen KJ, Rosaria-Chak KY, Baaijens FP, van der Schaft DW, Post MJ. Essential environmental cues from the satellite cell niche: optimizing proliferation and differentiation. Am J Physiol Cell Physiol, 2009; 296(6):C1338-45.
- Du Puy L, Beqqali A, Monshouwer-Kloots J, Haagsman HP, Roelen BA, Passier R. Cazip, a novel protein expressed in the developing heart and nervous system. Accepted for publication.
- Wilschut KJ, Haagsman HP, Roelen BA. Extracellular matrix components direct porcine muscle stem cell behaviour. Accepted for publication.

(publications recorded up to Oct 1, 2009; more manuscripts in preparation).

Press interest. Immediately from the start of the project the interest of the popular press, both national and international, has been rather overwhelming. Indeed interviews have been made and published in various media. In addition, particularly Henk Haagsman and Bernard Roelen have been invited for lectures, seminars and workshops on in vitro meat (both nationally and internationally) and have given a substantial number of interviews for all types of media. This has helped the researchers to get a feeling of the public opinion about in vitro meat. This information is crucially important when future strategies are to be designed for the correct commercialization of a product such as cultured meat.
Simultaneously, for public acceptance it has been and still is of importance to educate future consumers correctly about cultured meat. Part of the media interest may come from some form of sensationalism, but part of it is also driven by general interest and sincere concern for the environment and animal welfare. A selection of news media that interviewed the authors is indicated below.

National: Newspapers: NRC Handelsblad, Volkskrant, Algemeen Dagblad, Financieel Dagblad, Spits, De Pers; magazines: Intermediair, Natuurwetenschap & Techniek, Vrij Nederland; Radio: Radio 1 journaal, Vroege Vogels (VARA), Hoe? Zo! (TELEAC), Llink FM; TV: Nieuwslicht (VARA), Noorderlicht (VPRO), RTV Utrecht, Dierenduel (VPRO).
International: Newspapers: De Standaard, De Morgen; Gazet van Antwerpen (Belgium), Süddeutsche Zeitung; Frankfurter Allgemeine Zonntagszeitung, Westdeutsche Allgemeine Zeitung (Germany), The Times; Daily record (UK), Globe and Mail (Canada), The New York Times (USA).
International magazines: Der Spiegel, The Economist, Technology Review, Labor; Der Standard (Germany); Scientific American (USA). Forskning & Framsteg (Sweden).
International TV: Nanovision (Germany); SVT (Sweden), Tagesthemen; W wie wissen, Abenteuer Wissen (Germany), Kill it, cook it, eat it (UK), Future Food (Canada),
International Radio: (Belgium, Germany, Switzerland, Sweden, UK, USA).

International research. Although quite a lot of researchers have investigated, and still are investigating, the generation of embryonic stem cells, it has not yet been possible to establish an embryonic stem cell line from farm animal species. Most of these latter studies emerged from a biomedical interest, not with the intention of using these cells for food production. Nonetheless, there is a group of international researchers that investigate cultured meat from different angles.

In April 2008, a three-day 'in vitro meat' symposium was organized at the Norwegian Food Research Institute (Matforsk) in Aas, Norway and was hosted by the Norwegian Institute of Life Sciences (UMB) and Matforsk. Cell biologists, tissue engineers, engineers, entrepreneurs, NGO representatives and government officials from various European countries but also from the US, Canada, Australia and New Zealand discussed the key scientific challenges for the production of cultured meat. Issues that were discussed were e.g. production of culture media, stem cell generation and culturing, and muscle tissue engineering. Professor Stig Omholt (Chairman of the International In Vitro Meat Consortium, Norwegian University of Life Sciences, Norway) presented at this meeting a report made by eXmoor pharma concepts (UK) commissioned by him on behalf of the in vitro meat meeting steering committee (members: Prof. Stig Omholt, Norway; Prof. Henk Haagsman, Netherlands; Prof. José Teixeira, Portugal; Jason Matheny, USA, and Dr. Bernard Roelen, Netherlands). The study presented in the report deals with the question whether the production of in vitro meat can be financially viable as compared to the factory gate prices for cheap meat products such as chicken. As it is currently not possible to generate in vitro meat, several assumptions had to be made for this study:

1) Up front R&D en PR costs: Although these costs will be significant, they have been ignored in the model. Instead, it is assumed that these costs will be met by governments and charitable donations, but it is anticipated that these costs will be substantial.

2) Capital costs: As these are unknown until the technology is available, the model used factors from known technology.

3) Medium costs: Assumptions have been made based on medium that is currently available for biomedical research in relatively small quantities. These costs have been discounted to allow for larger volumes.

4) Financing costs: The model has assumed negligible business risks. Instead, it is assumed that research has demonstrated the proof of principle of the technology, and that the public opinion is positive (this will include acceptance by regulators that grant licenses and acceptance by consumers).

5) It was concluded that the costs have to be incurred and the technology has to be proven before manufacturers will invest in this technology. For the development of culture media it was concluded that either on site production or a separate media infrastructure would be necessary. Importantly, it was also concluded that the following challenges will have to be met:

- Sufficient knowledge of the biology of the stem cell and its differentiation into muscle cells.

- Tissue engineering on a very large scale.

- Maintenance of constant conditions around all individual cells in a large-scale reactor.

- Need of cell growth and differentiation and subsequent release from support without damage upon harvesting.

- Need for on-site cleaning and sterilization systems in the large-scale reactors.

- Sophisticated instrumentation for measuring and controlling conditions within large-scale reactors.

From the economic model it was concluded that a form of in vitro meat using mammalian cells in a 3D matrix could be produced at around Euro 3500/tonne. For comparison, the

production of chicken meat equals about Euro 1800/tonne (unsubsidized) or Euro 1400/tonne (subsidized), and the costs for production of beef (current market price) is around Euro 3550/tonne. Obviously, different capital cost and costs of the growth media do affect the forecast of the breakeven price for in vitro meat, and these costs can be reduced by for instance reducing the costs of the process plants, increasing the scale of production or reducing the cost of media preparation.

Experts' opinions (6): Role of The Netherlands in the development of cultured meat technology

- The Netherlands seems to be taking a leading role in this research at this time. If they stick with it they may emerge as the technology leaders in this area.
- The Dutch research effort into developing in vitro meat has the potential to revolutionize agriculture, saves human lives, and significantly reduce climate change. I sincerely hope that the Dutch government will expand its support of this research, positioning The Netherlands as a global leader.
- I believe that in vitro meat would be important for a densely populated country like The Netherlands with a large meat production. I hope the Netherlands will continue as a leading country in the area.
- The Dutch government should stimulate the development of cultured meat, including research on public reactions and acceptance.
- The Netherlands can be very important in the production of cultured meat as it is at the moment one of the frontrunners. However, it all depends on the amount of money invested.
- Positive, particularly when it leads to a reduction of millions of pigs in The Netherlands.
- The Netherlands can and must be a leading country in the development of this technology. The Dutch scientists have the required knowledge. It is important to act swiftly.
- The Netherlands have played a pioneering role so far, and by proper funding of the academic activity, the country will play a leading role for many years to come.
- I have high expectations of the Dutch research.
- I see culture meat development at the level of the academia. The Netherlands could play a role but this will depend on the choices that are made. The development of cultured meat is according to me not an essential investment for the Dutch government in order to support innovation.
- I think this is a technology that will take 10 – 20 years to be established. As a global leader in food and feed technology The Netherlands should at least assess the feasibility and potential of this technology through mathematical modeling.

Strengths and weaknesses of the current approach

Strengths. Although the development of in vitro meat is still in its infancy, a substantial group of international scientists is, indirectly, involved in this field (see first international in vitro meat symposium: http://invitromeat.org/content/view/14/29/). The scientists involved represent different disciplines, from cell biologists via process technologist to ethicists. The multidisciplinary approach already from the start ensures product development that fulfills all necessities.

The intense positive media interest that has, and still is, given to the Dutch in vitro meat project (see supplementary materials) indicates that there is a definite interest for (partial) replacement of traditional meat for cultured meat. The past decade has already seen a change in consumer habits leading to development of organic and other eco-labeled products, and tendencies towards vegetarianism and healthier diets. In this respect, it is anticipated that indeed there is a healthy market for cultured meat. This is further emphasized by the $1 million reward that has been offered by People for the Ethical Treatment of Animals (PETA) to the first scientist who produces cultured meat and brings it to market. However, there are some details attached to this contest. First it is stated that the cultured meat should be of chicken origin and should be ready to sell to the public by June 30, 2012. Further, the contestant must do both of the following: 1) produce a cultured chicken-meat product that has a taste and texture indistinguishable from real chicken flesh to non-meat-eaters and meat-eaters alike; 2) manufacture the approved product in large enough quantities to be sold commercially; 3) successfully sell it at a competitive price in at least 10 US states (http://www.peta.org/feat_in_vitro_contest.asp). It seems unlikely that anybody can meet these criteria by June 2012, but it does indicate the growing need for and interest in meat alternatives.

Weaknesses. Currently, research on making an edible product from in vitro cultured cells is basically only conducted in the Netherlands by the Dutch In Vitro Meat Consortium: Hellingwerf/Teixeira de Mattos with 1 technician, University of Amsterdam; Post with 2 PhD students, Technical University Eindhoven, Haagsman/Roelen with 2 PhD students, Utrecht University; with feedback from In Vitro Meat BV (van Eelen) and Stegeman (Verstrate). This research has been financed by a SenterNovem grant, started in the spring of 2005, and ended March 31st, 2009. The relatively small basis of this research consortium is of concern, and with the limited research that could be carried out it will be difficult to secure future industrial support.

Limiting factors in the development of an edible product are the generation of the stem cells that will be used, the characterization of these cells once generated and the optimal conditions how to culture them. The cells are essential for the generation of cultured meat, but in contrast to popular belief, correct bovine or porcine cells that can (a) proliferate indefinitely and (b) differentiate efficiently to muscle cells, have not been derived yet, nor are the culture conditions known to maintain such cells in a proliferative state. The cells that were generated with financial support by SenterNovem do not fulfill all the required criteria. Without the proper cells (the starting ingredients) it is difficult to design culture media for proliferation and differentiation. Similarly, tissue engineering research and the development of suitable bioreactors is limited if the proper cells are not available. To circumvent these problems, researchers have used related cells as model systems. The murine C2C12 myoblast cell line has been particularly useful in this respect. This approach has provided interesting and practical results, but it is expected that more concrete results should be available with cells of farm animal species before companies are interested to invest.

Experts' opinions (7): The most important criteria that have to be met in order develop marketable products

- Cost competitiveness with a suitable product, and very careful attention to public relations and advertising. Doing this wrong could be a disaster that makes GM foods appear to be "organic" by comparison.
- Low cost, safe and nutritious. Texture and taste of ground meat should not be a problem to duplicate, given what is already accepted by consumers.
- Products should be attractive to the senses. Additionally the products should have a proven safety and the sustainability should be clearly demonstrated. There should also be a social acceptance of technological food production. To my opinion, this should be achieved by a transparent production process.
- Societal obstacles are a very important issue. Many people react with emotional aversion on the subject of cultured meat. I will be very important to understand this aversion, and to investigate how it can be maximized and more importantly how it can me minimized.
- There are quite a number of criteria that are very important for the success of a new product. These are consumer acceptance, taste, continuity of production (it should be available all year round), and it should be available at a competitive price.
- Cost and consumer acceptance. Ethical concerns might be a point to consider.
- To show the necessity to produce cultured meat and benefit for both consumer and environment.
- The production of products with a good taste and 'bite',
- Comparable prices with ordinary meat, safe, tasty, healthy and having a resemblance with an ordinary high-quality piece of meat.
- Products have to be generated that the consumer will accept and wishes to buy.
- The technology must have been developed to a large extent. Societal acceptance is very important.
- Cost prize, equal or lower than real meat. Sustainability, significantly lower resource use per kg produced meat. Quality, equal or better taste etc. Public acceptance, avoid "Frankenstein food" image.
- Consumer acceptance, 'transparent' production process, not too expensive.

Opportunities. Man's impact on the planet Earth has become alarmingly clear in the past decades. Numerous reports have been produced with frightening scenarios of the future and indeed without a change of policies there is a distinct possibility that our planet will become inhabitable. Energy consumption and the livestock sector in particular have been identified as the leading drivers of climate change, deforestation, pollution and reduction of biodiversity. Simultaneously, the livestock sector is extremely important for the agricultural economy and obviously for the human diet. Different solutions can, and should be, developed to reduce the environmental impact of this sector, one of those being the (partial) replacement of traditional meat with an edible product made from animal proteins produced by cultured cells.

The possibility of making an edible meat product from cultured cells without the use of animals may provide a change in agriculture and society at large. The infrastructure that should accompany the implementation of such a technology is still lacking and has to be developed. At this point in time the Netherlands is considered to be the leading country with respect to the research on cultured meat. Although this notion may not be true, fact is that Dutch scientists have a very good reputation if it comes to food and agricultural

sciences, environmental sciences, microbiology, stem cell biology, and tissue engineering. Investment in research and development of this technology may create opportunities for Dutch companies to become leaders in a new 'green' technology.

In addition, the spin-off know-how and technology in the field of tissue-engineering can give Dutch universities and Dutch industry a significant scientific and technological advantage in the medical application of tissue engineering (tissue regeneration).

Threats. One major threat in the development of cultured meat is the relatively slow progress because of the limited number of scientists involved. Coverage by the media has been beneficial for public awareness and initiating discussions about innovative ways to produce animal proteins. On the other hand, media attention raised high expectations by citizens and media alike. Many journalists expect us to show and eat a piece of lab-grown meat and are surprised to hear that world-wide only four PhD students and one technician work full-time on cultured meat. If research continues at the present pace and progress remains slow, the present enthusiasm for the technology may dwindle. Most people that were interviewed by us indicated that, at the present state of knowledge (and the financial crisis), companies will not invest in cultured meat production. It is concluded that public funding is required at this point in time to increase the research efforts with respect to the production of animal proteins by cultured muscle cells. The major threat is that, if that does not happen, potential investors and companies will never invest because of a lack of tangible results. This threat may be thwarted by using the present momentum of public enthusiasm to justify the investment of public seed funds.

Another threat may be the lack of consumer acceptance. However, from the start of the project we have encouraged the involvement of representatives from consumer groups. Also, there has been considerable media attention for the project, and through radio/TV/newspapers/magazines the consumer has been (and will be) informed and educated about cultured meat production. In general, the media attention has been positive and supportive for an in vitro meat product. On the other hand, several media, in the internet in particular on various webblogs, have been more skeptical and even negative about the concept of cultured meat. 'Frankenfood' is a repetitively used word in this respect. Consumer acceptance is of utmost importance; without it there may be a product but no market. Knowledge of consumer's choices and factors that affect these choices is therefore important. Consumer's motivation, habits and choices should therefore be investigated. A proper feedback between those that study consumer's profiles with those that study cell biology/culture media/tissue engineering is crucial.

A major threat for the implementation of the technology comprises the production costs. Obviously the price of cultured meat should not differ too much from the price of traditional meat. For several reasons it is assumed that the price of in vitro meat will be similar to the price of regular meat. Firstly, the study by eXmoor pharma concepts concluded that the forecast costs for cultured meat products will be around €3300/ tonne. This is in the same order as the unsubsidized price of chicken meat that is around €1800/ tonne (see supplementary materials). Secondly, it has been predicted that the price of regular meat will rise significantly, as the meat demand will only increase with the growing world population while there is simply not enough feed for the animals. It is therefore expected that the price of regular meat will only increase, whereas it is predicted that the price of in vitro meat will decrease as the market becomes bigger.

Experts' opinions (8): The role of companies in the development of cultured meat

- Probably not until the risk has been mitigated substantially.
- Companies may invest but not until proof of principle is established by research funded by governments or foundations. I think private commercial funding at this stage may be less likely than public or foundation funding. Once proof of principle is established, it seems likely that private commercial funding will play an important role.
- Not in this first phase. There is still much basic science to be done. I believe the first phase has to be public.
- At the moment I do not think that companies will invest in research on in vitro meat. The product is simply too immature yet, first a number of uncertainties need to be answered.
- I would strongly suggest a private-public association. However, I consider that public institutions should have a key role in the launching of the project. In the second phase, private companies should join the project.
- I favor development on a public-private basis.
- At first companies will not invest in cultured meat. It is important to show that the technology is feasible.
- Companies will not invest before the technology is mature to allow very moderate R&D investment costs and before they see that governments will through legislation make a market for this. The FAO has already articulated favorable opinions about the concept, and they sent a high-ranking representative to the first international workshop on in vitro meat production held in Norway in 2008. They will probably continue to support this vision.
- It will take some time before this technology will become profitable, until this time public funding is preferable.

Spin-off. It is anticipated that research on cultured meat will have a significant spin-off, primarily for the biomedical industry. Four areas of spin-off can be discerned: 1) Generation of stem cells from pigs (and other farm animals); 2) Production of tissue culture media that do not contain animal products; 3) Increased knowledge on aspects of tissue engineering; 4) Specific know-how on (industrial scale) bioreactors.

Ad 1) Generation of porcine stem cells. The generation of stem cell lines from farm animal species also has the potential of generating genetically modified animals. Murine embryonic stem cells can be used to introduce specific gene modifications in mice and, as a result, numerous genes have been altered in mice. This has been a powerful method to help understand the functions of many genes and in 2007 the Nobel Prize in Physiology or Medicine was awarded to the discoverers of these techniques. Gene targeting is currently not common in mammals other than in mice, but the development of stem cell lines from farm animal species could facilitate to create pigs or cows with targeted gene modifications. Stem cells from farm animals can therefore be used for the generation of transgenic animals with improved production traits or disease resistance. Similarly transgenic farm animals, particularly pigs, can be extremely useful large animal model systems for human medicine. The pig is a more useful model for human medicine than the currently predominantly used mouse models, because pigs and humans have a comparative anatomy and physiology. For instance, the organ dimensions and life-span of pigs are more similar to those of man than those of rodents. It is therefore anticipated that porcine stem cells can be used to generate genetic models for human diseases. In

addition, stem cells can be used for the generation of animals that can be used in organ transplantation research or therapy (animal models or xenotransplantation).

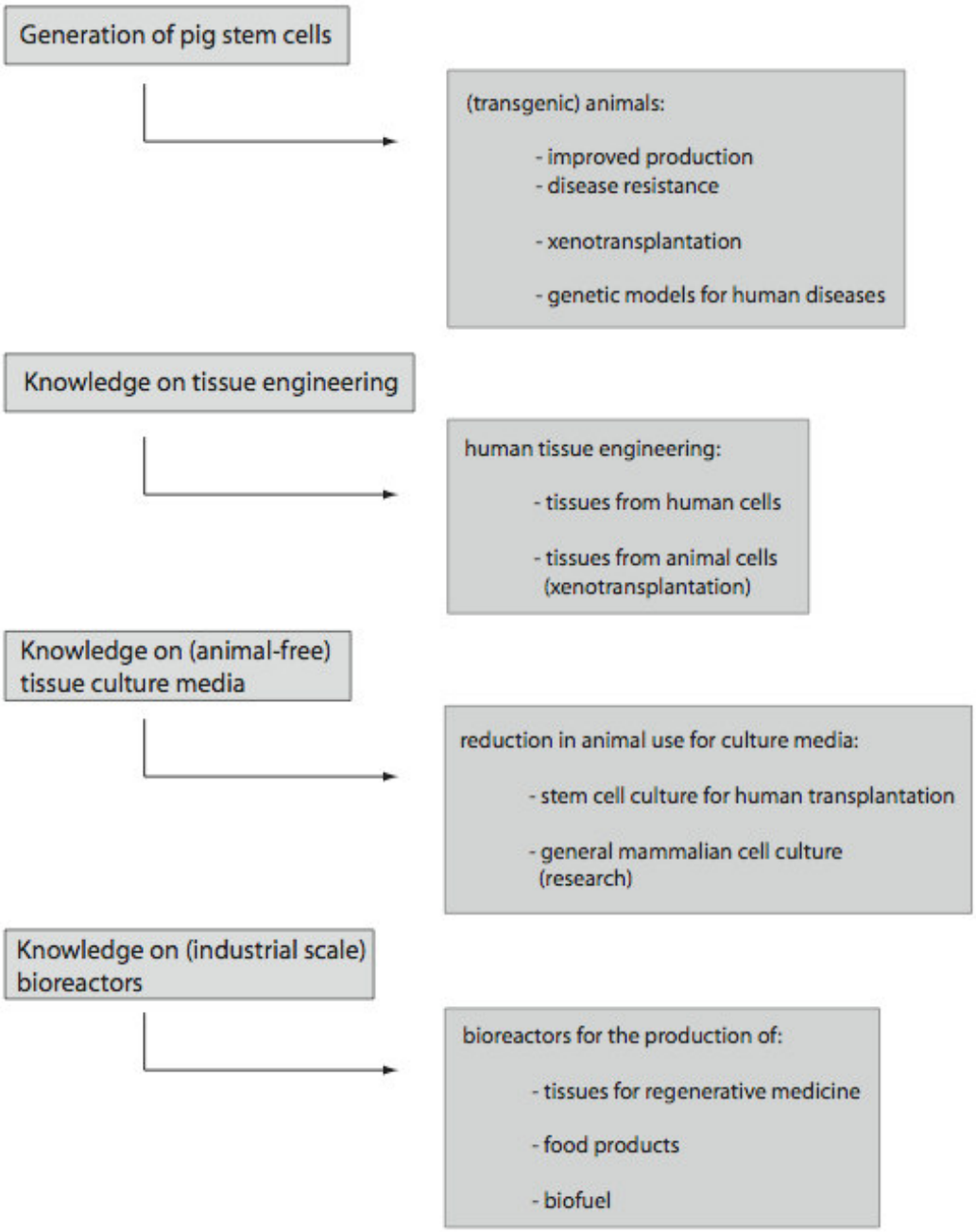
Ad 2) Production of tissue culture media that do not contain animal products. Currently most media used for the culture of mammalian cells contain fetal bovine serum. This serum, containing basic components to nurture cells, including hormones and growth factors, is derived from bovine fetuses of at least 3 months old by means of cardiac puncture. The availability of (affordable) culture media that do not contain animal products, i.e. for which no animals have been used will significantly reduce the use of animals. Indeed it has been proposed that fetal bovine serum should be replaced in cell culture both on scientific (batch differences) and moral grounds[44] . For safety reasons, it is crucial that cells and tissues used for human regenerative medicine have not been in contact with animal products. The development of animal-product free culture media will therefore also be beneficial for regenerative human medicine.

Ad 3) Increased knowledge on aspects of tissue engineering. Since the culture of muscle tissue from stem cells is in essence similar to tissue engineering as is being developed for the biomedical industry, the results and knowledge obtained are interchangeable. Knowledge on coatings, scaffolds, culture conditions and so forth is also advantageous for the in vitro culture of 3-dimensional human tissue to be used for regeneration studies. In addition, knowledge on in vitro culture of (engineered/transgenic) porcine tissues can be helpful for those interested in the use of porcine tissue/organs for xenotransplantation.

Ad 4) Specific know-how on (industrial scale) bioreactors. Many stem cell and tissue engineering studies are conducted with the ultimate goal of using in vitro cultured stem cells and tissues for regenerative medicine. It would be extremely beneficial for human medicine if tissue regeneration would become a commonplace therapy and, if this happens, bioreactors will be needed for tissue generation. The knowledge obtained from cultured meat studies can be used for the optimization of these bioreactors. Other cells, including plant and bacterial cells, can also be cultured using bioreactor technology. These can be used for the generation of food products other than cultured meat, but also for the generation of biofuel.

It has to be noted that the worldwide resources for human (regenerative) medicine are much higher than for cultured meat. Therefore, it is anticipated that, also in the future, knowledge transfer will be largely from the human medicine field to the cultured meat field.

Anticipated spin-off from cultured meat research



Experts' opinions (9): Potential spin-offs of cultured meat research

- If one understands how to propagate muscle stem cells and how to differentiate these into muscle cells, combined with the knowledge on muscle tissue engineering and muscle tissue formation, this knowledge can be helpful in basic research to minimize the use of animals.
- Cheap growth media for other tissue engineering applications, including those in biomedicine. Perhaps also insights into muscle tissue engineering that support medical therapies.
- Mostly in basic biology. Maybe also spin-off to medicine but more likely the other way around, because most probably much more funding will go to the medical endeavor to grow human organs for transplantation.
- As a spin-off, there is the strong potential that the role that technology can play in production of food, morality and sustainability is re-evaluated.
- Cultured meat is a spin-off of medical research into the differentiation of stem cells in body tissues.
- The technology will have an impact on tissue engineering and regenerative medicine.
- Fundamental knowledge on tissue engineering. Intellectual Property.
- As the development of the technology will demand contributions from several disciplines the spinoffs are likely to be considerable:

  - Chemical/biotechnological process plant construction and optimization
  - Materials technology
  - Biosensor technology
  - Instrument manufacturing
  - Advanced multivariate analysis methodology
  - Mathematical modeling of biological tissues and organs from gene to phenotype
  - Stem cell creation from domesticated animals
  - Large scale stem cell culturing
  - Cell co-culture methodologies and issues
  - Methodologies for directed stem cell differentiation into skeletal muscle
  - Bioprinting technology (myoblast sheets)
  - The developmental biology of muscle tissue
  - Experimental and theoretical biophysics of muscle tissue including the extracellular matrix
  - The developing and mature muscle tissue phenotype (in the wide sense)
  - Large scale genotyping technologies
  - Muscle tissue phenotyping technologies (in the wide sense)
  - (Large scale) 3-d cell (co-)culturing technology
  - Neutral lipid / flavor /muscle cell fatty acid biology
  - Enzymology of relevance to food processing
  - Cell culture diseases and associated diagnostic methodology
  - Production economist and process engineer to estimate costs under various regimes
  - Environmental economist with experience in life-cycle analysis to estimate environmental impacts relative to conventional meat
  - Food market analyses to assess consumer acceptance of in vitro meat

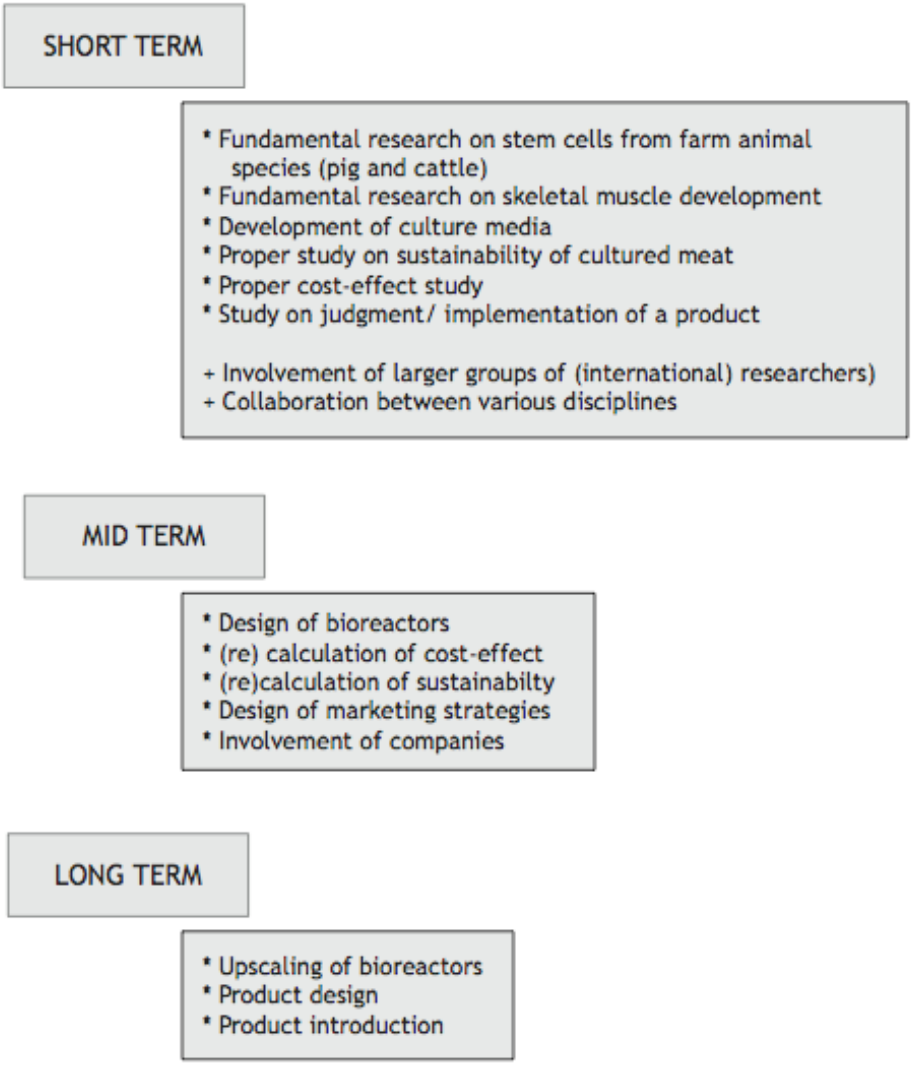
Policy making

Commercial production of cultured meat is as yet not possible, since knowledge is still lacking on the fundamental science level, as well as on the technological and societal levels. For one, suitable, well-characterized, stem cells from farm animal species are not yet available. In addition, there is a critical lack of information concerning culture conditions that would keep these stem cells undifferentiated and culture conditions that would steer these cells efficiently to skeletal muscle cells, bioreactors, etc. There is still too little known about consumer's judgment influences, consumer's choices etc. For reasons of credibility and for marketing strategies, it seems appropriate that a good communication exists between scientists/developers/industry and consumers (societal embedding).

For cultured meat to become realistic it is recommended that on a short term the focus must be on these lacunas in knowledge and lack of existing technology. With respect to long-term goals, if the technology has been established, attention should be directed towards scaling up of the bioreactors, not only for the culture of stem cells and production of cultured meat, but also for the production of culture media. The involvement of companies would be essential at this stage. During the whole process, transparency towards the consumers and societal embedding need to be warranted.

Experts' opinions (10): Investments in cultured meat research and technology

- Ball park - €25-50 million to establish labs and hire a team of ~5 scientists for 2-3 years.
- Realistic timelines on this research are unfortunately still very long: I estimate 15-20 years of intensive research at the very least. I would guess that for properly setting up research on in vitro meat, annually €4-$6 million over a 15-20 year period is needed.
- My guess is over €10 million will be needed to establish proof of principle. Then commercial firms would invest many millions to scale-up the technology.
- As a start at least €10 million is required to initiate research and development.
- If a broad but very concerted and tightly controlled R&D programme is made, I would think around €100 million is needed over a 3-4 year period before a pilot plant can be set up where the development to the next stage can be taken.
- About €30-50 million.
- Somewhere between €20-50 million.


SHORT TERM
* Fundamental research on stem cells from farm animal species (pig and cattle)
* Fundamental research on skeletal muscle development
* Development of culture media
* Proper study on sustainability of cultured meat
* Proper cost-effect study
* Study on judgment/ implementation of a product
+ Involvement of larger groups of (international) researchers)
+ Collaboration between various disciplines
MID TERM
* Design of bioreactors
* (re) calculation of cost-effect
* (re)calculation of sustainabilty
* Design of marketing strategies
* Involvement of companies
LONG TERM
* Upscaling of bioreactors
* Product design
* Product introduction

Summary

The global population is estimated to increase with 50% during the next 40 years. This population increase will be accompanied with an almost doubling of the greenhouse gas emissions if no actions are taken. It is anticipated that also the global meat consumption will double during the next 40 years if societies worldwide become more affluent. Meat production requires a relatively high proportion of land, energy, and fresh water use. Moreover, livestock contributes significantly to the emission of greenhouse gases and, in many countries, to the pollution of water and soil. An obvious solution to the problem would be to consume less products of animal origin. This may, certainly in Western societies where the consumption of (animal) proteins is very high, be part of the solution. Replacement of dietary animal (vertebrate) proteins by plant, fungal, or even insect proteins can be another part of the solution.

Yet another possibility is to culture large amounts of muscle cells derived from stem cells of farm animal species and to produce cultured meat (in vitro meat). The advantage of this technology is that for the making of most products of animal origin no animals are needed. Products prepared with the latter technology may combine a favorable ecological footprint with similar nutritional values as conventional products. Ultimately, cultured meat products could be made with similar sensory qualities of some of the conventional products. The present report is a survey of the current state of the required technologies, the life cycle assessment, ethical and societal issues, and economical aspects.

The first requirement for developing cultured meat technologies is a suitable, bona fide, stem cell line that can proliferate indefinitely. Both embryonic and adult stem cells may be suitable although adult stem cells must have a minimal self-renewal capacity since most adult stem cell types cannot be cultured indefinitely. One of the aims of the Dutch 'In vitro meat' project (2005-2009), funded by SenterNovem (an agency of the Dutch Ministry of Economic Affairs), was to generate a suitable stem cell line (embryonic and adult) from pigs. Adult stem cells have indeed been generated from porcine muscle but these cells do not very effectively differentiate into skeletal muscle cells. The generation of a suitable 'starter stem cell' from farm animals and its growth and efficient differentiation into muscle fibers still requires much research. Fortunately, the fast developments in stem cell biology will enable more directed research in this area.

A second requirement for the production of cultured meat is the availability of culture media that do not contain products of animal origin. Most media for culturing mammalian or avian cells contain animal serum. It is clear that for cultured meat production serum-free media must be used, a requirement that is also important if stem cells are used in regenerative medicine. Different media must be developed for growing the stem cells and for the differentiation media to produce muscle cells. A lot of progress has been made in recent years in the development of serum-free media and indeed several media are commercially available that do not contain animal products. The challenge is to produce the right culture media at prices that are compatible with food production.

A third requirement for cultured meat technologies to become successful is the formation of a three-dimensional structure of fused muscle cells (myofibers). In the absence of a blood flow, that provides oxygen and nutrients to the cells, the diffusion rates of these compounds limit the culturing of many layers of cells. Scaffolds or hydrogels may be used to circumvent this problem provided that these materials are edible and not from animal origin. Studies have been carried out as part of the 'In vitro meat' project to determine the optimal three-dimensional environment for muscle cells for culturing and differentiation. In addition, biochemical and biophysical stimuli (electrical and mechanical) were studied that may be important in the differentiation and maturation towards muscle tissue. While knowledge is accruing about the factors that are important for culturing muscle tissue, a lot of basic knowledge is still lacking. Nevertheless, the technology to grow small pieces of meat (as ingredients in complex food products) may be developed within a relatively short term, provided that the above-mentioned

requirements (suitable cells and medium) are met. The development of the technologies to produce larger pieces of meat may take considerably longer.

A fourth requirement is that cultured meat must be produced at prices that are comparable with factory gate prices for cheap meat such as chicken meat. A study commissioned by the 'International In Vitro Meat Consortium' indicated that cultured meat (from a three-dimensional culture) can be produced for €3500/tonne (compared to €1800/tonne for chicken meat and €3550/tonne for beef). It should be noted that several assumptions had to be made, since the technology to produce cultured meat has not been developed yet. Nevertheless, it is encouraging that the applied economic model predicts production costs that are comparable with that of current meat production.

A fifth requirement is that the production of cultured meat should have a favorable ecological footprint compared to the conventional production of meat. A preliminary life cycle assessment of cultured meat by dr. Tuomisto (University of Oxford) and dr. Teixeira de Mattos (University of Amsterdam) indicated that cultured meat production has a much lower environmental impact than conventional meat production. It was estimated that the average energy use is 45-60% lower; greenhouse gas emissions are 80-95% lower; land use is 98% lower and water use is 90-98% lower. Only poultry meat has a lower energy use per kilogram than cultured meat. Although also for this study several assumptions had to be made it is clear that the ecological footprint of the production of cultured meat is substantially lower than that of conventional meat.

A sixth requirement for the production of cultured to become successful is consumer acceptance. Until now no scientific study has been conducted about the ethical and societal aspects of cultured meat production and consumption. From the start of the 'In vitro meat' project in 2005 the research of the Dutch consortium has received a lot of media attention from all over the world. The coverage by the media provoked numerous discussions on the internet and, as discussed by Holmes and Dacey in their publication in the Journal of Agricultural and Environmental Ethics in 2008, the majority of the opinions is favorable towards cultured meat technology. One should be careful to draw conclusions from these anecdotes. Therefore, it is deemed of utmost importance to carry out research with respect to the ethics and societal aspects of cultured meat.

It is anticipated that research on cultured meat will have a significant spin-off, primarily for the biomedical field. Four areas of spin-off can be discerned: 1) Generation of stem cells from pigs (and other farm animals); 2) Production of tissue culture media that do not contain animal products; 3) Increased knowledge on aspects of tissue engineering; 4) Specific know-how on (industrial scale) bioreactors. It has to be noted that the worldwide resources for human (regenerative) medicine are much higher than for cultured meat. Therefore, it is anticipated that, also in the future, knowledge transfer will be largely from the human medicine field to the cultured meat field.

In the framework of our study we interviewed national and international experts from companies, research institutes and universities about many aspects of the development of cultured meat. Most experts indicated that there is an urgent need for dietary protein sources other than meat and that cultured meat may be an interesting option to produce meat in a sustainable way. However, a few experts never heard of cultured meat and were skeptical about developing this technology. The experts indicated several technological challenges; these challenges were also recognized by the authors and comprise an important part of this report. The experts were unanimous that at this point in time the traditional food companies will not invest in research on such a completely different commodity as cultured meat. All experts mentioned that, at this stage, investments in the technology should be done with public money. Although most experts see an important role for the Dutch scientists in cultured meat research, it is preferable that both funding and research efforts are coordinated internationally. The experts estimate that €10-€100 million is required to initiate an R&D program.

It can be concluded that commercial production of cultured meat is as yet not possible, since crucial knowledge is still lacking on the biology and technology. Furthermore,

knowledge is lacking with respect to ethical and societal issues. Companies will only invest if the technology has demonstrated to be viable on a small scale. It is recommended that on a short term the focus must be on filling these gaps in knowledge. During the whole process, communication with consumers and societal embedding is essential.

Nederlandse samenvatting

Geschat wordt dat de wereldbevolking gedurende de volgende 40 jaar met 50% zal toenemen. Deze toename van de wereldbevolking zal vergezeld gaan met een verdubbeling van de uitstoot van broeikasgassen als er niet ingegrepen wordt. Wereldwijd zal ook de consumptie van vlees in de komende 40 jaar verdubbelen vanwege het feit dat, naast de bevolkingsgroei, mensen ook meer te besteden hebben en de consumptie van vlees bij veel volkeren geassocieerd is met welvaart. De productie van vlees vereist relatief veel land, energie en water. Bovendien draagt de veehouderij verhoudingsgewijs sterk bij aan de emissie van broeikasgassen en in veel landen ook aan de verontreiniging van bodem en oppervlaktewater. Een voor de hand liggende oplossing zou de vermindering van de consumptie van dierlijke producten zijn. Dit zou zeker een deel van de oplossing in de Westerse wereld zijn waar de consumptie van (dierlijke) eiwitten hoog is. De vervanging van dierlijke eiwitten (uit gewervelden) in het dieet door eiwitten uit planten, schimmels of zelfs insecten zou een ander deel van de oplossing zijn.

Een heel andere oplossing is om niet de dierlijke eiwitten zelf maar de bron van dierlijke eiwitten te vervangen. Spiercellen, verkregen uit stamcellen van landbouwhuisdieren, zouden op grote schaal gekweekt kunnen worden. Aangezien vlees voornamelijk uit spiercellen bestaat kan op deze wijze 'kweekvlees' (in vitro meat) geproduceerd worden. Het grote voordeel is dat met deze technologie geen dieren meer nodig zijn voor de productie van vlees en vleesproducten. Kweekvleesproducten zouden op een milieuvriendelijke wijze vervaardigd kunnen worden terwijl de voedingswaarde vergelijkbaar is met die van conventionele producten. In eerste instantie zou de nieuwe technologie gericht kunnen zijn op het maken van ingrediënten voor samengestelde producten terwijl op de lange termijn smakelijke producten ontwikkeld kunnen worden die volledig vervaardigd zijn met de kweekvleestechnologie. Dit rapport is een studie naar de huidige kennis op het gebied van 1) de technologieën die nodig zijn voor de ontwikkeling van kweekvlees; 2) de milieugerichte levenscyclusanalyse; 3) de economische haalbaarheid en 4) ethische en maatschappelijke aspecten.

Een eerste vereiste voor de ontwikkeling van kweekvleestechnologieën is een geschikte stamcellijn die bij voorkeur oneindig door kan groeien. Zowel embryonale als niet-embryonale ('volwassen') stamcellen kunnen gebruikt worden hoewel de meeste volwassen stamcellen niet oneindig in kweek kunnen worden gehouden. Deze cellen moeten daarom voldoen aan bepaalde eisen wat betreft hun delingscapaciteit. Eén van de doelstellingen van het Nederlandse 'In vitro meat' SenterNovem project (2005-2009) was het verkrijgen van een geschikte stamcellijn (zowel een embryonale als volwassen lijn) van het varken. Volwassen stamcellen zijn inderdaad verkregen uit varkenspieren maar deze cellen bleken, in tegenstelling tot muizenstamcellen, niet op efficiënte wijze naar skeletspiercellen te differentiëren. Het verkrijgen van een geschikte 'startstamcel' uit landbouwhuisdieren en de groei en efficiënte differentiatie in spiervezels vereist nog veel onderzoek. Aangezien de ontwikkelingen in de stamcelbiologie snel gaan is het mogelijk steeds gerichter onderzoek te doen naar stamcellen van landbouwhuisdieren. De recente opheldering van de sequentie van het varkensgenoom is ook belangrijk in dit verband. Een tweede vereiste voor de ontwikkeling van kweekvleestechnologieën is de beschikbaarheid van kweekmedia die geen producten van dierlijke oorsprong bevatten. De meeste media die gebruikt worden om cellen van zoogdieren en vogels te kweken bevatten dierlijk serum. Vanwege de aard en veiligheid van het product is het duidelijk dat voor de bereiding van kweekvlees serumvrije media gebruikt moeten worden,

hetgeen overigens ook een eis is als stamcellen gebruikt worden in de regeneratieve geneeskunde. Verschillende media moeten ontwikkeld worden voor het opgroeien van stamcellen en voor de differentiatie en groei van spiercellen. De afgelopen jaren is veel vooruitgang geboekt met het ontwikkelen van serumvrije media en media die geen dierlijke producten bevatten zijn nu commercieel verkrijgbaar. De uitdaging is nu de juiste media te maken voor de productie van kweekvlees tegen een zo gunstig mogelijke prijs.

Een derde vereiste om de productie van kweekvlees succesvol te maken is de vorming van een driedimensionale structuur van een aantal lagen gefuseerde spiercellen (spiervezels). In de afwezigheid van een bloedstroom, die voedingstoffen en zuurstof voor de cellen beschikbaar maakt, is het niet mogelijk veel cellagen te stapelen. Een steigerwerk ('scaffold') of een hydrogel zou een oplossing zijn voor dit probleem waarbij de gebruikte materialen eetbaar moeten zijn en niet van dierlijke oorsprong. Ook zijn andere technologieën denkbaar om een driedimensionale structuur van spiervezels te maken. In het 'In vitro meat' project is onderzoek uitgevoerd om de optimale driedimensionale omgeving te creëren om spiercellen te kweken en te laten differentiëren. Bovendien werd onderzocht op welke wijze de differentiatie en rijping van spiercellen gestimuleerd kan worden. Hiervoor werden de cellen zowel biochemisch als fysisch (elektrisch en mechanisch) gestimuleerd. Ondanks het feit dat al veel bekend is over de factoren die van belang zijn om spierweefsel te kweken ontbreekt er nog veel kennis. Desalniettemin zal de technologie om kleine stukjes vlees te maken (als ingrediënt voor samengestelde producten) binnen relatief korte termijn ontwikkeld kunnen worden als de juiste cellen en media beschikbaar zijn. De ontwikkeling van technologieën om grotere stukken vlees te maken zal echter aanzienlijk meer tijd in beslag nemen.

Een vierde vereiste is dat kweekvlees geproduceerd moet kunnen worden tegen prijzen die vergelijkbaar zijn met de prijzen van gangbaar vlees, zoals goedkoop kippenvlees. Een studie, die in opdracht van het 'International In Vitro Meat Consortium' is uitgevoerd, wees uit dat kweekvlees geproduceerd kan worden voor €3500/ton (vergeleken met €1800 voor kippenvlees en €3550 voor rundvlees). Een kanttekening hierbij is dat verschillende aannames gemaakt moesten worden aangezien de kweekvleestechnologie nog niet ontwikkeld is. Het is echter bemoedigend dat het toegepaste economische model productiekosten voorspelt die vergelijkbaar zijn met de huidige productiekosten van vlees.

Een vijfde vereiste is dat de productie van kweekvlees duurzaam moet zijn en minder schadelijk voor het milieu dan de huidige vleesproductie. De voorlopige resultaten van een kwantitatieve, milieugerichte levenscyclusanalyse door dr. Tuomisto (University of Oxford) en dr. Teixeira de Mattos (Universiteit van Amsterdam) geven aan dat de productie van kweekvlees veel minder schadelijk is voor het milieu dan conventionele vleesproductie. De berekeningen wezen uit dat het gemiddeld energiegebruik voor de productie van kweekvlees 45-50% lager is, de emissie van broeikasgassen 80-95% lager, landgebruik 98% lager en watergebruik 90-98% lager. Hoewel ook voor deze studie verschillende aannames gemaakt moesten worden is het duidelijk dat de ecologische voetafdruk van kweekvlees aanzienlijk kleiner is dan die van conventioneel vlees.

Een zesde vereiste om de productie van kweekvlees succesvol te maken is consumentenacceptatie. Tot op heden is er geen wetenschappelijk onderzoek uitgevoerd naar de ethische en maatschappelijke aspecten van de productie en consumptie van kweekvlees. Vanaf de start van het 'In vitro meat' project in 2005 heeft het onderzoek van het Nederlandse consortium veel media-aandacht gehad vanuit de hele wereld. Deze aandacht heeft veel discussies uitgelokt (o.a. op internetfora). In een artikel van Holmes en Dacey in het tijdschrift 'Journal of Agricultural and Environmental Ethics' in 2008 werden deze discussies besproken. Het bleek dat de meerderheid van de meningen positief is ten opzichte van kweekvleestechnologie. Het zou echter onjuist zijn op grond van anekdotisch onderzoek verregaande conclusies te trekken. Het is daarom van groot

belang om gedegen wetenschappelijk onderzoek te verrichten naar de ethische en maatschappelijke aspecten van kweekvlees.

Het is de verwachting dat kweekvleesonderzoek een aanzienlijke spin-off kan hebben voor het biomedische veld. Er kunnen minimaal 4 gebieden geïdentificeerd worden die van dit onderzoek zouden kunnen profiteren: 1) Het verkrijgen van stamcellen van het varken (en van andere landbouwhuisdieren); 2) De productie van serumvrije kweekmedia voor cellen en weefsels; 3) Kennis over bepaalde aspecten van 'tissue engineering'; 4) Specifieke 'know-how' op het gebied van bioreactoren. Er moet gezegd worden dat er veel meer middelen voor onderzoek op het gebied van de (regeneratieve) geneeskunde zijn, zodat meer kennis van het domein van de geneeskunde naar de ontwikkeling van kweekvleestechnologie zal gaan dan vice versa.

Ten behoeve van dit rapport werden nationale en internationale experts geïnterviewd over verschillende aspecten van de ontwikkeling van kweekvlees. Deze experts zijn werkzaam bij bedrijven, onderzoeksinstituten en universiteiten. De meeste experts gaven aan dat er een sterkte behoefte bestaat aan alternatieve bronnen voor dieeteiwitten en dat gekweekt vlees een mogelijke manier is om op duurzame wijze in deze behoefte te voorzien. Enkele experts hadden echter nog nooit gehoord van kweekvlees en waren sceptisch over de technologie. De experts noemden verschillende technologische uitdagingen. Deze uitdagingen werden ook gezien door de auteurs en vormen een belangrijk deel van dit rapport. De experts waren eensgezind over het feit dat, met de huidige kennis, de traditionele voedingsindustrie niet zal investeren in een product als kweekvlees. Alle experts waren de mening toegedaan dat op dit moment investeringen in de technologie met publieke middelen zouden moeten worden bekostigd. De meeste experts zien een belangrijke rol voor Nederlandse wetenschappers in onderzoek en ontwikkeling van de technologie. Toch zal het de voorkeur verdienen om op termijn het onderzoek internationaal te coördineren, uit te voeren en te bekostigen. Volgens schattingen van de experts zou met het starten van een R&D programma €10-100 miljoen gemoeid zijn.

Er kan geconcludeerd worden dat de commerciële productie van kweekvlees nu nog niet mogelijk is omdat er nog lacunes zijn in de kennis wat betreft biologie en technologie. Bovendien is er nog geen onderzoek uitgevoerd naar de ethische en maatschappelijke aspecten van de productie en consumptie van kweekvlees. Bedrijven zullen pas grote investeringen doen als het op kleine schaal mogelijk is een product te maken. Aanbevolen wordt daarom dat op korte termijn de aandacht gericht moet zijn om de ontbrekende kennis te verwerven. De technologie kan vervolgens verder ontwikkeld worden door een gezamenlijke inspanning van overheid en het bedrijfsleven. Gedurende het gehele traject is het essentieel de consumenten goed voor te lichten en voor een maatschappelijk draagvlak te zorgen.

References

1. Churchill, W. Toughts and adventures (1932).
2. Benjaminson, M. A., Gilchriest, J. A. & Lorenz, M. In vitro edible muscle protein production system (MPPS): stage 1, fish. Acta Astronaut 51, 879-89 (2002).
3. Araujo Neto, J. S. & Ferreira Pinto, G. Nucleic acid and single-cell protein utilization in human feeding: a review. Arch Latinoam Nutr 25, 105-18 (1975).
4. Kihlberg, R. The microbe as a source of food. Annu Rev Microbiol 26, 427-66 (1972).
5. Pirt, S. J. The maintenance energy of bacteria in growing cultures. Proc R Soc Lond B Biol Sci 163, 224-31 (1965).
6. Oka, M. S. & Rupp, R. G. Large-scale animal cell culture: a biological perspective. Bioprocess Technol 10, 71-92 (1990).
7. Jochems, C. E., van der Valk, J. B., Stafleu, F. R. & Baumans, V. The use of fetal bovine serum: ethical or scientific problem? Altern Lab Anim 30, 219-27 (2002).
8. Hata, J. et al. Chemically defined medium for the production of biologically active substances of CHO cells. Cytotechnology 10, 9-14 (1992).
9. Deshpande, R. R. & Heinzle, E. Online monitoring of oxygen in spinner flasks. Biotechnol Lett 31, 665-9 (2009).
10. Srinivasa Babu, K. et al. Single step intein-mediated purification of hGMCSF expressed in salt-inducible E. coli. Biotechnol Lett 31, 659-64 (2009).
11. Chabanon, G. et al. Influence of the rapeseed protein hydrolysis process on CHO cell growth. Bioresour Technol 99, 7143-51 (2008).
12. Roelen, B. A. & Lopes, S. M. Of stem cells and gametes: similarities and differences. Curr Med Chem 15, 1249-56 (2008).
13. McKay, R. Stem cells--hype and hope. Nature 406, 361-4 (2000).
14. Atala, A., Bauer, S. B., Soker, S., Yoo, J. J. & Retik, A. B. Tissue-engineered autologous bladders for patients needing cystoplasty. Lancet 367, 1241-6 (2006).
15. Evans, M. J. & Kaufman, M. H. Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154-6 (1981).
16. Martin, G. R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78, 7634-8 (1981).
17. Thomson, J. A. et al. Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A 92, 7844-8 (1995).
18. Thomson, J. A. et al. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145-7 (1998).
19. Keefer, C. L., Pant, D., Blomberg, L. & Talbot, N. C. Challenges and prospects for the establishment of embryonic stem cell lines of domesticated ungulates. Anim Reprod Sci 98, 147-68 (2007).
20. Talbot, N. C. & Blomberg le, A. The pursuit of ES cell lines of domesticated ungulates. Stem Cell Rev 4, 235-54 (2008).
21. Buehr, M. et al. Capture of authentic embryonic stem cells from rat blastocysts. Cell 135, 1287-98 (2008).
22. Li, P. et al. Germline competent embryonic stem cells derived from rat blastocysts. Cell 135, 1299-310 (2008).
23. Slack, J. M. Origin of stem cells in organogenesis. Science 322, 1498-501 (2008).
24. Kues, W. A., Petersen, B., Mysegades, W., Carnwath, J. W. & Niemann, H. Isolation of murine and porcine fetal stem cells from somatic tissue. Biol Reprod 72, 1020-8 (2005).
25. Zeng, L. et al. Multipotent adult progenitor cells from swine bone marrow. Stem Cells 24, 2355-66 (2006).
26. Wilschut, K. J., Jaksani, S., Van Den Dolder, J., Haagsman, H. P. & Roelen, B. A. Isolation and characterization of porcine adult muscle-derived progenitor cells. J Cell Biochem 105, 1228-39 (2008).
27. Kook, S. H. et al. Satellite cells isolated from adult Hanwoo muscle can proliferate and differentiate into myoblasts and adipose-like cells. Mol Cells 22, 239-45 (2006).
28. Colleoni, S. et al. Establishment, differentiation, electroporation, viral transduction, and nuclear transfer of bovine and porcine mesenchymal stem cells. Cloning Stem Cells 7, 154-66 (2005).
29. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663-76 (2006).
30. Takahashi, K. et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861-72 (2007).

31. Yu, J. et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917-20 (2007).
32. Yu, J. et al. Human Induced Pluripotent Stem Cells Free of Vector and Transgene Sequences. Science 324, 797-801 (2009).
33. Wu, Z. et al. Generation of Pig-Induced Pluripotent Stem Cells with a Drug-Inducible System. J Mol Cell Biol 1, 46-54 (2009).
34. Esteban, M. A. et al. Generation of induced pluripotent stem cell lines from tibetan miniature pig. J Biol Chem 284, 17634-17640 (2009).
35. Zhang, X. et al. Shaken helical track bioreactors: Providing oxygen to high-density cultures of mammalian cells at volumes up to 1000 L by surface aeration with air. N Biotechnol 25, 68-75 (2008).
36. du Puy, L., Chuva de Sousa Lopes, S., Haagsman, H. & Roelen, B. Differentiation of porcine ICM cells into proliferating neural cells. Stem Cells Dev doi: 10.1089/scd.2009.0075 (2009).
37. Doetschman, T., Williams, P. & Maeda, N. Establishment of hamster blastocyst-derived embryonic stem (ES) cells. Dev Biol 127, 224-7 (1988).
38. Sukoyan, M. A. et al. Embryonic stem cells derived from morulae, inner cell mass, and blastocysts of mink: comparisons of their pluripotencies. Mol Reprod Dev 36, 148-58 (1993).
39. Graves, K. H. & Moreadith, R. W. Derivation and characterization of putative pluripotential embryonic stem cells from preimplantation rabbit embryos. Mol Reprod Dev 36, 424-33 (1993).
40. Piedrahita, J. A., Anderson, G. B. & Bondurant, R. H. Influence of feeder layer type on the efficiency of isolation of porcine embryo-derived cell lines. Theriogenology 34, 865-77 (1990).
41. Van Stekelenburg-Hamers, A. E. et al. Isolation and characterization of permanent cell lines from inner cell mass cells of bovine blastocysts. Mol Reprod Dev 40, 444-54 (1995).
42. Notarianni, E., Galli, C., Laurie, S., Moor, R. M. & Evans, M. J. Derivation of pluripotent, embryonic cell lines from the pig and sheep. J Reprod Fertil Suppl 43, 255-60 (1991).
43. Tian, H. B. et al. Factors derived from mouse embryonic stem cells promote self-renewal of goat embryonic stem-like cells. Cell Biol Int 30, 452-8 (2006).
44. Jochems, C. E., van der Valk, J. B., Stafleu, F. R., Baumans, V. The use of fetal bovine serum: ethical or scientific problem? Altern Lab Anim 30, 219-27 (2002).

Appendices

Interviewed experts

Name	Function
J. Chon	Director, Upstream Process Development, PERCIVIA (joint venture between Crucell and DSM Biologics), Cambridge, UK
R.G. Dennis	Associate Professor, Dept. of Biomedical Engineering, University of North Carolina, Chapel Hill, NC, USA
R.J. Hamer	Programme Director, TI Food and Nutrition; Professor, Agrotechnology and Food Sciences Group, WUR, Wageningen
T.H. van Kuppevelt	Head Section Matrix Biochemistry, Nijmegen Centre for Molecular Life Sciences, Radboud University, Nijmegen
J. Maat	Director External Research at Unilever, Vlaardingen and Managing Director TI Food & Nutrition, Wageningen
J. Matheny	Director of New Harvest and Johns Hopkins School of Public Health, Baltimore, MD, USA
C. Medlow	Technical Manager SAFC Biosciences, Sigma-Aldrich Corporation
C.L. Mummery	Professor of Developmental Biology, Chair of Department of Anatomy and Embryology, Leiden University Medical Center, Leiden
S. Omholt	Professor at the Norwegian University of Life Sciences (UMB) and Director of the Centre for Integrative Genetics (CIGENE), Aas, Norway
M.F.W. te Pas	Senior Researcher Genomics and Bioinformatics, Animal Production Systems, Animal Sciences Group, WUR, Lelystad
J. Teixeira	Professor, Department of Biological Engineering, Universidade de Minho, Braga, Portugal
H. Tobi	Associate Professor Research Methodology, Wageningen UR
H.A.P. Urlings	Director Quality Assurance, VION NV and Professor Chain directed animal production, Animal Nutrition Group, WUR
J. Vereijken	Expert Industrial Proteins, Agrotechnology & Food Sciences Group, WUR, Wageningen
P. Verstrate	Director Operations, Stegeman, Deventer
C. van der Weele	Assistant Professor, Applied Philosophy Group, Social Sciences, WUR, Wageningen
S. Welin	Professor Biotechnology, Culture, Society, Department of Medical and Health Sciences, Linköpings University, Linköping, Sweden
G. Zijlstra	Senior Scientist, DSM Pharmaceutical Products, R&D Department, DSM Biologics Company BV, Groningen

Statements of the experts are personal views and do not reflect the opinion of the organizations that they represent.

Alternative (animal) cell systems for protein production

Since the early sixties, when research in 'single cell protein' production was at its peak, we know that eukaryotic cells in general - in contrast to the cells of prokaryotes - are suitable as a edible source of food for humans. The latter, apart from the toxic products they may contain, like endotoxin, generally have a too high nitrogen content, because of the relatively high content of ribosomes - and therefore RNA - so that uric acid, and by consequence kidney stones, are formed in the human consumer [1]. A well-known exception to this rule are some cyanobacteria, like Spirulina platensis, which - in part because of its slow growth rate - has been in use for centuries as a traditional source of food in tropical regions of Africa [2]. It should be added, however, that bacterial biomass from various species - when present in large amounts - readily elicits allergic reactions [3].

The human diet should ideally contain a balanced amount of molecules from multiple classes, most notably, sugars, amino acids, fatty acids, vitamins and minerals. The first three of these are usually derived from their polymeric precursors: polysaccharides, proteins and lipids. All eukaryotic cells contain these components, in varying proportion. Furthermore, when cells are consumed as a 'tissue', specific components may be over-represented, like (phospho)lipids in brain tissue, fatty acids in seeds and fibrous cell wall components in plants. The fibrous material constitutes a class of its own, because it may function 'catalytically' in the food-intake process, i.e. by having a positive, stimulatory effect with being consumed itself.

Besides a balanced overall elemental composition, for a healthy human diet specific attention should be paid to the relative enrichment of the (l-)amino acids. This is because humans are unable to synthesize a limited number (8) of these themselves, so that these have to be provided directly through food intake [4]. The best known example is l-lysine, but several more are essential or important*. Therefore the advise generally is given that, if a diet is vegetarian, it should be composed of a combination of products from two differen plant species, i.e. a monocotyl, like corn or wheat (for: methionine, valine, threonine, phenylalanine, leucine en isoleucine) and a dicotyl, like beans (for: valine, threonine, phenylalanine, leucine, isoleucine, tryptophan en lysine). The suitability of a food ingredient can be expressed as its essential amino acid index (EAAI; i.e. amino acid content relative to that in eggs, or: LAAC, the 'limiting amino acid concept'; see e.g. [5]).

In many eukaryotic cells, particularly in those of muscle, the amino acid composition is much more similar to the optimal composition for human nutrition than the one of other cell types, i.e. they are relatively enriched in the essential amino acids, in particular in l-lysine. Hence the advise to make meat an intrinsic part of the human diet. However, this optimality in amino acid composition may be offset by a less optimal composition of one or more of the other key ingredients, like e.g. (unsaturated) fatty acids or vitamins. Because of the above, the (non-)suitability of a specific cell type as a major source of amino acids for the human diet can only be decided within the context of the overall composition of that particular diet and the WHO has guidelines as to the minimum content of specific foodstuffs with respect to the essential amino acids [6].

The above considerations lead to the conclusion that almost all toxin-free eukaryotic cell types are suitable to form part of the ingredients of the human diet. This then includes microbial-, plant-, and animal cells and tissues. This being said, it is clear that consumer preference will have a dominant impact on the choice from this range of possibilities. The recent extensive publicity on Dutch TV on the use of insects as a suitable protein source is an illustration that in a significant fraction of the Dutch population, consumer preference begins to move away from traditional meat sources.

Very recently additional insight has been obtained into the mechanism by which the amino acid composition of our diet may have effects on our health, in particular related to aging [7]. This mechanism is based on variation in the rate of gene transcription which modulates the level of expression of DNA repair enzymes (see also [8]). Accordingly, our diet directly affects the amount of DNA damage inflicted upon our genomes. These insights may in the future raise the interest of the consumer in specifically engineered food (to be obtained either through genetic- or through physiological engineering) in which the (amino acid) composition has been optimized with respect to its effect on health (see e.g. also [9]).

From a technical point of view there is quite a difference between large-scale culturing of a free-living unicellular eukaryotic microorganism and that of a mammalian myocyte that can only grow on a solid support, with many eukaryotic cell types intermediate between these two extremes. A good example are the insect cells that are extensively used for the heterologous overproduction of human proteins. This is routinely performed with the Sf 21 cell line that was originally prepared from the ovaries of Spodoptera frugiperda [10]. Scale-up of growth of these cells in chemically defined media is straightforward up to the m^3 scale and the technical facilities

required do not much extend beyond those for single-celled (eukaryotic) microorganisms. This is largely due to the considerable shear-stress resistance of these cells. Besides shear-stress resistance, two other major factors in the scale-up of the growth of animal cells is their dependence on (i) specific growth factors and (ii) a solid support.

Literature

1. Giesecke, D., S. Gaebler, and W. Tiemeyer, Purine Availability and Metabolism in Dogs Fed Single-Cell Protein or Rna. Journal of Nutrition, 1982. 112(10): p. 1822-1826.
2. Colla, L.M., et al., Production of biomass and nutraceutical compounds by Spirulina platensis under different temperature and nitrogen regimes. Bioresour Technol, 2007. 98(7): p. 1489-93.
3. Sikkeland, L.I., et al., Complement activation and cytokine response by BioProtein, a bacterial single cell protein. Clin Exp Immunol, 2007. 148(1): p. 146-52.
4. Berg, J.J., Tymoczko, J.L., Stryer, L., Biochemistry. Sixth Edition ed. 2006, New York: W.H. Freeman and Company.
5. Woodham, A.A. and E.M. Clarke, Nutritive value of mixed proteins. 2. As determined by net protein utilization and protein efficiency ratio tests. Br J Nutr, 1977. 37(3): p. 309-19.
6. Moon N.J., H.E.G., Glatz B.A., Conversion of cheese whey and whey permeate to oil and single-cell protein. Dairy Science, 1978. 61: p. 1537-1547.
7. Evenblij, M., Veroudering en DNA schade. Mediator, 2009. 20(3): p. 8-9.
8. Garinis, G.A., et al., Persistent transcription-blocking DNA lesions trigger somatic growth attenuation associated with longevity. Nature Cell Biology, 2009. 11(5): p. 604-U370.
9. Gudbrandsen, O.A., et al., Dietary single cell protein reduces fatty liver in obese Zucker rats. Br J Nutr, 2008. 100(4): p. 776-85.
10. Chan, L.C., P.F. Greenfield, and S. Reid, Optimising fed-batch production of recombinant proteins using the baculovirus expression vector system. Biotechnol Bioeng, 1998. 59(2): p. 178-88.

*essential amino acids: Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan, Valine

*semi-essential (in young children): Tyrosine, Cysteïne, Histidine, Arginine

List of speakers at the first in vitro meat symposium

This symposium was held at the Norwegian Food Research Institute (Matforsk), Aas, Norway, hosted by the Norwegian University of Life Sciences (UMB) and the Norwegian Food Research Institute (Matforsk), April 9-11 2008. The authors of this report are not included.

Expertise: Founder of New Harvest (www.new-harvest.org)
Jason Matheny, New Harvest & Johns Hopkins University, Baltimore, MD, USA

Expertise: Myogenic cells/Biotechnology
Prof. Dr. Tor Erling Lea
Prof. Dr. Stig W. Omholt
Norwegian University of Life Sciences, Aas, Norway

Expertise: Market motivation
Prof. Dr. Elizabeth L. DeCoux, Florida Coastal School of Law, Jacksonville, FL, USA

Expertise: Large scale fractionation of cell extracts
Dr. Arild Johannessen, International Research Institute of Stavanger, Stavanger, Norway

Expertise: Synthetic serum-free media for cell cultures
Dr. Kjell Bertheussen, University of Tromsø, Tromsø, Norway

Expertise: Bioreactors for tissue engineering
Prof. Dr. Jose Teixeira
Dr. Manuela Gomes, University of Minho, Braga, Portugal

Expertise: Muscle tissue engineering
Robert G. Dennis, UNC NCSU, University of North Carolina, Chapel Hill, NC, USA

Expertise: Experimental and theoretical biophysics of muscle tissue
Dr. Poul Nielsen, Department of Engineering Science, The University of Auckland, Auckland, New Zealand

Expertise: Large and complex bio-engineering plants
Dr. Gunnar Kleppe, Norwegian Bioindustry Association, Oslo, Norway

Participants in a European FP7 application (2009)

IMPROVESS (In vitro Meat PROduction is Vital for Environmental and Societal Sustainability); Call FP7-KBBE-2009-3; Activity 2:2 Fork to farm ; Subactivity KBEE-2-2-3 Sustainable food and feed processing. The authors of this report are not included.

Expertise: Biotechnology and society; Bioethics
Prof. Dr. Stellan Wellin
Prof. Dr. Anders Norgen
Dr. Maria Hilling
Linköping University, Linköping, Sweden

Expertise: Ethics in science and technology
Prof. Dr. Mathias Kaiser
National Committee for Research Ethics in Science and Technology
Bergen University, Bergen, Norway

Expertise: Food technology
Dr Johanna Berlin
Katarina Lorentzon
Dan Melin
SIK, The Swedish Institute for Food and Biotechnology, Göteborg, Sweden.

Expertise: Biophysics
Dr. Julie Gold
Prof. Dr. Peter Apell
Department of Applied Physics, Chalmers University of Technology, Göteborg, Sweden

Expertise: Innovation and development of polysaccharide products for biomedical applications.
Dr. Peter Fyhr
Dr. Bo Ekman
Magle. Magle is a privately held company with administrative headquarter in Lund, Sweden and its pharmaceutical manufacturing plant in Kristianstad, Sweden

Expertise: Function of markets and institutions in Europe
Dr. Georg Licht
Dr. Mark O. Sellenthin
Industrial Economics and International Management at the Centre for European Economic Research (ZEW), Mannheim, Germany

Expertise: muscle differentiation
Dr. Marinus F.W. te Pas
Animal Science Group of WageningenUR, Lelystad, The Netherlands

Publications about in vitro meat

links can be found on the following webpages:

http://invitromeat.org/content/view/27/45/

and

http://www.new-harvest.org/resources.htm

About the authors

Henk P Haagsman (1954) graduated from Utrecht University in 1978 with a degree in chemistry. He defended his thesis in January 1983. From 1978 onwards he teaches at the Faculty of Veterinary Medicine. In 1982 he started to work on the pulmonary surfactant system and studied mechanisms that cause alterations in the surfactant system in toxic and diseased states. As an experimental system he used isolated type II pneumocytes. This work was supported by a grant from the Netherlands Asthma Foundation. In 1985 he was the recipient of the Constantijn en Christiaan Huygens Award bestowed by the Netherlands Organisation for Scientific Research (NWO). This award, intended to save excellent scientists for the academia and given to only a few biochemists, enabled the recipients to do research and to teach for five years at universities of their choice. From 1986 to 1988 he worked as a visiting scientist at the Cardiovascular Research Institute, Faculty of Medicine, University of California at San Francisco, USA. Here he started his work on the structure and function of pulmonary surfactant proteins in the laboratory of Professor J.A. Clements. After his return he continued working on these proteins. His publications on this subject include more than 90 scientific papers. In 1998 he was appointed Professor of Meat Science at the Faculty of Veterinary Medicine of Utrecht University. From 1998 until 2003 he also had an appointment at the TNO Nutrition and Food Research Institute. From 2001-2005 he was a member of the University Council. Gradually the emphasis on applied meat research and regulation of muscle homeostasis shifted towards innate host defence and stem cell biology. His credo is: "Healthy animals, Healthy food, Healthy people".

Klaas J Hellingwerf (1950) graduated in 1975 from the University of Groningen with a degree in chemistry. He obtained his PhD degree in 1978 at the University of Amsterdam and subsequently became an assistant professor at the Department of Microbiology of the University of Groningen. In 1982 he was a visiting scientist at the Department of Physics and Biology of the University of California at San Diego, and in 1987 he was a visiting scientist at the Institute for Zoology of the Chinese Academy of Sciences, Beijing, China. From 1988 he is professor in general Microbiology at the University of Amsterdam. His research interests are signal transduction mechanisms and bioenergetics of pro- and eukaryotic microorganisms.

Bernard AJ Roelen (1968) studied Biology at Utrecht University where he graduated in 1992. He then worked as a PhD student at the Hubrecht Laboratory in Utrecht under the supervision of dr. Christine Mummery, and obtained his PhD degree in 1997. Subsequently he worked as a junior scientist at the same Hubrecht Laboratory until 2001. From 2002-2002 he worked as a post-doctoral researcher at Harvard University in Boston and after that at the Netherlands Cancer Institute in Amsterdam. In 2003 he was appointed assistant professor at the Faculty of Veterinary Medicine of Utrecht University. His research subjects are development of germ cells and stem cells.