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It’s all About Stem Cells

From modern medicine to novel foods

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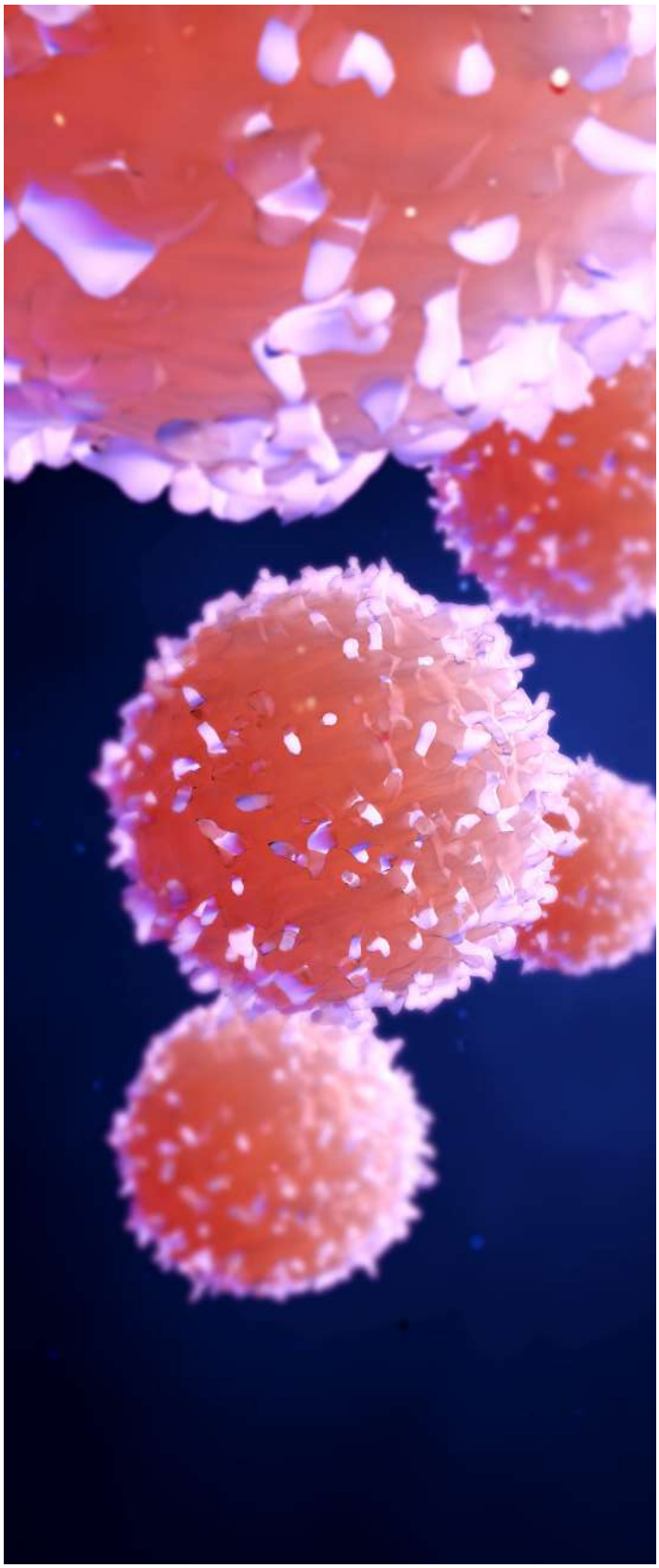

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WE CAN CHANGE THE WORLD

How Stem Cell Technology is revolutionizing 21st century industries from personalized medicine to meat production

ADDITIONAL CONTENT

- eBook: Bioprocessing Basics

Introduction

The term "stem cell" is a broadly encompassing designation, referring to an undifferentiated cell of a multicellular organism which possesses the capacity to generate more cells of the same type. Furthermore, stem cells, when appropriately activated, can produce a wide variety of differentiated cell types.

Today, three broad categories of mammalian stem cells are recognized. (a) Totipotent cells are found only in early embryos and possess the programing to produce a complete organism. (b) Pluripotent stem cells are more restricted in their potential abilities. They occur in the inner cell mass of the blastocyst and can form upwards of 200 different cell types present in the adult individual. (c) Multipotent stem cells are derived from fetal tissue, cord blood and adult stem cells. They have been employed for many years for the treatment of a wide range of blood disorders.

Our modern concept of stem cell biology is credited to the observations of Till and McCulloch in the early 60s, who provided the first evidence of the existence of blood stem cells. In the decades that followed an army of researchers and clinicians carried out volumes of work on the properties and possible applications of stem cells. So moving into the 21st century, they have become a vital tool in the treatment of disease. And this demand has accelerated the development of a wide choice of stem cell lines.

In this report we will consider bioreactors and their challenges, including the demand for stem cells in current medical science which calls for robust systems for their cultivation and handling. Stem cells can be obtained from donors but the actual numbers are quite low. A solution for the generation of adequate numbers is to scale of the culture of these cells *in vitro*.

Although it was commonly thought that stem cells could not be cultivated in stirred tank bioreactors dues to high shear stress, this was found to be incorrect. Thus today bioreactor technology, including critical culture parameters, bioreactor configurations, and pursuit of pioneering technologies in the bioprocess development stage is now hotly pursued.

Stirred-tank bioreactors of all sizes have been adopted for production of stem cells on a large scale. This report will compare them to uncontrolled systems such as shaker flasks and detail how they provide dramatic improvements for monitoring and control of key cultivation parameters. In this regard, facilitating scale-up and ensuring consistency throughout the project's development phases are of primary concern. Controlling agitation and gassing conditions will provide smooth performance while minimizing shear forces, essential for maximum yields of product.

The most dynamic sector of drug development is in the realm of biologics. Antibodies are perhaps the most important product, but many other protein-based therapies are recognized today for their contribution to successful new pharmaceuticals.

Personalized medicine today includes a range of agents that may be configured to fit individual histories. Much research is directed toward compartmentalizing patients to optimize their treatment with innovative protein therapeutics. In this regard, one of the most important current applications of stem cell technology is in the area of cardiovascular disease modeling. In particular, induced pluripotent stem cells (iPSCs) are under study as a platform to better understand cardiomyopathies, rhythm disorders, valvular and vascular disorders at the cellular level. They lend themselves especially as tools in the study of precision medicine and therapeutic screening. Because they can be expanded without limit in bioreactors, they provide a convenient source of experimental material to evaluate pharmaceutical agents on a cellular level, although this requires sub cloning and monitoring of their properties to ensure the absence of wanted variation in the lines over time.

Another technology under consideration are stem cell-derived neurospheres, of great interest to neuroscientists. Bioreactors may allow tight regulation of process parameters, thereby improving homogeneity of neural spheroid cultures for novel pharmacological devices such as Multi-Organ Chip systems.

In this time of rapid change there are dramatic application on the horizon for stem cell technology. One of the most important in terms of its impact on society is focused on cultured meat, which could have extraordinary economic, health, animal welfare and environmental benefits over traditional meat. Appropriate stem cell lines, grown in bioreactors, could in principle produce unlimited quantities of excellent quality meat products. When widely adopted, the commercial production of this new source of protein will upend whole industries, with dramatic effects on our entire society.

REFERENCES

1. Ramalho-Santos, M. and Willenbring, H. 2007. On the Origin of the Term "Stem Cell". Cell Stem Cell 1 (1) 35-38.

ADDITIONAL CONTENT

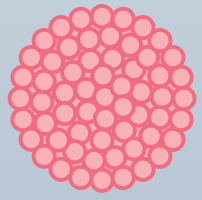
Our Top 5 List of Stem Cell Bioprocessing Papers:

1. Abecasis *et al.*, 2017.
2. Panchalingham *et al.*, 2015.
3. Kropp *et al.*, 2017.
4. Sart *et al.*, 2014.
5. Kempf *et al.*, 2015.

Stay Informed

Stem Cell Expansion in Bioreactors

Stem cell culture in stirred-tank bioreactors makes scale-up easier and allows comprehensive monitoring and control of parameters like temperature, pH, and dissolved oxygen. Here are some tips to help you transfer your stem cell culture from dishes and flasks to bioreactors.



Cell-only aggregate

Cells grown on microcarriers

1 Culture surfaces

In bioreactors, adherent stem cells can be expanded in suspension as cell-only aggregates or on microcarriers. The size of cell-only aggregates can be influenced by seeding density, stirring speed, and the bioreactor impeller design. Culture on microcarriers under restrictive cell culture conditions (e.g. a serum-free medium) requires coating them with peptides or proteins like fibronectin or collagen.

2 Inoculation

Some guiding values for culture on microcarriers:*

Description	Value
Cell seeding density	2,000–10,000 hMSCs/cm²
Microcarrier loading density	1–4 g dry beads/L
Cell-to-bead ratio	min. 3–5 cells/bead

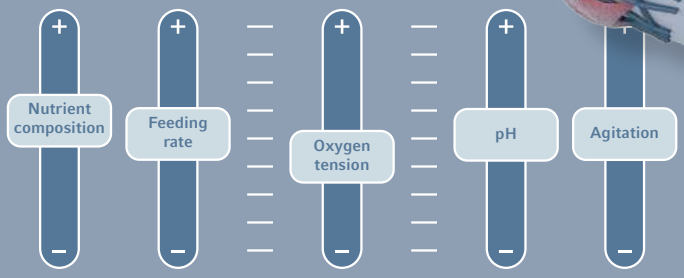
* Case-by-case optimization required

To improve cell attachment:
> Reduce initial culture volume
> Do not agitate during the first few hours

4 Case-by-case optimization needed

Due to cell heterogeneity (tissue sources, storage conditions, preexpansion conditions, culture medium, and others) and the large number of interactive process parameters (dissolved oxygen, pH, stirring speed, cell substrate, bioreactor type, and the like), each process will require individual optimization.

> Software-aided monitoring and control of critical process parameters helps to improve process stability and reproducibility.



3 Cell expansion

Bead-to-bead transfer: The progressive addition of fresh microcarriers increases the surface area for growth while avoiding dissociating cells from the beads (passage step).

> Visually monitor the carrier occupation percentage closely to determine the optimal timing for addition of fresh carriers.

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CONTROLLED, LARGE-SCALE MANUFACTURING OF hiPSC-DERIVED CARDIOMYOCYTES IN STIRRED-TANK BIOREACTORS

Yang Jiang, Karin Langenberg, Viola Borgdorff, Marta Duńska, Robert Post, Oscar Bartulos, Mieke Doornbos, Stefan Braam, Arie Reijerkerk

Abstract
Effective drug discovery and development relies in large part on the availability of predictive preclinical model systems. Application of human cellular models from tissues which are difficult to access, such as cardiomyocytes and neurons, is still challenging. Technologies based on human induced pluripotent stem cells (hiPSC) hold great promise to overcome this challenge. Their routine use in industrial drug research requires a constant supply of stem cell-derived cells of consistent high quality. Researchers from Ncardia® developed a bioprocess for the large-scale manufacturing of cardiomyocytes derived from a variety of healthy and diseased hiPSC lines for implementation into their DiscoverHIT platform.

They expanded hiPSCs as cell aggregates, in a DASbox® Mini Bioreactor System. Aggregate size, hiPSC-marker expression during the expansion phase, and differentiation to the desired cell type were reproducible in three batches. In a proof of concept study, the researchers scaled-up the process using a BioFlo® 320 bioprocess control system. The cells retained key iPSC markers during the expansion phase, providing confidence that stirred-tank bioreactors are suitable to scale-up the production of hiPSC-derived cellular models.

The production of several billion cardiomyocytes per batch will enable the screening of large libraries of compounds against phenotypic cellular models representing human biology.

Introduction

Human stem cell technologies enable an end-to-end solution for efficient high-throughput phenotypic screening in drug discovery and development. State-of-the-art hiPSC technologies allow the generation of relevant human cell models for diseases derived from virtually any patient. HiPSC-based cellular assays for drug research need to be robust and reliable, and compatible with automated high throughput screening (HTS) platforms. To mass produce such assays, hiPSCs need to be expanded and differentiated into the desired cell type in a reproducible and scalable manner.

Stem cell characteristics and differentiation are influenced by the culture conditions, as defined by the medium composition, physiochemical environment, physical forces, cell-cell and cell-matrix interactions, and others. The ability to control and reproduce culture conditions is therefore critical to obtaining a standardized cell population. Cultivation in bioreactors opens up new possibilities for process monitoring and control compared with conventional cell culture flasks. pH, temperature, and DO, among other parameters, can be monitored and controlled in real time, making it easier, on the one hand, to achieve conditions resembling the physiological situation, and on the other hand, to reproduce cultivation conditions from batch to batch.

Transfer of cell-based assays from the development phase to commercial production requires producing more cells with the same characteristics. When using cell culture plates or flasks, increasing the amount of cells usually involves "scale-out", meaning increasing the number of culture vessels. Stirred-tank bioreactors also allow for "scale-up", meaning increasing the culture volume by using larger vessels. Stirred-tank design allows similar vessel geometries and capabilities at different scales, facilitating a smooth transition to larger volumes.

Ncardia
Stem cell experts

Ncardia is a stem cell drug discovery and development company developing highly predictive human cellular (disease) assay systems for drug discovery. Its DiscoverHIT is a full service HTS-screening solution based on human induced pluripotent stem cell technology, comprising disease model development, manufacturing, assay development, and high throughput screening[1].

ADDITIONAL CONTENT

- Webinar: From Small-scale Stem Cell Production to Large-scale hiPSC-based Drug Development
- AppNote: Scalable Expansion of Human Pluripotent Stem Cells in Eppendorf BioBLU® 0.3 Single-Use Bioreactors
- AppNote: Production of Human Induced Pluripotent Stem Cell-Derived Cortical Neurospheres in the DASbox® Mini Bioreactor System

To enable robust and scalable production of cardiomyocytes for cardiovascular high-throughput screening applications, Ncardia has implemented a bioprocessing pipeline comprising state-of-the-art bioreactor systems which allows them to optimize processes at small scale, to validate the most promising conditions at mid scale of 100–250 mL, and to bring the process to the necessary 1–10 L scale. The resulting process provides a relatively mature cardiomyocyte model, recapitulating a human cardiomyocyte's contractile and electrophysiological profile with high predictivity in cardiac toxicity and efficacy assays.

With the current results, Ncardia demonstrates robust large-scale manufacturing of functional human cardiomyocytes derived from a diverse set of (disease) hiPSC lines.

Material and Methods

For a detailed method description and more information, please download the full-length application note 409.

HiPSC lines

Throughout the study, researchers at Ncardia used their standard hiPSC line for commercial manufacturing of cardiomyocytes, a patient-derived line with a homozygous mutation in KCNQ1 (potassium voltage-gated channel subfamily Q member 1), a CRISPR/Cas9-corrected version of this line, and a patient-derived line with a mutation in MYH7 (myosin heavy chain 7).

Bioprocess systems

Researchers at Ncardia used differently scaled bioreactors for process development and scale-up. They used a DASbox Mini Bioreactor System (Eppendorf) for the validation of the most promising conditions at small scale (250 mL). For manufacturing at the 1 to 10 L scale, they used a BioFlo 320 bioprocess control station (Eppendorf). The DASbox system and the BioFlo 320 were equipped with BioBLU® c Single-Use Vessels (Eppendorf).

HiPSC expansion and cardiomyocyte differentiation

Researchers at Ncardia cultivated hiPSCs and hiPSC-derived cells as cell aggregates. They applied their proprietary differentiation methodology for directed differentiation of hiPSC-derived cardiomyocytes. The workflow for hiPSC expansion, cardiomyocyte differentiation, cryopreservation, and functional testing is represented in Figure 1. At all steps, fully defined media free of serum and growth factors were used.

Scale-up of hiPSC production and differentiation

To further demonstrate the robustness of the hiPSC-derived cardiomyocyte manufacturing process, the researchers scaled it up from the DASbox Mini Bioreactor System, with a working volume range from 100 mL to 250 mL, to the BioFlo320 bioprocess control system equipped with a BioBLU 3c Single-Use Vessel (working volume from 1.25 L to 3.75 L). Culture media and bioprocess parameters, like tip speed, pH, temperature and DO setpoints, were translated from small to bench scale without major modifications.

Results

hiPSC expansion in the DASbox Mini Bioreactor System

Researchers at Ncardia expanded their hiPSC line for cardiomyocyte manufacturing in a DASbox Mini Bioreactor System equipped with BioBLU Single-Use Vessels. Bright field images of cell aggregates sampled just before start of cardiomyocyte differentiation revealed the formation of spheroids with a diameter of 50–100 µm in multiple batches (Figure 2A). More than 90% of the cells expressed the stem cell markers SOX-2, OCT-3/4, and NANOG (Figure 2B).

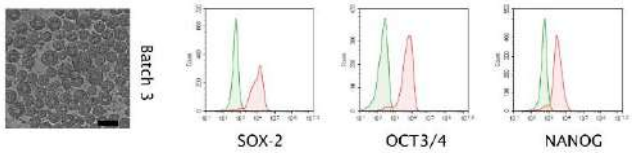

Figure 2: Characterization of hiPSC aggregates and stem cell cardiac marker expression in DASbox bioreactors. A: Phase contrast images of hiPSC aggregates. B: FACS profiles of hiPSC marker expression at the start of pre-cardiac specification phase.

Cardiac differentiation

The fate of pluripotency, pre-cardiac, and cardiac markers during the whole differentiation process was assessed in more detail by flow cytometric measurements (data not shown).

At the time of inoculation and after expansion in the bioreactor, high levels of pluripotency markers were observed. Upon start of differentiation into the pre-cardiac lineage, expression of the stem cell marker SOX-2 rapidly declined and was almost absent after one day of culture. OCT-3/4 was completely diminished after three days from start of differentiation. The development of cardiomyocytes was demonstrated by the appearance of the cardiac marker cTNT, reaching maximum levels at the day of harvest.

Ncardia's quality control standard is >70% batch purity with respect to cTNT. Successful cardiomyocyte production meeting this standard was achieved in batches with a cTNT levels of >40% at an intermediate culture time point. This result provides a means to abrogate unsuccessful productions already at an early stage. Thus far, Ncardia's selected process has resulted in the generation of multiple cardiomyocyte batches with a mean purity of 87.4 ± 5.9% and a mean yield of 1.05 ± 1.11 x 10^6/mL.

Functional characterization of bioreactor-derived cardiomyocytes

First, the quality of bioreactor-derived cardiomyocytes was confirmed by the presence of beating cardiomyocyte monolayers expressing

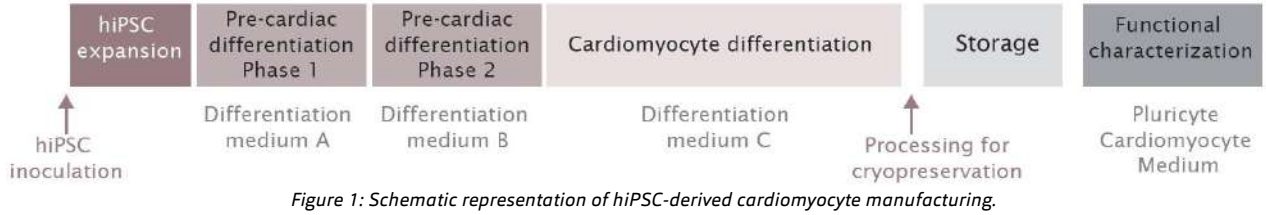

Figure 1: Schematic representation of hiPSC-derived cardiomyocyte manufacturing.

ventricular-like cardiomyocyte markers (cTNT and MLC2v) after 8 days of culture. 91.7 ± 1.5% of cultured and dissociated cells from the three batches were cTNT positive. There was a high level of cTNT+MLC2v+ cells (84 ± 5.6%), indicating the maintenance of a pure and ventricular-like cardiomyocyte phenotype. Immunofluorescence analysis of cTNT and alpha-actinin showed a high degree of ultra-structural organization in the cells (Figure 3).

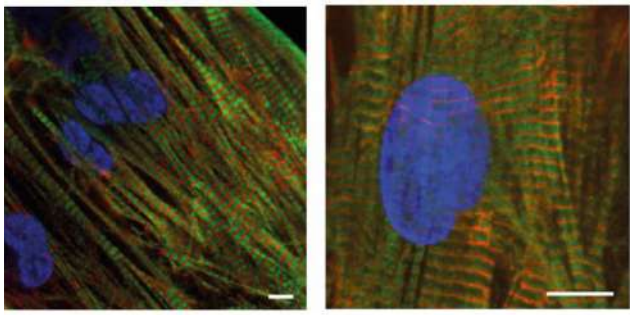

Figure 3. Phentotypic characterization of hiPSC-derived, cryopreserved cardiomyocytes produced in bioreactors. Immunofluorescent staining. Alpha-actinin in red, cTNT in green, nuclei in blue, scale bar: 5 µm.

Second, the quality of the cells in the three batches was corroborated by electrophysiological properties. Bioreactor-derived cardiomyocytes showed reproducible field potential signals with pronounced de- and repolarization peaks which allowed accurate assessment of beat rate and field potential duration. The mean beat rate was very regular at 2.1 ± 0.14/s (irregularity in each batch was <2%), mean field potential duration was 560 ± 47 ms, and mean sodium spike amplitude was 1.7 ± 0.1 mV (Figure 4A–D).

Third, the responses to a set of model compounds which mainly target beta-adrenergic receptors and sodium, calcium and potassium channels of the hERG-type, respectively was assessed. The concentration-dependent response to these cardioactive reference compounds further confirmed the functionality of bioreactor-derived cardiomyocytes. The beta-adrenergic receptor agonist isoproterenol enhanced beat rate, the sodium channel blocker mexiletine reduced sodium spike amplitude and prolonged field potential duration (FPD), the Ca_2+ channel blocker nifedipine decreased FPD, hERG channel blockers dofetilide and E4031 prolonged FPD and caused TdP-like arrhythmias (Data available in the full length application note 409).

Cultivation of other (disease) hiPSC lines in stirred-tank bioreactors

Ncardia's DiscoverHIT Platform offers the creation of disease models using advanced gene editing technologies. To realize full potential of hiPSC-based disease models in phenotypic screening, broad application of Ncardia's manufacturing process is a critical component. An efficient workflow requires fast implementation and scale-up of differentiation from any hiPSC line relevant for the disease or application of interest. This can only be done with a robust manufacturing approach. In general, the majority of published protocols for cardiomyocyte differentiation from hiPSCs requires cell line- and cell culture-dependent optimization and can easily lead to heterogeneous differentiation results[1,2]. To further assess the robustness of their protocol for a bioreactor-based manufacturing process, the researchers tested it for the expansion of a patient-derived line with a homozygous mutation in KCNQ1, a CRISPR/Cas9-corrected version of this line, and a patient-derived line with a mutation in MYH7. The three lines were maintained, inoculated, and differentiated into cardiomyocytes in BioBLU 0.3c Single-Use Vessels using the bioprocess conditions and culture media selected for commercial manufacturing of the standard hiPSC line, without any modification. After differentiation, cardiomyocytes were obtained by enzymatic dissociation of cell aggregates and single cells were cryopreserved using Ncardia's standard procedure. Cardiomyocyte purity in batches derived from the different hiPSC lines were 91%, 90%, and 70% as assessed by cTNT analysis by flow cytometry (Figure 5A). The quality of bioreactor-derived cardiomyocytes was confirmed by the presence of beating monolayers after 8 days culturing in Pluricyte Cardiomyocyte Medium according to Ncardia's standard protocols. FACS analysis of these cultured cardiomyocytes obtained from the three hiPSC lines revealed high levels of cTNT+ MLC2v+ cells (Figure 5B), indicating the maintenance of a pure and mainly ventricular-like cardiomyocyte phenotype. Phase contrast imaging at this time point revealed a confluent monolayer of beating cardiomyocytes (Figure 5C).

Bioprocess scale-up

To further demonstrate the robustness of the hiPSC-derived cardiomyocyte manufacturing process, the researchers scaled-up the expansion and differentiation of a selected hiPSC line from a BioBLU 0.3 c Single-Use Vessel (working volume 100–250 mL) to a BioBLU 3c Single-Use Vessel (working volume 1.25 L–3.75 L). In-process assessment of previously identified intermediate critical quality attributes revealed high expression levels of pluripotency marker genes (SOX-2, OCT3/4 and NANOG) before induction of cardiomyocyte differentiation and intermediate cTNT levels of 63%. Final batch size was 7.5 x10^9 (2.5 x 10^6/mL, conversion rate 12.5) comprising 87% cTNT+ cells (Figure 6). Further characterization of the cells upon 8 days culture in Pluricyte Cardiomyocyte Medium showed a confluent monolayer of beating cardiomyocytes and high levels of cTNT+ MLC2v+ cells, indicating the maintenance of a pure and ventricular-like cardiomyocyte phenotype (Figure 6D, E).

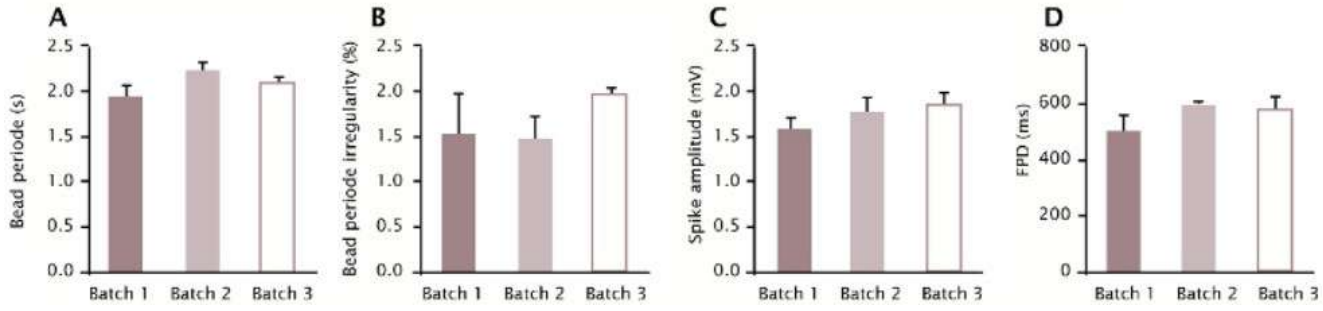

Figure 4. Functional characterization of three batches of hiPSC-derived, cryopreserved cardiomyocytes produced in bioreactors. A–D: Functionality of the cardiomyocytes was assessed in Maestro MEA system at different timepoints after plating. A: Beat period. B: Beat periode irregularity. C: Sodium spike amplitude. D: Field potential duration (FPD).

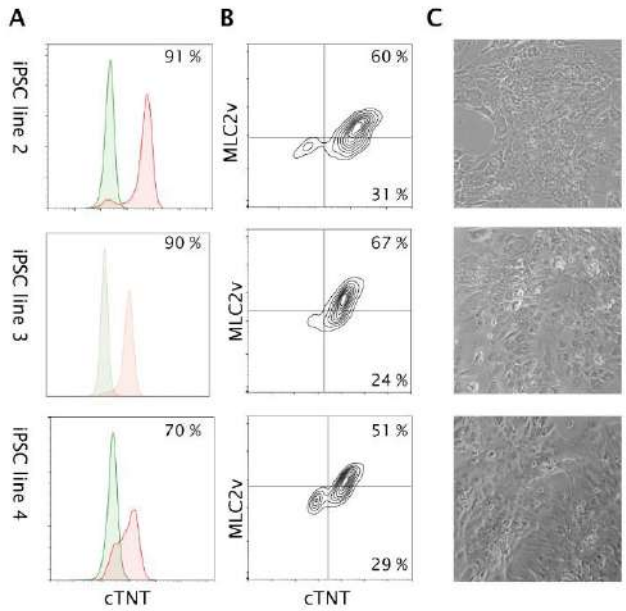

Figure 5: Characterization of cardiomyocytes derived from three additional hiPSC lines. A: Percentage of cTNT+ in three cryopreserved batches B: cTNT+/ MLC2v+ co-expression and C: Phase contrast images of hiPSC-derived cardiomyocytes 8 days after culture in Pluricyte Cardiomyocyte Medium

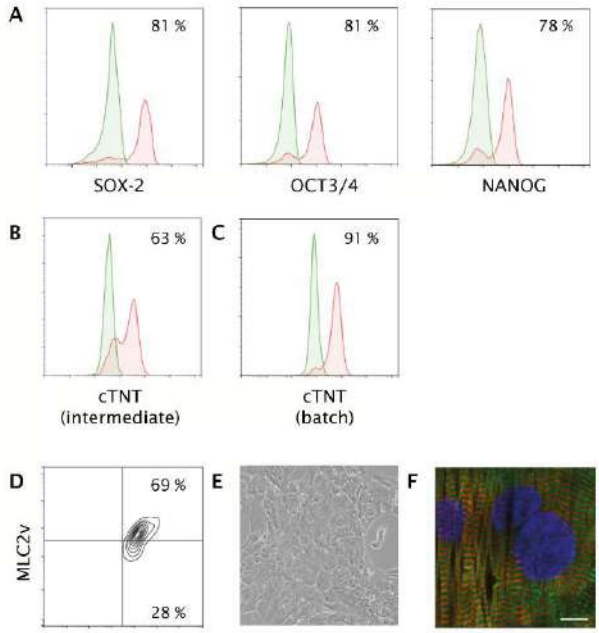

Figure 6: Fast upscaling of Ncardia's stirred-tank bioreactor process using a selected hiPSC line in Bioflo 320 bioprocess control station. A: FACS profiles of hiPSC marker expression at the start of pre-cardiac specification phase. B: FACS profile of intermediate cTNT. C: FACS profile of final cTNT. D: FACS plot showing cTNT+/MLC2v+ coexpression, E: Phase contrast image and F: Immunofluorescence staining of alpha-actinin (red), cTNT (green) after 8 days culture of a batch of Bioflo 320 bioreactor-derived iPSC-cardiomyocytes. Nuclei are in blue; scale bar represents 5 µm.

Immunofluorescence analysis of cTNT and alpha-actinin showed a high degree of ultra-structural organization in the cells (Figure 6F).

Conclusion

Using a controlled, stirred-tank bioreactor approach Ncardia manufactures functional human cardiomyocytes derived from a diverse set of (disease) hiPSC lines at large scale. This enables the high-quality and large-scale manufacturing of cardiovascular cells required for phenotypic screening of potential drugs in any relevant biological system at a high throughput. Ncardia's fully controlled bioprocess for manufacturing of hiPSC-derived cardiomyocytes (serum free, growth-factor free differentiation) is highly reproducible in independent batches originating from one hiPSC line. The proprietary process was successfully used, without modifications, for manufacturing of cardiomyocytes from any other hiPSC line tested. With this, the manufacturing process has great potential for the development of a variety of disease-specific models (patient-derived or genetically introduced) for use in Ncardia's DiscoverHIT Platform. The yield of ~1 x 10^6 hiPSC-derived cardiomyocytes per milliliter in each bioreactor showed that the process is capable of generating large-scale batch sizes in a robust manner. Ncardia's bioprocess capabilities allowed easy and fast upscaling to 10^{10} hiPSC-derived cardiomyocytes per batch, enabling application in high-throughput phenotypic drug discovery campaigns.

This, in combination with a quality-by-design approach, will allow fast process development for manufacturing of other hiPSC-derived cell types and use in DiscoverHIT Platform applications.

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4. Sanchez-Freire et al. Effect of human donor cell source on differentiation and function of cardiac induced pluripotent stem cells. J Am Coll Cardiol. 64(5):436-48. Aug 5, 2014

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STEM CELL EXOSOME PRODUCTION ON THE SCIVARIO® TWIN BIOPROCESS CONTROLLER

Jorge L. Escobar Ivirico and Ma Sha, Eppendorf, Inc., Enfield, CT, USA

Abstract
Exosomes derived from mesenchymal stem cells (MSC) are a new alternative to restore tissues and organs, avoiding the limitations associated with stem cell therapy. Therefore, increasing the mass production of exosomes in a controlled environment is the necessary next step. In this study, we used a combination of SciVario® twin bioreactor control system and BioBLU® 1c Single-Use Vessels for large-scale exosome production in bioreactors.

Introduction

Regenerative medicine is a multidisciplinary field that engineers the structure and function of tissues and organs. Due to their ability to migrate to the site of injury and promote tissue regeneration through paracrine factors (secretome), mesenchymal stem cells have become the most widely used stem cell type for such investigations[1–3]. However, problems associated with inadequate cell localization and low cell survival rate within the target tissue make the MSC less attractive.

Recently, paracrine factors have attracted increasing interest due to their potential at overcoming the limitations of MSCs. Extracellular Vesicles (EV), including exosomes, are one of the most important paracrine effectors involved in intracellular communication and trafficking[4].

Exosomes are lipid bilayer vesicles with a diameter ranging from 30 to 200 nm, which can be easily confirmed by surface markers such as CD9, CD63, CD81 of tetraspanins[5]. They offer several advantages compared to cell-based approaches, including exceptional stability and biocompatibility. Moreover, they can be delivered to target tissues easier than whole cells and can migrate across the blood brain barrier[6]. In addition, since exosomes can conduct immune modulation, there is much less risk for tumor formation or inhibition of inflammatory cell migration[7,8]. Moreover, the lack of complex metabolism of exosomes and the influence of the environment on target tissues reduces complications upon use.

ADDITIONAL CONTENT

- Poster: The Potential of Stirred-Tank Bioreactors in Exosome Production
- White Paper: Controlled Cultivation of Stem Cells
- Webinar: Stem Cell Derived Exosome Production in Stirred-Tank Bioreactors

In this study, we produced human Induced Pluripotent Stem Cells (hiPSC)-derived MSC exosomes using a SciVario twin bioreactor control system equipped with BioBLU 1c Single-Use Vessels. We analyzed cell growth, viability, and metabolic activity, as well as the exosomes abundance at different times through CD63 Elisa assay.

Material and Methods

Cell culture in T-flasks and multilayers flasks
For bioreactor inoculum preparation, we cultured iPSC-derived MSCs (ATCC®, ACS-7010™) in T-flasks and multilayer flasks as described in the Eppendorf application note 435[9].

Microcarriers preparation
We used collagen-coated cross-linked polystyrene microcarriers (Pall Corporation, C-221–020) as support matrix for stem cell culture in bioreactors. Preparation of the microcarriers is described in Eppendorf application note 435.[9]

Bioreactor control system, vessel, and process parameters
We used the SciVario twin bioreactor control system to perform medium exchange-batch culture (Figure 1). We equipped the control system with BioBLU 1c Single-Use Vessels with a single pitched-blade impeller. The vessels were equipped as described[9]. We placed the assembled vessels in their respective temperature control blocks to maintain constant temperature. Finally, we introduced the DMEM/F12 complete medium or ATCC complete medium[9] into each vessel and conditioned the vessels for at least 24 hours under the parameters and setpoints listed in Table 1.

Cell culture in BioBLU 1c Single-Use Vessels
We harvested cells from multilayer flasks, seeded them onto collagen-coated microcarriers, and transferred them to a BioBLU 1c Single-Use Vessel as described[9]. We set the overlay N_2 gas flow

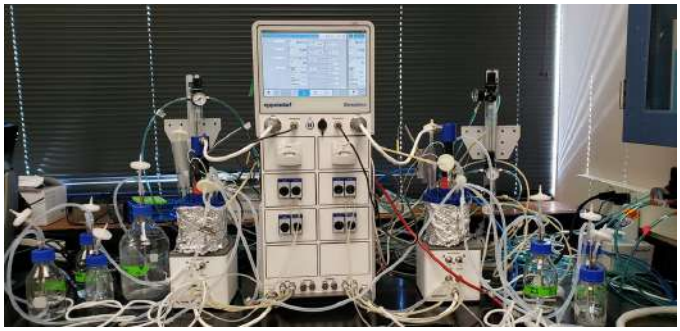

Figure 1: Human iPSC-derived MSC exosome production in a medium exchange-batch culture using the SciVario twin bioreactor control system and BioBLU 1c Single-Use Vessels.

	First Experiment	Second Experiment
Parameters	Setpoints	
Starting volume	700 mL	
Ending volume	1 L	
Initial agitation	80 rpm (0.2 tip speed)	
Temperature	37 °C	
Inoculation density	3 x 10^4 cell/mL	10.4 x 10^4 cells/ mL
Cell culture medium	DMEM/F12 medium	ATCC complete medium
DO Setpoint	40% (P=0.1; I=0.001)	
pH Setpoint	7.2 (deadband = 0.1), cascade to CO_2 (acid) cascade to 0.45 M sodium bicarbonate (base)	7.6 (deadband = 0.1), cascade to CO_2 (acid) cascade to 0.45 M sodium bicarbonate (base)
Overlay N_2 gas flow	0.20 SLPM	0.25 SLPM
Gassing range	0.1 SLPH-30 SLPH	
Gassing cascade	Set O_2 % at 30 % controller output to 21 % and at 100 % controller output to 21 %. Set flow at 0 % controller outputto 0.5 SLPH, and at 100 % controller output to 30 SLPH.	

Table 1: Process parameters and setpoints

between 0.2 and 0.25 SLPM to maintain the DO level at 40%. We cultivated the cells using the setpoints described in Table 1 and as described in more detail in Eppendorf application note 435.[9]

After day 5 of cell culture, we performed a medium exchange every two days until day 9 and then on a daily basis as required. Moreover, in the experiment using ATCC complete medium, additional glucose solution with a concentration of 1 g/L was added on day 11 of the culture to the fresh medium to increase glucose levels in the cell culture with subsequent medium exchanges.

Exosome collection and purification

After day 5, 8, 11 and 14 of culture, we collected 50 mL of iPCS-derived MSC/microcarriers/medium as described[9].

We enriched exosomes from the culture medium following the exosomes enrichment by the ExoQuick-TC PLUS protocol with some modifications[9]. We quantified tetraspanin-containing exo-somes through ExoELISA-ULTRA CD63 Kit (System Bioscience, EXEL-ULTRA-CD63-1)[9].

Cell viability and metabolic activity

We collected a sample every day in the course of the experiment using DMEM/F12 complete medium or every two days in the experiment using ATCC complete medium from the bioreactors to determine metabolites concentrations (glucose, ammonia, and lactate) using a Cedex® Bio Analyzer (Roche).

After detaching the cells from the microcarriers using trypsin, we measured the cell viability and density using a Vi-Cell® XR Viability Analyzer (Beckman Coulter).

We analyzed the morphology of the human iPSC-derived MSCs growing on microcarriers during the cell expansion process using bright-field microscopy[9].

Results and Discussion

After the initial expansion of the cells in T-flasks culture conditions, we analyzed their stemnnes capacity by flow cytometry noting that they displayed the typical phenotype of MSCs. Specifically, cells were positive for CD90 and CD29 (typical MSC markers) and negative for hematopoietic markers such as CD34 and CD11b[9].

Next, we established the optimal conditions for iPSC-derived MSC culture in BioBLU 1c Single-Use Vessels. In the first experiment we used DMEM/F12 complete medium as the cell culture medium for initial expansion and bioreactor culture. We inoculated the BioBLU 1c Single-Use Vessel at an initial cell density of 5 x 10^3 cells/cm^2 (3 x 10^4 cells/mL). We added the anti-foaming agent Pluronic®- F68 surfactant (0.1%) to the medium.

After the initial cell expansion, the cell count was erratic, especially after each collection day[9]. We attributed this behavior to the low inoculation density and the use of DMEM/F12 cell culture medium whose composition may be suboptimal for iPSC-derived MSC expansion. Overall, cell growth increased 4-fold more than the initial cell density on day 15 of culture, but the final density was low.

We performed a second experiment to increase the iPSCs-derived MSCs inoculation density and the exosomes production yield from each sample. To achieve that, we changed the cell culture conditions of the iPSC-derived MSC relative to the first experiment. First, we performed the cell expansion and the initial cell attachment to the microcarriers in ATCC complete medium. Then, we increased the inoculation cell density to 17 x 10^3 cells/cm^2 (10.4 x 10^4 cells/mL), maintaining the microcarrier concentration at 17 g/L. We kept the rest of the conditions the same as for the first experiment, with the exception that the

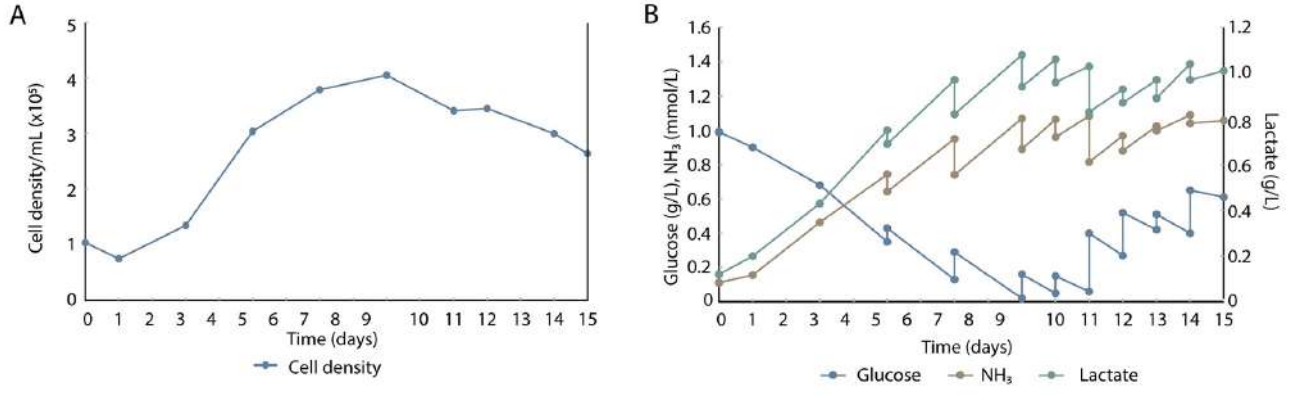

Figure 2: iPSCs-derived MSCs growth profile in BioBLU 1c Single-Use Vessel with ATCC complete medium. A: iPSCs-derived MSC density. B: Metabolic profile.

overlay gas flow was increased to 0.25 SLPM to provide a better balance to the minimum air flow. We performed a medium exchange daily starting from day 5 of the culture. We observed an initial lag phase 24 hours after the inoculation followed by a steady increase of cell growth between days 1 and 9 of culture.

We then determined the consumption of glucose and production of lactate and NH_3 while maintaining the concentration of lactate and NH_3 below 1.2 g/L and 1.2 mmol/L, respectively during the whole run (Figure 2). The glucose level was significantly lower in the ATCC complete medium than in the DMEM/F12, necessitating the addition of 1 g/L of glucose.

The culture reached the stationary phase around day 9, followed by a subsequent decrease in cell density until day 15 (Figure 2). At day 9, the iPSC-derived MCS reached a maximum cell density of 4.1 x 10^5 cells/mL. However, we observed substantial microcarrier aggregation later during the run. Therefore, we based the cell count in the later stages on only the floating microcarriers, thus the true average of the cell expansion in the vessel is undoubtedly higher.

At day 5, 8, 11 and 14, we collected the exosomes from the ATCC complete culture medium containing ED-FBS and purified them using the ExoQuick-TC PLUS kit. As shown in Figure 3A, exosomes are highly enriched in proteins in which tetraspanins (membrane proteins: CD63, CD9, CD81, CD82) play a key role in cell invasion, penetration and fusion events. We used a direct Enzyme-Linked ImmunoSorbent Assay (ELISA), specifically the ExoELISA-ULTRA CD63 Kit to quantify the exosome abundance. The results show that the number of iPSC-derived MSC exosomes constantly increased from day 5 (2.6 x 10^{10}) to day 16 (8.6 x 10^{10}, Figure 3B). In addition, we found a direct correlation between cell density and secreted exosomes up to day 9 while the decrease in cell density in the bioreactor did not influence the exosome secretion after the stationary state.

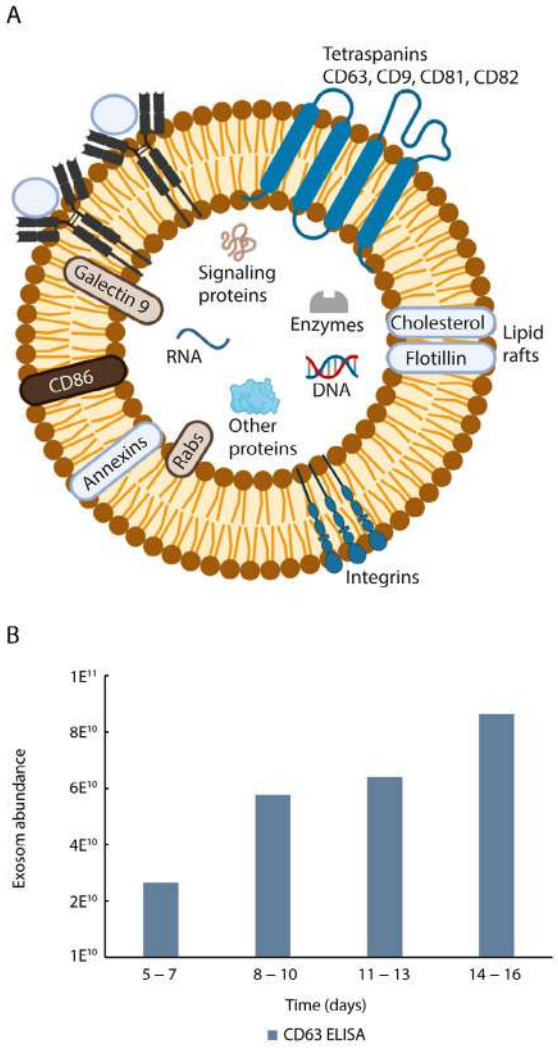

Figure 3: A. Schematic representation of an exosome composition. (Created with BioRender.com) B. Exosome abundance secreted by iPSC-derived MSC on each collection period.

To study the three-dimensional cell morphology on collagen coated microcarriers, we collected samples at days 5 and 9 of culture and visualized them through bright-field images. Taking into account the limitations of using bright-field systems for live cell imaging, we evaluated the morphology of human iPSC-derived MSC, demonstrating that at the early stage of the cell growth profile, a few cells attached on the microcarriers surface and progressively formed inter-microcarriers cellular bridges and aggregates.[9]

Conclusions

We have established the feasibility of producing MSC-derived exosomes using the SciVario twin bioreactor control system and BioBLU 1c Single-Use Vessels. The design of the control system allows the precise manipulation of the cell culture environment, leading to rapid adhesion and proliferation of human iPSC-derived MSC to the microcarrier's surface. These experiments are preliminary studies and have not yet been optimized to ascertain the maximum exosome production levels. However, our observations can serve as a guideline for further improvements in MSC-derived exosomes isolation, purification, and scale-up protocols.

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CELLULAR AGRICULTURE – THE VERSATILITY OF STEM CELLS

Dr. Mark Post, MD, PhD, received his medical degree from the University of Utrecht in 1982 and, at the same University, his PhD in 1989. In his scientific career, he conducted research in the field of vascular biology and more specifically on neovascularization at the Royal Dutch Academy of Science and the Harvard Medical School, Boston, MA . Since 2002, Dr. Post was appointed as a professor for Vascular Physiology at the Maastricht University and of Physiology at the Maastricht University. His main research interests are vascular biology and tissue engineering of blood vessels and skeletal muscle. These subjects are studied from their basic molecular aspects and cellular mechanisms up to preclinical models and eventually, patients and consumers. In addition, he pioneered the creation of meat from stem cells and presented the world's first hamburger from cultured beef in 2013. As a result, he was awarded the World Technology Award for solutions that benefit the environment at the World Technology Network summit in 2013. He recently co-founded and is CSO of Qorium and Mosa Meat, two start-ups respectively commercializing the technologies to produce bovine leather and cultured meat using tissue engineering.

Q: Where do you see the potential of stem cells outside of the clinical sector?

Mark Post:
If you ask me, the biggest application for stem cells is obviously not going to be in the medical area. I'm a medical doctor by training, so I started thinking mostly about the medical applications. But I think the non-medical applications probably are going to be much bigger. I think the cellular agriculture applications are really exciting and have the potential to outgrow the medical applications by far. What we are doing now will not only have a social impact, e.g. how we see food or agriculture. It will also have a tremendous impact on the clinical sector and even kind of all the boundary conditions that the production system has to take into account.

Q: In which way will your research influence stem cell-based research and development?

Mark Post:
The major challenge in working with stem cells are the high costs. The average working volume for cell and gene therapy applications is in the range of a few liters but can cost already around 200.000€. To produce enough cultivated meat in stirred-tank bioreactors we are talking of working volumes of several thousands of liters. If you start thinking about that problem and you start thinking about the scale that is required for the production, you immediately see that there is quite a disconnect from the medical field in terms of scale and in terms of cost considerations that you need to have. I believe that this will challenge all of us, especially companies such as the growth factor producers or things that are traditionally developed in the pharmaceutical industry or in Advanced Therapy Medicinal Products (ATMP) production.

All those concepts will be challenged by these cellular agricultural applications in terms of scale, cost, but maybe also really on how things are done. When you start to force yourself to work with a platform that is very costly already at small scale, but you are aiming for lower costs and way larger volumes, you need to start thinking about: How am I going to source the feedstock for the cells? How am I going to source the bioreactors for the cells? Do we really need to adhere to those stringent pharmaceutical criteria for all these components, or can we get this to work in a more food or feed grade system?

Q: Especially the costs are a major challenge on the way to commercialize lab grown food. One of your major focus is to reduce the costs of the growth media. How will this affect the medical field?

Mark Post:
If you think of the costs of current gene therapy treatments with

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regulatory T-cells for example, these can lay in the range of 220,000€ per treatment, which is just crazy. I cannot really analyze the costs, but if you just think of the costs of the growth factors in the medium which are sold for a million euro per gram, whereas enzymes in the feed industry in a fermentation setting are produced for less than 10€ per gram. It is of course not the same protein, but it is a similar one, produced with the same technology. How can it be that one costs 1 million euro and one just 10? With our research, we are challenging that part of the industry. They have high-quality products and I believe that they have been doing the right thing, but they now see that they are going to have competitors who produce a very similar product with similar effectivity for one thousand of the price. Most of us will benefit from this development. Think of the costs of a regulatory T-cell therapy treatment – In a few years from now, this will cost only 10.000€ instead of 220.000€.

Q: Outside of the clinical sector, how can stem cells revolutionize, how we see food?

Mark Post:
There is already a lot of technology in the food and agricultural industry, but people don't necessarily realize this. Let's take the example of milk: The milk you buy in a supermarket is seen by most people as the product that comes out of a cow, but of course, anybody who is working in the food and feed industry knows that the thing that comes out of the cow is just an ingredient of milk, and perhaps not even the most important one.

With stem cells, or cellular agriculture, for instance, we are using recombinant proteins or stem cells to create a tissue that you can eat instead of having to grow in a cow. This is a scary concept for people, or at least an unfamiliar concept. But at some time, they will get familiar with it. In my vision, in 20–30 years from now, I believe that we will all eat meat that was primarily made in a setting outside of a cow, based on stem cell technology. This will enable us to implement a lot of standardizations, and recycling steps that we just cannot do with living organisms, which are mostly very inefficient in converting feed into proteins.

Q: What are the challenges and limiting factors in the development of lab grown meat?

Mark Post:
It will take a lot of more time and efforts to bring lab grown meat to the market. One of the major challenges will be the scaling issue. If you think of cell therapy applications, working volumes in the low liter range are sufficient, but one hamburger has 10 billion cells. We need to scale up the production tremendously and this process needs to be very robust and industrial. This is the point where bioreactor design and process optimization come into play. There's a need for technologies to develop single-cell suspension or maybe modified planar systems like packed-bed bioreactors. So far, working in these large volumes where you harvest the cells has never been done in that scale with mammalian cells. The yield of course depends on the achieved cell density per mL in the bioreactor, which is currently in a range of 10.000.000 cells per mL, but could be even higher if the cells are grown as aggregates. Despite of the scaling issue, there are innovations missing for the large scale-differentiation of the stem cells. The end-product consists of differentiated cells which already are in the form of tissues. You want to define how your tissue is shaped, how big it is, and how it is being made. For this, you need customized solutions, which are not available on the market yet. And this part also needs to be automated, so you need to think about automated tissue dispensing, whether this is 3D printing or extrusion or basically injecting and assembling that at high density into a bioreactor.

Q: Do products of the cellular agriculture fall under special regulations?

In Europa it is considered a novel food, which means that safety has to be demonstrated and accepted. This is uncharted territory. Nobody has ever done that, also the European Food Safety Authority (EFSA) has never done this before. They also do not exactly know exactly what they need to do and what they need to know. But fortunately, Europe has defined the pathway of regulatory approval quite extensively and with that are ahead of most other nations. The regulatory guidelines mostly focus on product composition and on the process itself. The production process needs to be described and defined. But there will be additional questions since everyone in this field is learning right now.

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