Final report of HESUB – FP7 – #601700

Overview of HESUB final achievements

Stem cells bear an enormous promise for future therapy and have already shown their efficacy in numerous clinical trials. Today's research in stem cell-based therapy focuses on identifying the appropriate cells with the targeted properties of biological efficacy, differentiation, phenotype and safety. The state-of-the-art methods for stem cells expanding and differentiation rely on 2D static culture protocols, which are lacking reproducibility, are highly labour consuming and inefficient. To meet the demand of health care addressing life-threatening diseases by cell therapy and tissue engineering, new methods and equipment to enlarge the manufacturing capability of these cells under controlled conditions are urgently needed.

There are several incurable diseases associated with muscular dystrophies (MD), heritable neuromuscular disorders, which can manifest as progressive muscle weakness, muscle wasting and potentially death. Skeletal muscle regenerates new muscle fibres from myoblasts, quiescent cells in the muscle. Stem cells are preferred for regenerative medicine due to their capacity for self-renewal and differentiation potential. The treated pathologies include muscular dystrophies, heart failure associated with myocardial infarction (HFMI), and stress urinary incontinence (SUI). One of the applications of HESUB project is the amplification of human myogenic stem cells/precursor for a Proof-of-Concept of novel bioreactors in cell therapy. Another application is the expansion of human pluripotent stem cells and their differentiation into neural fate. HESUB has created two new bioreactors for perfusion operations: a stainless steel bioreactor of 3 mL working volume, 'mini-bioreactor', and single-use bioreactor of 50 mL working volume, 'SUB-SC'. These novel bioreactors are supporting human stem cell proliferation and differentiation in view of therapeutic applications and study in model systems. In particular, the project has focused on the production of myogenic precursors, but includes also other of cell fate as described below. An overview of HESUB project is given here:

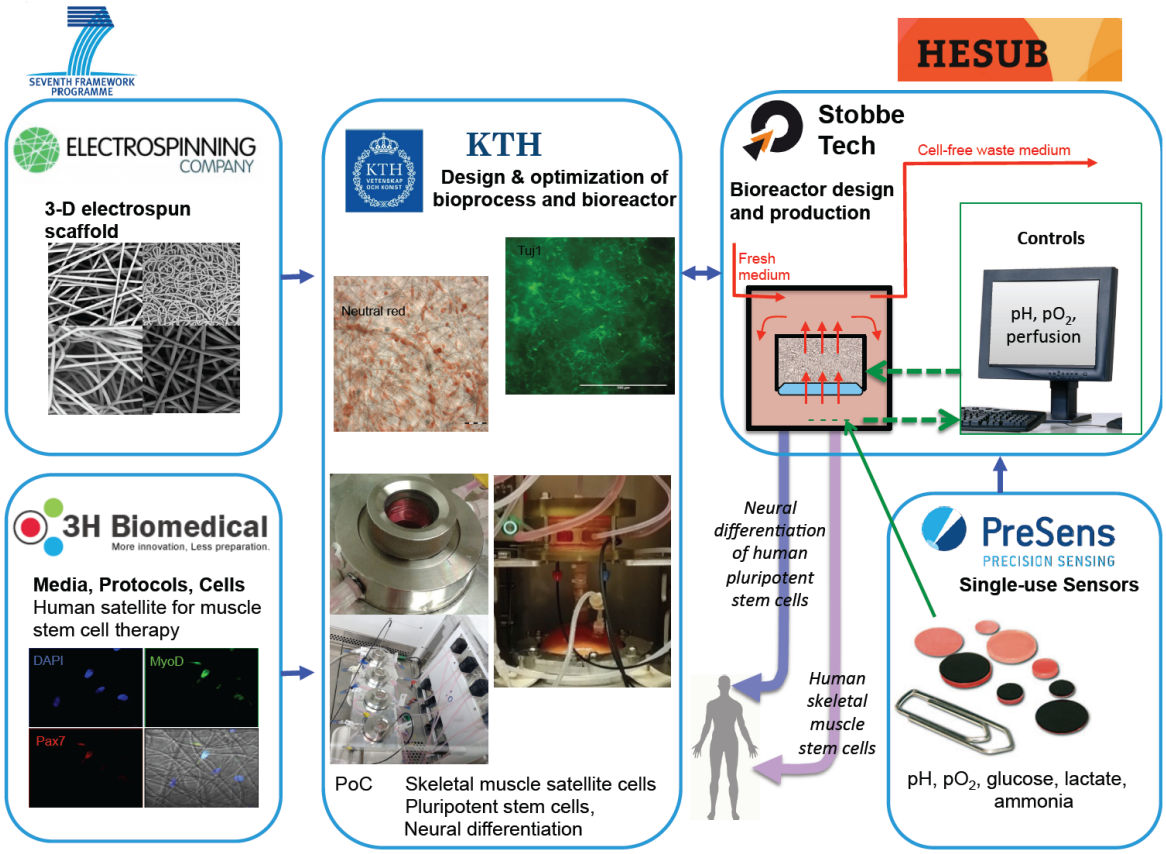

Figure 1: Overview of FP7 HESUB project – Perfusion bioreactor for human stem cells in 3D electrospun nanofibre scaffold

The main final objective of the whole HESUB project was the realisation of a new perfused Single-Use-Bioreactor suitable for human stem cell culture or SUB-SC, equipped with electrospun nanofibre (ENF) scaffold harbouring the cells and monitored by Single-Use-Sensors (SUS's), see Figure 1. HESUB's consortium includes a university and 4 SMEs: KTH (Kungliga Tekniska Högskolan – Royal Institute of Technology, Sweden) is Coordinator and responsible for the development and optimization of the perfusion process. The SUB-SC is created by Stobbe Tech (ST), Denmark, in collaboration with KTH. The Electrospinning Company (TECL), UK, develops the electrospun nanofiber scaffold. Presens Precision Sensing (PS), Germany, creates the SUS's. 3HBiomedical (3H), Sweden, is specialised in myogenic system, including production, culture media, assays, etc.

The ENF scaffold, developed in WP1 by partner TECL, is made of biocompatible polymer. The SUS's, developed in WP2 by partner PS, ensure the measurement of pH and dissolved oxygen concentration (DO). Furthermore to support the on-line characterisation of the culture, sensors of metabolites, glucose, lactate and ammonia have been developed in HESUB. Two bioreactors, the mini-bioreactor and the SUB-SC, have been developed in WP3 by partner ST in collaboration with partner KTH and applied to human stem cell types in WP5 and WP6, by partners KTH and 3H. The development of HESUB's bioreactors has been performed by studying first-hand the proliferation and/or differentiation of human myogenic precursors/stem cells, but also the differentiation of human embryonic stem cells (hESC) into neural cells, taken as benchmarking examples important for the regenerative medicine.

Partner 3H is an SME specialised in myogenic systems and particularly focussed on the treatment of muscular dystrophies. In WP4, 3H has developed methods (isolation, storage, manufacturing, culture media, quality control assays, etc.) for the production of myogenic precursors in large-scale under strict control of cell quality. 3H has aimed at translating, optimizing and validating the protocols obtained in a previous FP6 project, MYOAMP, for the proliferation of myogenic stem cells. 3H has been therefore an ideal end-user of the bioreactors of HESUB project.

HESUB achievements

The ENF scaffold chemistry and architecture have been optimized for the culture in perfused bioreactor in terms of porosity, surface treatment and material. The ENF scaffold manufacturing capacity of TECL has been substantially scaled-up to achieve a production capacity suitable for the needs of bioreactors with the manufacture of scaffolds up to 3.5 mm thickness. Manufacturing reproducibility under strict quality control and material stability of the ENF have been demonstrated.

Stainless steel small bioreactor systems of four parallel bioreactors ('mini-bioreactors') of 5 mL working volume, have been created, with the possibility to encage scaffold(s) of different thicknesses up to 6 mm, see Figure 2. The mini-bioreactors include the monitoring of pH and dissolved oxygen concentration by SUS's as well as continuous perfusion operation of the ENF scaffold where the cells are harboured. We have shown that these are excellent tools for tissue engineering for the production of dense culture of cells anchored in ENF scaffold. This has been consistently demonstrated for the proliferation of human skeletal muscle satellite cells, human mesenchymal stem cells, human embryonic pluripotent cells, as well as the differentiation of these latter into neural fate reaching a tissue-like density, see Figure 3. The cells have excellent phenotypic properties demonstrated by immunocytochemistry and RTq-PCR. In the mini-bioreactors, the SUS's are integrated by a new procedure clearly superior to the use of liquid glues due to absence of poisoning of the pH sensor spot by vapours of the glue or glue diffusing into the spot, and the risk of air gap formation is significantly reduced, avoiding delamination, bad performance, and risk of bio burden.

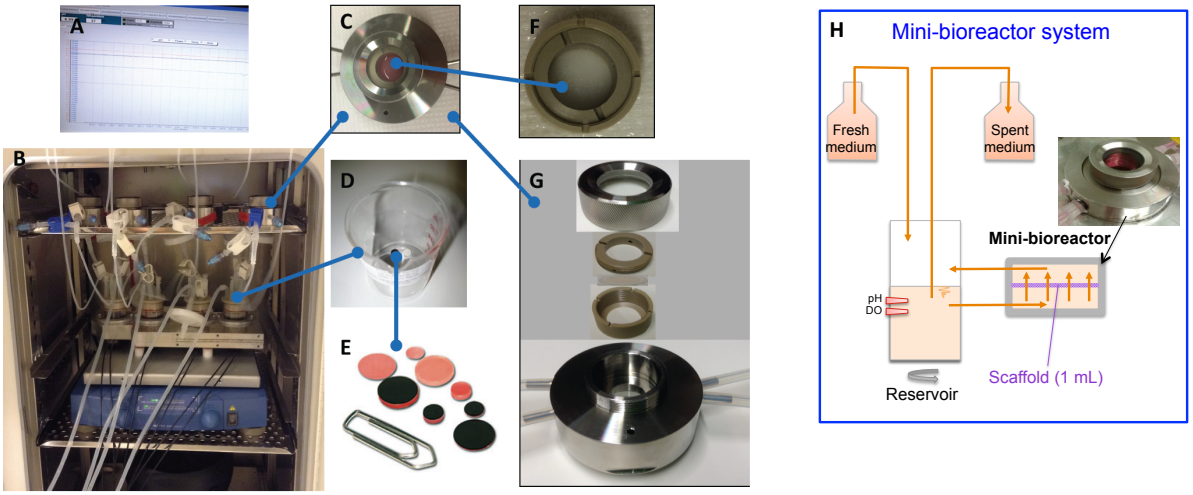

Figure 2: Mini-bioreactor showing (A) Online monitoring of pH and DO. (B) Experimental set-up showing four parallel bioreactors, medium reservoirs on a shaking table and perfusion loops in a 37°C and 5% CO2 incubator. (C) Bioreactor chamber containing an ENF construct fixed in a bioreactor insert. (D) Reservoir with pH and DO sensors fixed at the bottom. (E) pH and DO sensor spots. (F) Scaffold clamped in a bioreactor insert. (G) Parts used to assemble the bioreactor chamber. (H) Schematic overview of the mini-bioreactor culture system

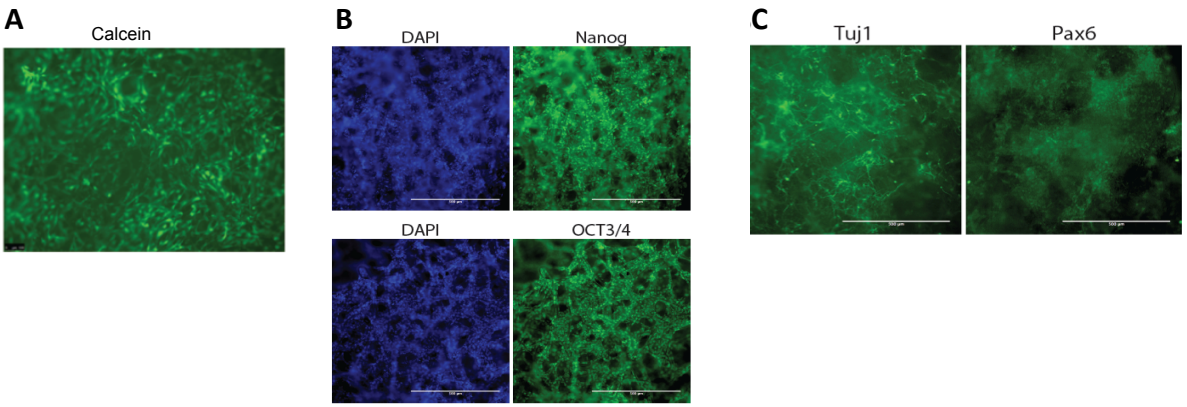

Figure 3: Cultures of human stem cells in poly-caprolactone electrospun fibre scaffold in HESUB's perfused mini-bioreactor: (A) Staining of live skeletal muscle satellite cells using calcein (B) Staining using antibody against Oct3/4 and Nanog, and counter stain of the nuclei with DAPI of human embryonic stem cells (C) Staining using antibody against neural markers Tuj1 and Pax6 at day 15 of neural differentiation of of human embryonic stem cells. Scale bars 500 µm.

The design and realisation of a new single-use bioreactor ('SUB-SC') has been supported by the studies performed in mini-bioreactors as scale-down for the perfusion process development of the proliferation of myogenic cells, and neural differentiation, and the optimization of the scaffold properties. The SUB-SC includes a matrix of ENF scaffold harbouring the cells and perfused with culture medium actuated by a new diaphragm pump with high precision. The SUB-SC monitoring includes pH, DO and novel SUS's to measure the concentrations of glucose (see Figure 4), lactate and ammonia. The novel sensors for glucose and ammonia displayed very good performance in SUB-SC culture. For all the sensors, absence of toxicity was demonstrated in tests using human embryonic pluripotent stem cells. The processes developed in the mini-bioreactors were applied to the SUB-SC bioreactor in which expansion of myogenic precursors was successfully demonstrated. An open platform for the monitoring and control of the SUB-SC bioreactor was created, enabling the integration of any instrumentation and control system by external manufacturers via Modbus communication.

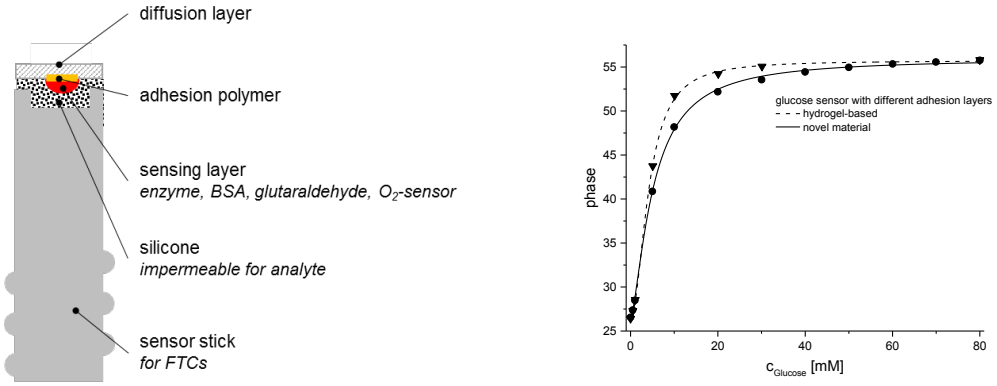

Figure 4: Left: schematic drawing of the principle sensor setup. Right: response curves of glucose sensors with hydrogel-based and improved adhesion lay

Several new products supporting muscle repair therapy and systems for disease model study have be created within HESUB: human muscle satellite cells (MyoSatellite Cells™) characterized by the expression of myogenic marker, CD56, Pax7, Pax3, Myf5, MyoD and Myogenin. MyoSatellite Cells (Pax7+, MyoD+) are suitable tools to study the biology of muscle stem cells and for screening studies using a muscle stem cell model. Several human muscle stem cell media supporting the growth and proliferation of $Pax7^+$, $MyoD^+$ human satellite cells have been developed: xeno-free medium (MyoMedium-HS™) and low FBS medium (MyoMedium-FBS™). Furthermore, 3H is first to have successfully developed serum-free and chemically defined media (MyoMedium-SF™) to support human Pax7 positive satellite cells growth and proliferation.

All the HESUB industrial partners are ISO9000 and/or ISO 13485 certified.

Commercial exploitation, impact and dissemination

The global autologous cell therapy market will be 2.2 b$ by 2017 growing at more than 20 % rate. It was estimated for 2009 that the potential market for all cell-based therapies in the USA alone was well in excess of 100 million patients. Beyond therapies for MD, which has been a high focus in HESUB, the applications potentially include life-threatening and handicapping diseases like heart failure, neurodegenerative diseases, musculoskeletal disorders, spinal cord injury, stroke, etc. Today a major limitation for the application of cell therapy is the absence of technologies generating large enough volumes of stem cells in controlled environments that ensure cell quality is high enough for commercial treatments. Bioreactors are recognized to be tomorrow's solution to this limitation.

During HESUB, the mini-bioreactors were originally developed only as scale-down for the larger SUB-SC; however these bioreactors have proven to be excellent systems for tissue engineering to grow satellite cells and pluripotent cells and to differentiate these latter into neural fate becoming a tissue mimic with desired retained phenotype. Importantly for tissue engineering, the cells grown or differentiated in the mini-bioreactors are very difficult to dislodge from the scaffold, implying that after transplantation of these tissue mimics, the cells will not 'escape' so easily. The maintenance of the cells at the transplantation site is a very common issue often jeopardizing the transplantation outcome. The tissue mimic produced in the mini-bioreactors will not risk this effect and furthermore the high porosity achieved in the ENF scaffold will allow the blood circulation and thus the vascularisation of these transplanted cell material. The immediate applications of this new technology will be muscle or brain repair, for which tissue-mimics have been obtained in HESUB. Ulterior applications will include for instance co-cultures thanks to the design of the insert holding the scaffold in the mini-bioreactor allowing variable scaffold thicknesses and multiple layers. The new scaffolds now available from HESUB efforts can be produced in high porosity, in large-scale with proven long-term stability.

In HESUB project, the SUB-SC has been created and proof-of-principle has showed that this technology has very good potential. As described above, due to the difficulty to dislodge the cells from the scaffold, its application in tissue engineering is highly suitable. Brought by the new SUB-SC technology, a new disposable diaphragm pump Erato (see Figure 5) with high precision has been created, with numerous applications in pharmaceutical industry, where there is high demand for disposable pump systems alternative to the peristaltic pumps. Within the project, new sensors for glucose, lactate and ammoniac were established. These sensors can widely be applied in any applications of cell therapy production, tissue engineering, biologics production, and in particular in other perfusion based processes. Glucose sensors will be shipped to next testers and users shortly after the project.

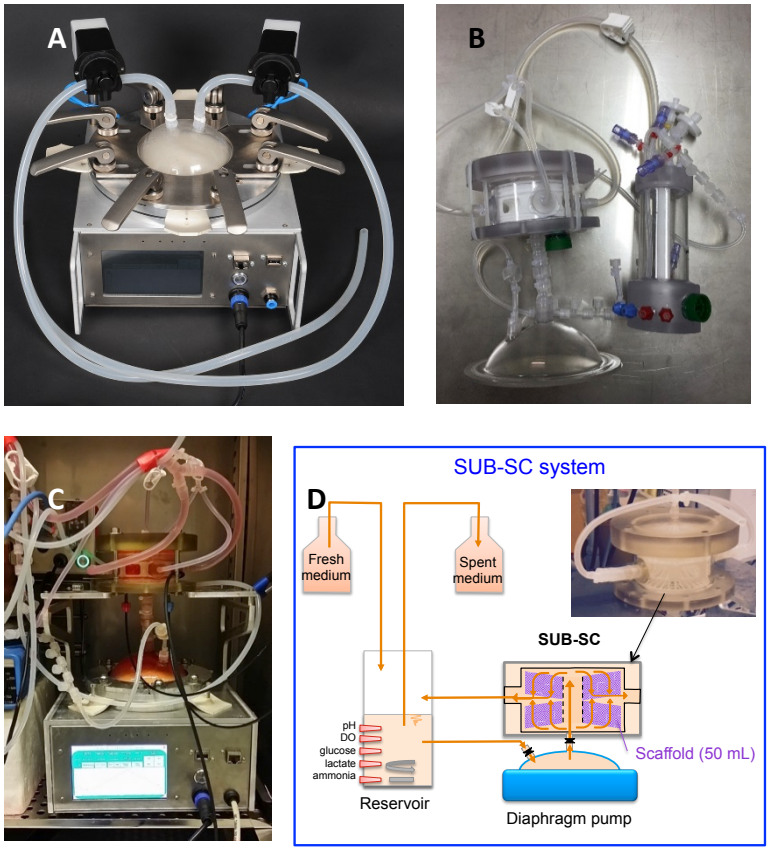

Figure 5: (A) High precision disposable diaphragm pump Erato, Stobbe Tech. (B) SUB-SC equipped with the scaffold, the SUS's and the reservoir for the medium re-circulation. (C) SUB-SC in operation mounted on the Erato pump. (D) Schematic overview of the SUB-SC bioreactor culture system

Concerning the myogenic system, it is expected that MyoMedium-SF™ will be rapidly adopted for manufacturing under GMP condition and use for the future clinical trial of human muscle satellite cell therapy in a near future. Enhanced Pax7 expression at both mRNA and protein levels has been shown in satellite cells cultured with the serum-free MyoMedium-SF™ medium. Commercial products including MyoSatellite Cells and satellite cell media have been sent to the research community for testing.

We expect that the different novel products created in HESUB will bring new solutions for the expansion of therapy by tissue engineering enabling healthy ageing and independent living for EU citizens and worldwide, and alleviating the societal challenge of the ageing European population. Furthermore, housing ENF scaffold, HESUB mini-bioreactor and SUB-SC will also be an excellent tool to study the stem cells in e.g. developmental biology. The HESUB project will not only leverage the exploitation of ENF scaffold for large scale SC culture in perfused bioreactor environment but it will also enable the production of tailored organoid grafts using ENF and cultured stem cells, exploiting the biocompatible and ECM-

mimicking features of the ENF nanostructure. The monitoring and control environment created by the new sensors will ensure a higher quality level.

The work and the new products issued from HESUB project have been presented in lectures, posters, exhibitor stands, leaflets, etc. at DASCS Symposium, Denmark, 2014 Mar 7-8; Horizons in Human Cells Conference, Edinburgh, UK, 2014 May 26-28; Regener8, Leeds, UK, 2014 Sept 17; EPIC, London, UK, 2014 Oct 02; Frontiers in Life Science Technologies at KTH - Technologies for Personalised Medicine and Health, KTH, Stockholm, Sweden, 2014 Nov 27; World Stem Cells congress, London, England, 2015 May 20-22; ESACT, Barcelona, Spain, 2015 May 31-June 3; Danish Stem Cell Society, Denmark, 2015 June 4-5; Gordon Research Conference on Myogenesis, Lucca (Barga), Italy, 2015 June 21; ISSCR, Älvsjö, Sweden, 2015 June 24-27; Bioprocess Submit, Boston, MA, USA, 2015 August 3-7; Biotechnica, Hannover, Germany, 2015-Sept-6-8; TERMIS World Congress, Boston, MA, USA, 2015-Sept-8-11; EMBO Conference on Cell Therapy, Manchester, UK, 2015 Sept 9-12; NIH Research Festival, NIH Bethesda Campus, U.S.A, 2015 Sept 16-18; Cell Biology ASCB Annual Meeting, San Diego USA, 2015 December 12-16; Cell Culture World, Munich, Germany, 2016 February 23-24; ISRAstem, Tel Aviv, Israel, 2016 April 5-6; Interphex, Jawitz Center, New York, USA, 2016 April 26-28; TERMIS, Uppsala, Sweden, 2016 June 28-July 1. One article is accepted in Journal of Biomaterials and Tissue Engineering and three other articles are under review.

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