Final report

Grant Agreement number: 223098

Project acronym: OPTISTEM

Project title: Optimization of stem cell therapy for degenerative epithelial and muscle diseases

Executive summary

Optistem was a partnership of 18 different leaders in stem cell research and clinical translation from academia and industry that collaborated from January 2009 to December 2013 on a series of projects and concepts that focused on the development and implementation of clinical trials for genetic and acquired diseases of epithelia and skeletal muscle, by transplantation of donor or genetically corrected, autologous adult stem cells. The project addressed clinical experimentation, pre-clinical tests in large animals and in immune deficient mouse models, cell biology of stem cells as well as fundamental research on the biology of epithelial and skeletal muscle regeneration, the control of angiogenesis, inflammation and the immune response in the host.

The whole project concept was feasible because despite their different self-renewal rate, epithelia and skeletal muscle share a number of structural and functional features; both tissues contain very little extra-cellular matrix so that adhesion of the cell membrane to the basal lamina is crucial to maintain the tissue cyto-architecture. The disruption of this link between these two components leads to profound alterations of cell function and survival, being the cause of two devastating diseases of skin and muscle: epidermolysis bullosa and muscular dystrophy, respectively. Correction of these diseases by transplantation of stem cells would depend upon a number of factors that are crucial in both cases: survival and integration of transplanted cells in the host tissue, a self-renewing residual potency of donor cells sufficient to maintain tissue homeostasis for a long period, control of the inflammation and consequent scarring, control of blood and circulatory systems, and inhibition of possible immune reactions against donor cells. The outcome being that results obtained with one tissue will benefit work on the other, and many therapeutic design strategies would be in common.

The partnership composed of globally renowned academic scientists and companies in stem cell biology, clinical translation and implementation, disease modelling, and developmental biology, who leveraged significant prior know-how and intellectual property to generate:

- Over 100 publications of which 15 were in very high impact journals, such as the New England Journal of Medicine, Nature and Cell.

- 6 Patents

- 2 start up company, Gymetrics SA, co-founded by Yann Barrandon based on his work on stem cell expansion and culture, Promimetic SA co-founded by Pura Munoz Canoves working on anti-fibrotics.

These tangible outcomes were all focused on addressing traumatic and degenerative diseases of two specific tissues, from which information could be obtained, gleaned and applied to a significant proportion of chronic, progressive and often fatal diseases with profound human and societal costs that could potentially be treated with stem cell therapeutics. Effective, applicable and reimbursable therapeutic approaches are few for many of these diseases and innovative approaches need to be considered.

These diseases create a life-altering experience for the afflicted person, for their partner, parents, siblings, and children. The progressive diminishment of body functions associated with the diseases can cause depression and loss of self-esteem while the diversity of the diseases can give rise pathological manifestation at any age: either as a child, during an individual's most productive years, or more frequently as an aged person. The increased prevalence of degenerative diseases with the growing aged population, is well documented with significant socio economic impact which has precipitated the need for effective, affordable and widely applicable therapies. Over the past 50 years, average life expectancy at birth has increased globally by over 20 years, from 46.5 years in 1950-55 to 65.2 years in 2002.

Today there are 600 million people in the world aged 60 years or over, and this will double by 2025 and reach 2 billion by 2050. The direct healthcare costs of organ replacement are about € 240 billion globally (about 8 per cent of global healthcare spending) arising from therapies that keep people alive (such as kidney dialysis), implanted replacement devices, and organ transplants. There is a great disparity between transplantable organs available and patient need with the present market built on first generation tissue and organ therapy products and substitutes; with the development of affordable and reproducible therapeutics regenerative medicine has a potential to exceed € 600 billion by 2030 and be of great benefit to all people suffering from what will be curable disease.

Table of contents **Page**

Project context and main objectives

The context of regenerative medicine

Degenerative diseases represent a significant proportion of chronic, progressive and often fatal diseases with profound human and societal costs for which there are no effective therapeutic approaches and are associated with a progressive decline in tissue function that share many hallmarks of ageing. Moreover, in addition to degenerative disease that are acquired during life and ageing, there is a very large number of inborn genetic diseases: although their prevalence is rare, together they affect a substantial fraction of the population, lack an efficacious therapy, and severely compromise the quality of life for many patients and their families, thus representing a major challenge for any National Health System. Since their rarity lowers the interest of large pharmaceutical industries, funding research for individual rare diseases is often extremely difficult if not impossible. For all of these reasons, defining and optimising a therapeutic strategy that may be applied, with tissue-specific modifications, to many rare genetic diseases of the mesoderm would of paramount importance in terms of both medical and economic impact. There has been an increased prevalence of degenerative diseases, both genetic (due to better diagnosis) and acquired (due to a growing aged population). This situation has stimulated a urgent need for effective, affordable and widely applicable therapies. Given the very large and increasing cases of degenerative diseases, the economic and social impact of morbidity in this population represents a significant burden, which requires effective and rapid solutions.

Solutions are being developed representing combinations of biomaterials, cells, and bioactive growth factors which can be tailored to treat these diseases, however in the context of the real world these patient focused therapies must have the following characteristics:

- **Cost effective** (resolve direct and indirect costs)
- **Reimbursable** (it is affordable for customers; governments and HMOs)
- **Reproducible** (so its worth reimbursing)
- **Broadly applicable** (a platform to be tailored and expanded)
- **Exportable** (it works for everyone, everywhere)
- **Generating a Return on Investment greater than 3%** (so its worth it)

This ‘must have’ list to some extent represents the holy grail of all medical development, with the exception that this grail can be achieved, although some diseases (e.g. epithelia and blood) are closer to (or already have) an efficacious therapy than disease of tissues such as the heart, the brain or the skeletal muscle. . To be clear what is being referred to above is an apothecary, which contains either singularly or as a combination, the tissue specific cell, restorative genetic introduction or modification of signalling pathways and functional tissue restoration through targeting of supporting cell structure. They are clinically approved products in their own right, but more importantly have been approved to be used in any number of combinations based on the patients need as determined and tailored by the doctor following diagnosis.

The cell

Human tissues can self-repair in response to moderate injuries, but are not able to regenerate when significant loss of tissue occurs in extensive trauma or surgery. Similarly, they cannot sustain repeated cycles of degeneration/regeneration. Reconstructive strategies, such as autologous cell transplantation and injection of progenitor cells yield only modest therapeutic outcomes, mainly because the tissue often presents an inflamed or sclerotic environment that results in poor survival and only modest integration of engrafted cells that are also targets of an immune reaction. Moreover, the in vitro cultivation history of the grafted cells can also negatively affect the efficacy of cell transplantation, although this may be prevented by culturing cells on appropriate biomaterials. Among the new therapeutic strategies for several age related and degenerative diseases, stem-cell transplantation is becoming a promising clinical option.

There is therefore the need to focus on optimising the use of engrafted stem cells as therapeutics which are best complemented by advancing our understanding of the basic biology of stem cell that addresses these key issues. The potential successes and applications of engrafted stem cells, needs to be matched with those aimed at optimising regenerative potential once the cells have been transplanted. As stated above, injury or disease often produces inappropriate re-patterning of the tissue culminating in scar tissue formation (fibrosis), inadequate blood vessel creation, or chronic inflammation; none of which are beneficial. Therefore we anticipate that the cell of choice for regenerative medicine and strategy to restore tissue functionality is totally dependent on a detailed knowledge of the specific pathological situation at the stage of the disease when the therapeutic is administered, with the approach tailored based on this information. For example, a

cell which is transplanted (isolated from the patient, developed as an iPS, or obtained from potential donors) following expansion in the laboratory has to maintain its original restorative characteristics in tune with the stage of development and repair of the target tissue itself that upon integration interacts with the resident cells to restore function in the degenerating tissue.

Molecular control of stem cells function: from development to adult

Like all other cell types, stem cells are sensitive and responsive too stimuli that arise outside of the cell (extracellular) and inside the cell (intracellular). Physiological damage to any tissue results in a very large number of signals that can be portrayed as a 'white noise' to the cell itself, with each individual cell having the potential to behave in a wide variety of manners depending on its location, the other cells it is interacting with, the concentrations of various growth factors and nutrients. This is not an ideal scenario as too the greater extent as successful stem cell therapy is dependent on the transplanted cells behaving in a coordinated fashion.

One approach to address this is to analyse developmental associated signals and leverage these to surpass the confusion. Developmental biology, a science that looks at how organisms change from a collection of cells into a multi-organ and complex living being reveals that developmental signals provide very strong direction and instruction which can be leveraged in an adult scenario to replicate the same effect and direct the cells towards the required fate. This has been extensively performed for developing muscle in invertebrates and lower vertebrates; for example detailed knowledge of the factors that control the choice a muscle stem cell makes between undergoing myogenic differentiation or self-renewal is crucial. Sustaining a viable muscle stem cell pool ensures ongoing muscle regeneration that will create the conditions for donor cell integration into newly regenerated fibers.

A second approach is to analyse inside the cell itself and looking for variations in gene expression linking cell signalling, with internal transcription factors (biomolecules which turn on the expression of DNA) and the specific gene expression. By defining genetic programs that underlay stem cell self-renewal and regeneration, this progresses the insights towards providing molecularly defined conditions for in vitro expansion of the target cell prior to transplantation. It also potentially provides molecular targets during tissue regeneration that can be stimulated with biomolecules or already available medicines and further alleviate the confusion of signals that prevent the stem cell from doing what it has been transplanted to do.

Microenvironmental control of stem cell behaviour

It is nice to conceive that stems cells operate in isolation and that by injecting them they behave as a panacea for the tissue damage; unfortunately this is not the case and the stem cell must survive, self renew, differentiate and integrate into a hostile pathological scenario. Chronic inflammation, reduced blood vessel supply, scarring and immune reaction, all reduce the benefits that the stem cells can elicit.

Inflammation accompanying cell death is characterised by macrophages and T cell lymphocytes infiltration and is followed by dense connective and adipose tissue accumulation which leads to a marked reduction of the ciculatory system and consequently reduces oxygen supply to surviving cells and also reduces the possibility for the cellular therapeutic to reach the damaged tissue. There is therefore the need to stimulate tissue angiogenesis (blood vessel growth) which should improve donor cell homing in the regenerating area and cell survival. Furthermore, proliferating blood vessels create a favorable niche for donor cell implant and proliferation and, most importantly, reduce extension of fibrosis. The growing vessels also produce growth factors able to improve cell division, provide resistance to cell death and allow a correct delivery of oxygen and nutrients to the damaged area.

Fibrosis, resulting in scarring, also plays a prominent role in the clinical decline following tissue damage and represents a major obstacle for successful engraftment of donor cells. It is therefore necessary to reduce fibrosis which should facilitate stem cell engraftment and, hence, regeneration. It has already been demonstrated that when stem cells were transplanted after treatment of tissues with anti-fibrotic agents, the stem cells engrafted better and generated functional tissue demonstrating that reduction in fibrosis and stimulation of angiogenesis improve stem cell implantation.

Finally, the body's immune systems (innate and adaptive) are exquisitely sensitive to changes in homeostasis in any of the body's tissues. An inflammatory immune response involving both cellular and soluble components of the innate immune system is triggered in response to injury even in the absence of an antigenic stimulus. The introduction of stem cells or stem cell derived tissues in vivo is likely to cause some degree of injury at the site of

implantation, either as a result of the underlying tissue damage or as a result of the procedures required to introduce or promote the implantation of the stem cells themselves.

In addition to the injury response the source of stem cells, autologous (derived from the patient) or allogeneic (derived from a donor) with varying degrees of histoincompatibility (bodies recognition of non-self), will contribute to the evolution of the immune response. Autologous cells and tissues will most likely not elicit a sustained immune attack unless they have been modified to express 'new molecules' to which the host is not immunologically tolerant, i.e. will not be considered as 'self' by the host immune system. For each underlying disease state and site of implantation, characterising the extent of either pre-existing or induced inflammatory responses is an essential insight required to enable appropriate intervention steps to be taken to reduce the level of inflammation induced, and permit tissue engraftment.

Optistem

The design of effective cells therapies is therefore critically dependent on the understanding of how to manipulate the therapeutic cell during its transition from initial removal from the body, expansion in a clinical setting and reimplantation at the required site to regenerate tissue, alleviate disease and restore functionality. The aim was to replicate and clinically validate approaches in which the stem cell loses none of the key characteristics that defines it as the ideal therapy for tissue regeneration and refabricate the basics of the damaged tissue and initiate a cascade of interactions within the host tissue resulting in a functional restoration.

To achieve this we assembled a partnership of 18 globally renowned specialists in the stem cell field who were to design and develop therapies that:

1. Clinically validated the selected approaches using stem cells targeted to muscular and epithelial disorders.
2. Integrated patient insight and need, matched with regulatory requirements for developing a cost effective stem cell product.
3. Leveraged new insights into stem cell control at the molecular and cellular level, which could be translated into second generation and optimised cell therapies.
4. Targeted the diseased tissue and the inflammatory and fibrotic response to enable cell therapy to restore function.
5. Enhanced host angiogenesis and modulated the immune system to permit effective tissue growth and prevent rejection of the implanted cells/

To achieve this, the work was divided into six interlocking but distinct **strategic focuses** in which individual partners would bring in specific expertise and resources that were collectively leveraged and funneled over the 5 year duration.

The first focus was on clinical trials (CT) with epithelial and muscle stem cells (Mesangioblasts/MAB). CT represented the core of the OPTISTEM project: they would benefit from discoveries during preclinical trials in large animals and their results would lead to the identification of new therapeutic approaches through further studies in small animals. These clinical trials would move towards the treatment of *(i)* muscular dystrophies using allotransplantation of normal mesoangioblasts and intra-muscular transplantation of systemically deliverable stem cells, *(ii)* ocular disorders through an innovative, simultaneous transplantation of corneal and conjunctival cells and *(iii)* skin stem cells deficiencies *via* transplantation of cultured oral mucosal stem cells.

The second focus was on preclinical large animal models. It relied on results produced in small animals and constituted a key translational interface between stem cell biology and the implementation of clinical protocols. In this regard, dystrophic dogs would be used to test long-term efficacy of wild-type and dystrophic mesoangioblasts transplantation using *(i)* lentiviral vectors or *(ii)* engineered small nuclear RNAs. Pigs would also be used as a model in experimental ophthalmology and we will develop single cell analysis and transplantation of transduced stem cells.

The third focus was on developing mouse models for cell therapy including immune-deficient animals for human cell transplantation. The injection of human keratinocytes into immune-deficient mice would provide a quantitative assay for the effects of different signalling pathways on epidermal lineage selection. These xenografts of modified human epidermal cells were to be used in conjunction with different transgenic mice in order to *(i)* modulate the Notch pathway involved in determining epidermal or hair follicle fate in the skin and *(ii)* to define the optimal conditions of neo-dermis formation.

The fourth focus was on the factors that control stem cell activation and renewal at a mechanistic level. Using cutting-edge molecular and cellular approaches we studied the different signalling pathways and transcription factors that control *(i)* satellite cell behaviour and repair of skeletal muscle, *(ii)* muscle interstitial cells and *(iii)* mesoangioblasts.

The fifth focus related to tissue remodelling and engraftment of stem cells. Besides cells, modification of the muscle environment is crucial for the successful engraftment of donor cells. Here we aimed to *(i)* unveil the mechanisms that control the induction of angiogenesis *(ii)* increase stem cell homing through pharmacological approaches and *(iii)* improve fibre survival by reducing fibrosis using plasminogen activation and pharmacological depletion of fibrinogen.

Finally, in the sixth focus we covered the immunological aspects that result from stem cell engraftment. The regulation of the immune response is a key success factor for stem cell therapy, and we determined *(i)* to what extent mouse and human stem cells, prior or after manipulation, elicit or not an immune response, *(ii)* how this response was modulated and *(iii)* how we could induce tolerance.

High impact results

The project design and implementation generated significant innovations with high impact in the stem cell, regenerative medicine, and degenerative disease field of endeavour. Below we indicate those innovations which will have paradigm changing impact in the development of state of the art therapeutics; given the space limitations we do not report on the extensive amount of insights and fundamental knowledge generated by the partnership which will serve as a long term reinforcement for our continued innovation in this sector.

A) Clinical trials and patient treatment

i)Treatment of patients suffering from Muscular dystrophy with Mesangioblasts

We planned to start a phase I clinical trial with donor MABs from HLA-matched donors to be transplanted in patients affected by Duchenne Muscular Dystrophy (DMD). Muscular dystrophies are clinically and molecularly heterogeneous diseases, characterized primarily by wasting of skeletal muscle that compromises patient mobility and, in the most severe cases, such as DMD, respiratory and cardiac functions, leading to wheelchair dependency, respiratory failure and premature death. In many cases, the mutation affects proteins that form a link between the cytoskeleton and the basal lamina. Mutation in one of these proteins often causes disassembly of the whole complex, leading to increased fragility of the sarcolemma, especially during intense contractile activity. This in turn results in increased calcium entry (though the molecular mechanisms are not yet elucidated in detail), and focal or diffuse damage to the fibre. Damaged or dead fibres can be repaired or replaced by muscle progenitor cells, up to a few years ago the only known myogenic cell present in post-natal life. However, these newly generated fibres share the same molecular defect and so are also prone to degeneration. With time, the satellite cell population becomes exhausted and the muscle tissue is progressively replaced by connective and adipose tissue that follow the chronic inflammation elicited by fibre degeneration. The rationale for performing this clinical trial with MABs was based on the efficacy that the same treatment has demonstrated in preclinical models (mice and dogs).

The clinical trial started on March 15th, 2011 and MAB infusions were completed on December 21st, 2011 in first three (DMD0301, DMD0302, DMD0303) out of the six selected patients. Patients DMD0301 and patients DMD0303 received four MABs infusions in lower and upper limbs. With the exception of patient DMD0302, the others were far above the estimated body weight of 30 Kg, further reducing the dose of cells administered that, in the end, turned out to range from 10% to 40% of the dose that had been administered to dystrophic dogs.

Finally, due to the time elapsed between recruitment (February 2009) and initial of the trial (March 2011) all the three patients were undergoing a crisis resulting in a progressive and dramatic decrease of their motility and force of contraction. Indeed patient DMD0301 was already wheelchair bound at the start of the infusions and MRI revealed a dramatic loss of muscle mass.

Patient DMD0302 received an additional MABs infusion in lower limbs in December 2012 based on:

1. Observation of increase in walking stability, reduced stiffness of muscles and, most importantly, absence of spontaneous falls during six months after the last infusion.
2. Protein analysis revealed a faint but clearly detectable band, at the same molecular weight of normal dystrophin. This protein must have derived from donor cells since the patient has a deletion of exon 4-44. No presence of dystrophin was detected instead in the other two patients DMD0301 and DMD0303.
3. Both patients DMD0301 and DMD0303 have T-cells reactive against dystrophin and donor cells, whereas patient DMD0302, while still able to respond to viral antigens, has no immunological response to dystrophin or donor cells.
4. The neuromuscular magnetic resonance data of lower limbs of patient DMD0302 (updated on 28.05.2012) showed from April 2011 (starting date of the cell therapy-trial) a substantial stability of fibro-fatty degeneration of all muscles of the pelvis, thigh, legs, except a minimal increase in correspondence of the hamstrings muscles.
5. The functional measures of patient DMD0302 remained stable from April 2011 to August 2012 when an initial decline was observed, concomitant with a rapid growth in height of about 6 cm. At the same time he has begun again to walk unstably and to fall frequently.

Unfortunately, despite the additional MABs infusion of December 2012, one month after this infusion, we observed in this patient a severe decrease in muscle force and ability to walk.

Patient DMD0301 had already lost walking ability six months before starting the trial. After the infusions there were no noticeable changes in patient DMD0301 but a stabilization of functional measures in first six months after last infusion. Subsequently the patient showed an irreversible decrease in muscle force.

In patient DMD0303 we observed a clinical modest amelioration after the first three infusions, also confirmed by increase in walking stability, reduced stiffness of muscles and, most importantly, absence of spontaneous falls. However Patient DMD0303 underwent a severe adverse event after the last infusion in December 2011 (a small ischemia of the thalamus, whose cause remains unexplained, and had no clinical consequences). Moreover patient DMD0303 developed a severe scoliosis which prevented him to walk autonomously and is mainly confined to a wheelchair.

In consideration of severe adverse event occurred in patient DMD0303, a substantial amendment was performed to the clinical protocol in August 2012 with the following significant changes that were introduced for the last two patients treated from November 2012 to June 2013:

1. We infused MABs in the next scheduled patients only in lower limbs with increase in cells doses This change was mainly motivated by very small amount of the donor's dystrophin detected in biopsies of the first three patients performed after the four infusions. We believe that infusion of a higher dose of cells in the lower limbs, increases the chances of a greater accumulation of dystrophin. We also preferred to evaluate a clear effect in one anatomical region, rather than disseminate cells in more districts. On this occasion, we recall that the positive effects observed (maintenance of muscle strength at the same level for more than a year, less muscle stiffness, lack of falls, increased bone density) are probably due to a paracrine effect of the mesoangioblasts.
2. Cardiac monitoring for 24 hours post-infusion was performed to monitor the possible development of arrhythmias, such the one that had preceded the ischemic event in patient DMD0303.

Infusions in the remaining two of the other three patients planned (one patient developed a viral myocarditis during August 2011 and failed to match the inclusion criteria) started in November 2012 and were concluded in June 2013. No SAE were observed. However, because patient DMD305 is still stable we obtained permission with a last amendment (February 14, 2014) and performed two more infusions at high cell dose, in February 20 and April 2014.

The results of this trial are summarized below:

1. The five patients are well and at home, following their normal life schedule. No SAE due to immune suppression. Patients DMD301 and DMD305 were wheel chair bound when the trial started.
2. Patients DMD302, and DMD305 showed clinical signs of stabilization of the disease: muscles were less rigid, they no longer fell spontaneously, posture and motility appeared stable until August 2012 (16 months, for DMD302). Patient DMD305, who started later is still stable.
3. Patients DMD302 showed stabilization in his functional tests that were rapidly worsening before starting cell infusions. Stabilization lasted from spring 2011, despite the fact that they are growing, to August 2012. Patient DMD305 is still stable. Figure 1.

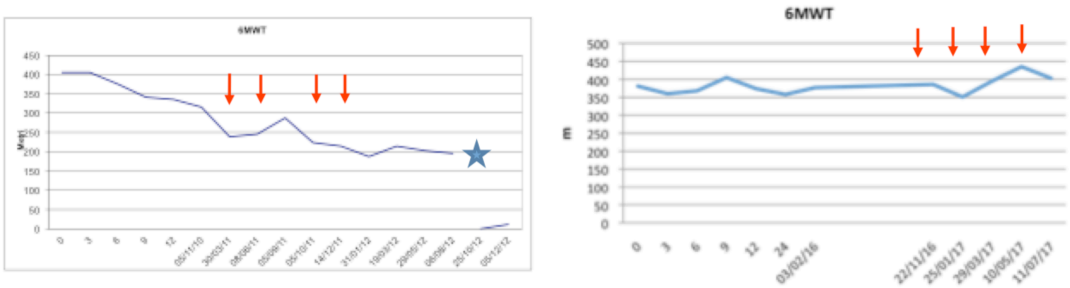


Figure 1: 6 minutes walk test of patient #02 (left) and #05 (right)

4. Biopsies of patients # 2 and 3 show presence (0.1- 0.7%) of donor DNA and low expression of dystrophin in certain areas. WB analysis confirmed the presence of donor dystrophin in patient DMD302. Biopsies of patients DMD304 and DMD305 showed the presence of donor DNA and significant amount of dystrophin,

However, since the last two patients have a point mutation RNA seq is being run to distinguish between donor derived and revertant dystrophin. Patient DMD302has a 40 exon deletion and therefore full MW dystrophin may only derive from donor (Figure 2)

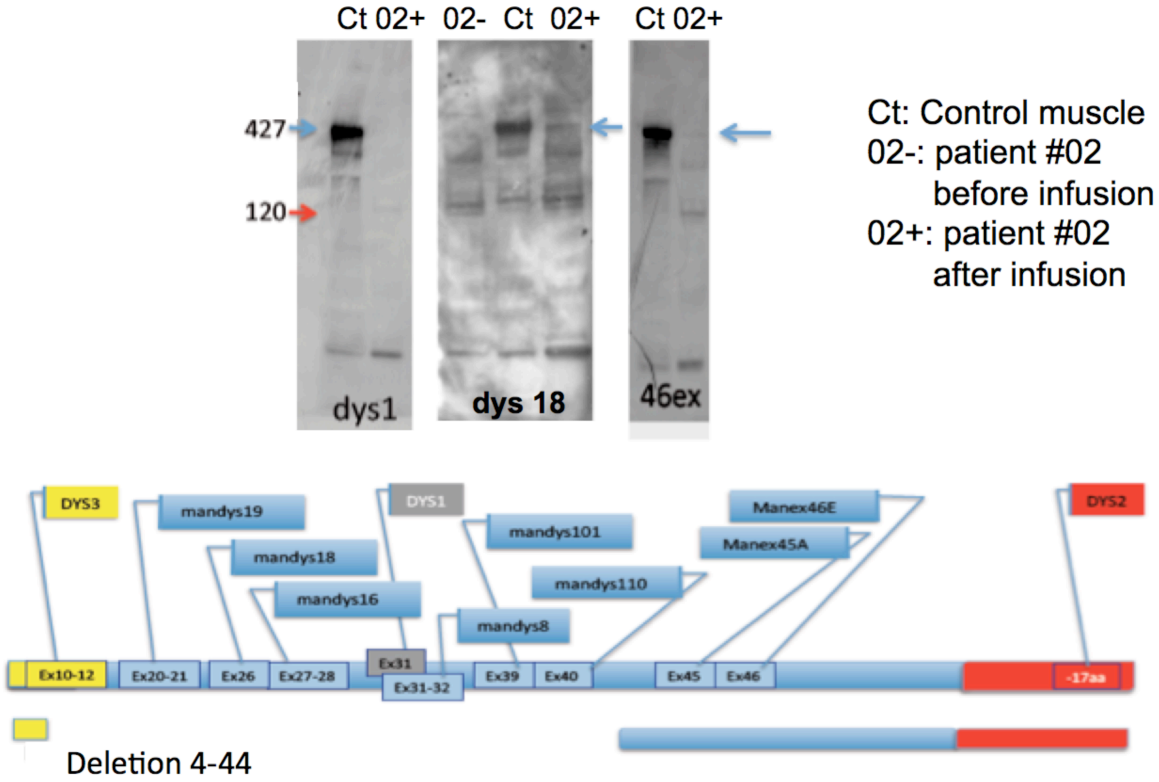


Figure 2: Western blot analysis of biopsies of patient #02, showing low levels of HMG dystrophin (arrow) detected with different antibodies. The mutation and the antibodies detecting different exons are shown below.

In conclusion:

1. The trial was safe (but 1 SAE).
2. Efficacy was transient and lower than those observed during the preclinical work, possibly because:
   a) cell dose was too low
   b) disease was too advanced when trial started
   c) mice and dogs use four limbs to walk, humans two and need support from pelvic girdle and back muscles that we did not target. To address these issues we plan to:

1. start infusions in younger patients as suggested also by our SAB.
2. model in mice delivery in more anatomical districts.
3. develop gene therapy methods that will amplify the production of dystrophin.

ii)Treatment of patients suffering corneal degeneration by autologous cultures containing limbal stem cells

Limbal Stem Cell Deficiency (LSCD) is a corneal condition that is characterized by lack of corneal epithelium replacement, which is initially characterized by recurrent or persistent epithelial defects, inflammation and, subsequently, by migration of the conjunctiva over the cornea and the formation of a neo-vascularization pannus.

The level of LSCD is defined and quantitatively measured on the basis of a score associated with the clinical and the cytological status of the corneal surface as follows; Opacity of the corneal epithelium and/or recurrent epithelial defects: 1; Persistent epithelial defects: 2; Total conjunctivalization of the cornea: 3.

Medical quantification of the degree of limbal deficiency (Total limbal deficiency: both scores equal to 3, total score 6; Severe limbal deficiency: both scores equal to 2, total score 4; Mild limbal deficiency: both scores equal to 1, total score 2).

These quantifications are aimed at providing the attending doctor with a measure for the analysis of the condition of the deficit affecting the patient. In addition to the assessment of the limbal deficiency, the subjects are required to be in good overall health. These conditions are a favourable prognostic factor with regard to the favorable clinical outcome of the therapeutic treatment.

Diagnosis and grading of LSCD was based on clinical evaluation (recurrent or persistent epithelial defects, presence of a fibrovascular pannus over the cornea and chronic inflammation). LSCD was arbitrary divided in three grades: (i) moderate, when conjunctivalization was sectorial with less than 2 quadrants involved (>180°) and did not reach the central cornea; (ii) severe, when recurrent or persistent epithelial defects occurred, peripheral corneal conjunctivalization involved more than two quadrants (<180°) and central corneal opacification was present; (iii) total, when the cornea was completely vascularized and opacified. Disorders of eyelids and conjunctiva (entropion, ectropion, occlusion, trichiasis, symblepharon) were treated beforehand and procedures were recorded. Inflammation was considered a negative prognostic factor, and was treated with topical steroids weeks before surgery; when possible, surgery was postponed until its complete resolution. Eye examination included visual acuity, tonometry, slit-lamp examination, fundus, ecography, Schirmer test and photographs.

Not all patients suffering from Limbal Deficiency can be treated with stem cell therapies as there can be significant risk factors which can negatively impact the success of the therapeutic treatment, which served as exclusion criteria for this clinical trial. These criteria include ocular inflammation, active systemic infectious diseases, local or systemic neoplasia, limbal deficiency occurring as a result of radiation therapy, and non compensated diabetes

Following a preliminary infectious profile analysis (analysing for HAV, HBV, HCV, HIV1 and 2, HTLV, WNV, and Treponema Pallidum) and confirmation that none of these agents are present autologous limbal cells were obtained from 1-2 mm^2 biopsy taken from the patient's uninjured eye and cultured on a feeder layer of lethally irradiated cells established in a certified structure, according to the ICH guidelines.

Once the first cell culture was established growing cells were dissociated and frozen. For graft preparation, the cells were thawed and grown on a circular fibrin substrate (3 cm diameter) in the presence of a feeder layer. At confluence, fibrin-cultured epithelial sheets were washed and transferred at room temperature to the hospital in order to be transplanted onto the diseased eye. Limbal biopsies were processed within 30 hours of removal from the limbus. Grafts were applied within 24–36 hours after their transfer to the hospital.
The surgical procedure was performed under local-regional anesthesia or general anesthesia. A 360° limbal peritomy was performed and the fibrovascular corneal pannus carefully removed. The fibrin-cultured epithelial sheet was placed on the prepared corneal wound bed spanning the limbus about 2-3 mm, to reduce competition with conjunctival ingrowth. The conjunctiva was then sutured over the peripheral fibrin sheet in order to protect the border of the sheet and help it to adhere on the surface. The eyelids were kept closed with medical gauze and patched for one week. During the first 2 weeks after grafting, topical treatment was avoided to prevent mechanical damage when opening the eyelids and secondary toxic effects while the cells were engrafting.

Systemic post-operative therapy (antibiotic and anti-inflammatory therapy) was administered from the day of the surgery for a further two weeks. Topical preservative-free dexamethasone was started at day 14 and topical ofloxacin was administered only in case of incomplete re-epithelialization. Frequent use of preservative-free lubricants was advocated. Patients were seen at day 3, 14 and at month 1, 3, 6, 12.

After at least 1 year follow-up, standard lamellar or penetrating keratoplasty or phototherapeutic excimer laser keratectomy (depending on the depth of the stromal involvement) was performed on patients considered to have been successfully treated, but whose visual acuity was still impaired because of stromal scarring.

Penetrating keratoplasty-associated donor cornea rejection was graded as (i) mild (epithelial rejection or endothelial precipitates with clear graft); (ii) moderate (endothelial preciptates and edema of the graft with visible iris details); (iii) severe (diffuse edema with non-visible iris details). Mild and moderate rejection was treated with topical dexamethasone, every 4 and 1 hour(s), respectively. The regimen was tapered depending on clinical amelioration. For severe rejection, systemic methylprednisolone was added. Antibiotic therapy and anti-inflammatory therapy were usually ended at day 25 and 45, respectively.

Each follow-up included patient history, recording of symptoms and complete eye examination. In particular: evaluation of adverse events and collection of medical data; general medical visit; ophthalmology visit (assessment of epithelial defects and assessment of neovascularisation; ocular tonometry; examination with a Slit Lamp; assessment of conjunctival inflammation; assessment of corneal sensitivity and opacity; assessment of symptoms such as: pain, burning and photophobia); digital photograph of the eye.

The limbus (narrow zone between the cornea and the bulbar conjunctiva) harbors the stem cells of the corneal epithelium. Limbal cultures restore the corneal epithelium in patients with ocular burns.

Biological parameters instrumental for clinical success were investigated in 152 patients carrying corneal destruction due to severe ocular burns, treated with autologous limbal cells cultured on fibrin and clinical-grade feeder cells. Clinical results were statistically evaluated both by parametric and nonparametric methods. This long-term multicenter study – together with a careful evaluation of many biological parameters – allowed us to identify some of the factors determining the clinical success of limbal cultures, obtain insights on the biology of limbal stem cells and address safety concerns.

Clinical outcomes were scored as full success, partial success and failure in 66.05%, 19.14% and 14.81% of eyes, respectively (Figures 3 and 4).

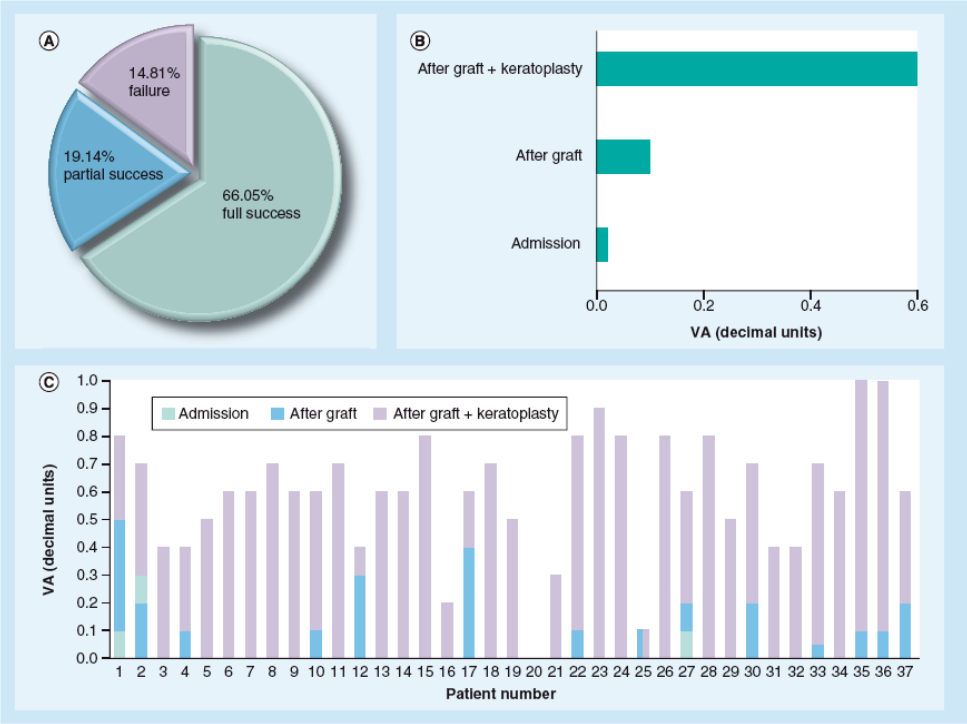


Figure 3: Clinical outcome and visual acuity. (A) The first conclusive clinical evaluation was done after at least 1 year of follow-up. Clinical outcomes were scored as full success (green), partial success (blue) and failure (purple) in 66.05, 19.14 and 14.81% of eyes, respectively. In all full success patients, complete and stable re-epithelialization occurred, and the corneal surface appeared clear, smooth and covered by a transparent normal-looking epithelium. Due to the concomitant stromal scarring, however, the average VA improved only slightly after culture grafts. (B & C) To improve VA, lamellar or penetrating keratoplasty were performed on 56 patients, 37 of whom had no additional ocular pathologies. At admission, mean ± standard deviation VA of these 37 patients was 0.03 ± 0.05 ([B], and green bars in [C]). After graft, VA improved only slightly, 0.08 ± 0.11 ([B], and blue bars in [C]), but only in some of the patients. Indeed, this improvement was not significant, with the median value (0.02) being identical in the two groups of patients. However, VA strongly improved after keratoplasty and reached a median value of 0.6 after keratoplasty ([B], and purple bars in [C]). Of note, 15 of these patients (40.5%) recovered 0.7–1.0 VA (purple bars in [C]).VA: Visual acuity.

Figure 4: Corneal restoration. (A) Left eye (at admission) of a 42-year-old patient who had total limbal stem cell deficiency due to acid burn. Note corneal neovascularization (asterisks) and stromal scarring with consequent complete corneal opacification. The patient received a first graft of autologous limbal cultures 4 years after injury, which failed because of the severity of the ocular damage and severe postgraft inflammation. Two years later (during which the eye was stabilized by proper anti-inflammatory topical therapy), the patient received a second graft of autologous limbal cultures. This time, the cultures were able to regenerate a fully functional corneal epithelium, hence the clinical outcome was now ranked as full success. (B) Because of stromal scarring, cultures were not sufficient to improve the patient's visual acuity. To remove stromal scarring and improve his vision, 1 year after the graft we performed a penetrating keratoplasty. The engrafted limbal stem cells were able, a second time, to generate the corneal epithelium necessary to resurface the donor stroma, which resulted in complete restoration of visual acuity (0.8). This panel shows the eye of the patient at the last follow-up, 6 years after graft and 5 years after keratoplasty. Note that the cornea is covered by a transparent, normal-looking epithelium and the complete absence of vascularization.

The total number of clonogenic cells, colony size, growth rate and presence of conjunctival cells could not predict clinical results. Instead, the clinical data provided conclusive evidence that graft quality and likelihood of a successful outcome rely on an accurate evaluation of the number of stem cells detected before transplantation as holoclones expressing high levels of the p63 transcription factor (Figure 5).

Clinical data provided conclusive evidence that the most important biological criterion to assess graft quality and the likelihood of a successful treatment was a rather precise evaluation of the number of stem cells detected as holoclones (expressing high levels of p63), which are the only epithelial clonal types possessing self-renewing properties and long-term regenerative potential. No adverse effects related to the feeder layer were ever observed and the regenerated epithelium was completely devoid of any feeder cell contamination.

Neither total number of clonogenic cells nor colony size could predict clinical outcomes, confirming that the vast majority (≈95%) of clonogenic keratinocytes behave as transient progenitors. However, a proper number of clongenic cells was a necessary, although not sufficient, parameter to define the potency of the culture.

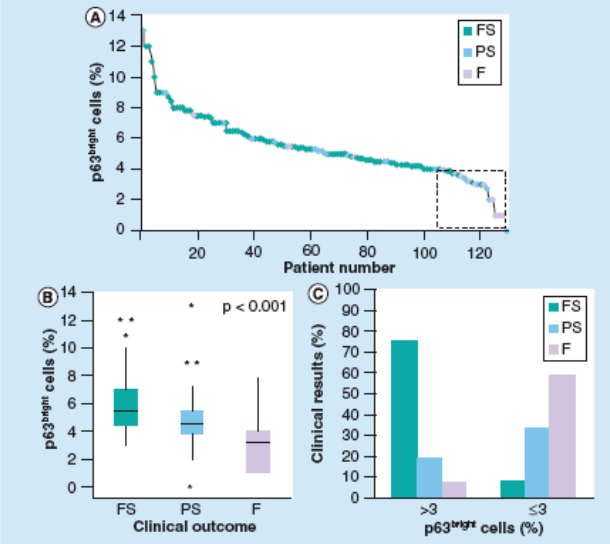


Figure 5: Relationship between clinical results and number of stem cells in limbal cultures. (A) The percentage of p63bright cells found in each culture was plotted in decreasing order. Most cultures containing a high percentage of p63bright cells were FS (green dots). In sharp contrast, most cultures containing the lowest percentage of p63bright cells (dotted square) failed (F, purple dots). PS cultures were scattered but mainly located at the center/right part of the graph (blue dots). (B) Relationship between the percentage of p63bright cells and the three clinical oucomes. FS (green), PS (blue) and F (purple) cultures contained 5.5, 4.3 and 3.2% p63bright cells, respectively. The horizontal line within each box represents the median value; the bottom and top lines of the box represent the 25th and 75th percentiles (with lowest and highest values), respectively. The asterisks indicate the excluded outliers. (C) The correlation study conducted by means of the χ^2 contingency table method shows that cultures containing >3% of p63bright holoclone-forming cells were FS in 74% of eyes and F in 6.8% of eyes, whereas cultures containing ≤3% of such cells were FS in 8% of eyes and F in 58.3% of eyes. Thus, the percentage of p63bright holoclone-forming cells can be used to predict the clinical outcome of limbal cultures. F: Treatment failure; FS: Fully successful; PS: Partially successful.

Epithelial cultures containing holoclones cannot be distinguished from those devoid of them. This makes it quite difficult to define the quality of the graft. Clonal analysis and/or the evaluation of the number of cell doublings generated during serial cultivation were the most rigorous criteria to evaluate stem cells in all of epithelial cultures. However, both assays are cumbersome as routine procedures and unsuitable as preoperative quality controls since their results become available only at least 1 month after grafting. However, clinical data provided conclusive evidence that holoclones can be identified by quantitative immunodetection of p63, confirming the notion that p63 is a determinant of the regenerative potential of epithelial stem cells. This does not mean that any positivity for p63 should be considered as a marker for limbal stem cells since transition from holoclone to paraclone is accompanied by the progressive disappearance of the protein.

The strict correlation between clinical performance and level of expression of p63 indicates that cells expressing high levels of p63, should first be validated by a quantitative assay and correlated with the number of holoclone-forming cells contained in a reference culture, then assessed by quantitative immunocytochemistry in every culture used for transplantation. Determining the frequency of cells containing high levels of p63 should now effectively prevent failures, since the assay can be performed before grafting.

The presence of a defined amount of stem cells in the graft was not the only factor determining clinical success. Indeed, some cultures containing ≤3% of p63bright cells were successful, while some cultures containing an appropriate number of holoclones failed. Such failures were significantly associated with severity of ocular damage, inflammation and postoperative complications.

In conclusion, despite many years of successful clinical application, we still have no sense of the number of stem cells that can engraft on the wound bed. It is conceivable that stem cells of some cultures were lost

owing to a hostile *in vivo* microenvironment. Similarly, grafts containing a low number of stem cells could reproducibly succeed, provided the presence of an optimal microenvironment. Thus, proper selection of patients, definition of stringent inclusion and exclusion criteria and proper preparation of the damaged ocular surface during the weeks/months preceding the grafting procedure and defined surgical procedures might be as critical as the stem cell content.

B) Preclinical work

Preclinical work mainly oriented around the development of scale up procedures and validation of cell therapy products for application to epithelial (skin, eye) related diseases, integrating the regulatory requirements necessary for translation to clinical trials and validation of the therapies in humans.

Virtually all of this work was performed in Swizterland, whose authorities do not allow animal experiments if an innovative strategy dealing with the 3R (reduce, refine and replace) is not put forward. The experimental design can be moved to live animals to demonstrate experimental feasibility once the proof of concept has been achieved ex vivo.

In the context of treating eye disorders, to address these understandable requirements, the first series of breakthrough's were performed using discarded pig eyes obtained from the abbatoir. Pig eyes have the dimensions as human eyes thereby providing valuable insight into assessing the precise cell number and type that needs to be used for application. The first step was the successful generation of complex micro incubator, (Figure 6) which permitted the eyes themselves to be maintained as integral units in organ type cultures for several weeks and retain many of their regenerative characteristics that can be observed in living animals. Specifically in the context of cell therapy, they permitted live imaging and analysis of transplanted cell movement within the tissue.

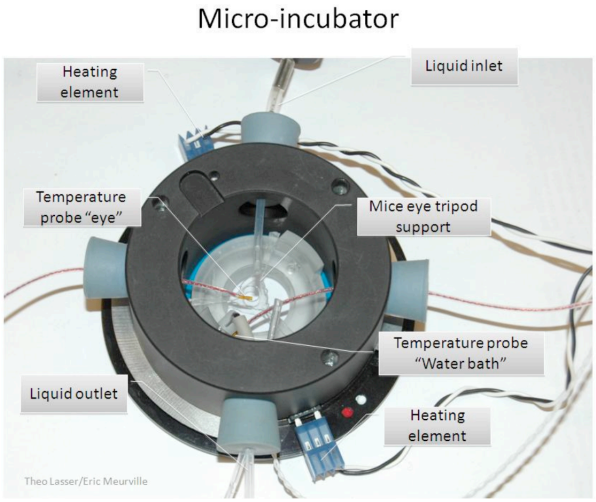


Figure 6: Micro-incubator for ex vivo organ culture of isolated eyes and stem cell transplantation on the ocular surface

Using these devices, it was demonstrated that 20 000 expanded cells from a different eye, seeded in central part of a pig cornea are enough to entirely reconstruct the corneal epithelium in two weeks. This achievement allows the reliable and reproducible transplantation of cultured stem cells onto the cornea. This work was further enhanced via genetic modification of the transplanted cells with a marker that fluoresces: this is a standard but very important tool for the development of cell therapeutics, and through it, it was feasible to image and trace the fate of a small number of stem cells transplanted onto the cornea.

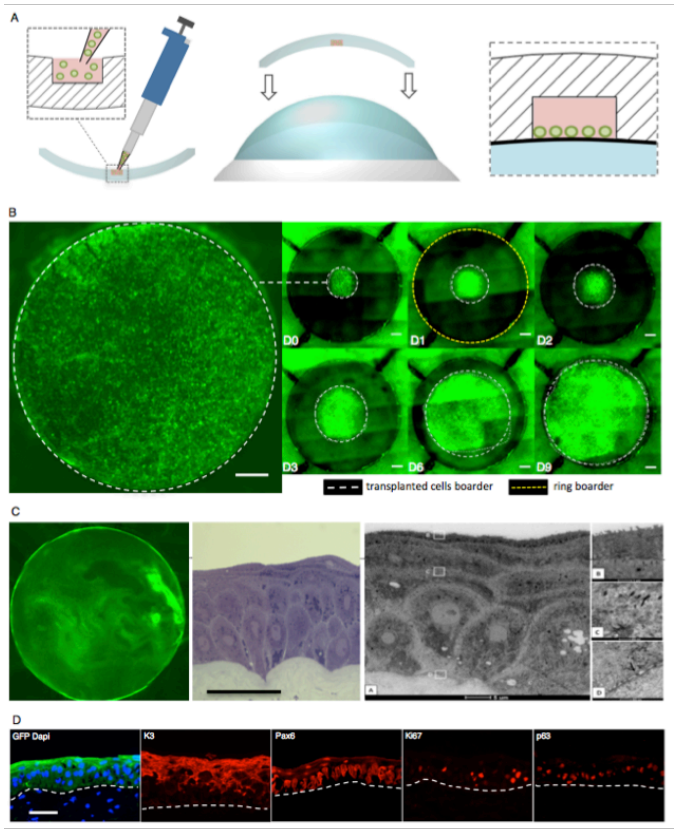


Figure 7: cultured corneal stem cells adhere rapidly a denuded corneal stroma and rapidly reconstitute a genuine corneal epithelium both through migration and proliferation.

As an extension of this work, and to broaden applicability it became essential to isolate epithelial stem cells from other tissue sources. The rationale behind this is that in the case of eye damage it is highly likely that the patient themselves will not be able to provide enough stem cells from their own eyes, thereby limiting the approach. However epithelial stem cells themselves are found in all epithelial tissues: hair, eye, skin. To prove this this stem cells were obtained from the ocular surface, the thymus, the tooth and the hair follicle of the rat, labelled with the fluorscent marker and transplanted on pig cornea to evaluate their migratory capacity and their ability to reconstruct a corneal epithelium.

Using the lens device described above, various epithelial cells were transplanted and their rate of attachment, proliferation and migration studied by live imaging. This work demonstrated that a number of non-ocular epithelial cells can migrate and proliferate onto a corneal matrix, thereby opening the avenue for treating eye disease with a patients own cells isolated from a different part of their body.

For the development of skin therapies clones of stem cells from the epidermis and the hair follicles of the pig were isolated. After dissociation, clonogenic keratinocytes were cultivated using the same protocol for the treatment of extensive burn wounds in the human. This is important for clinical translation as the method of culture has to be as highly validated and quality controlled as the cell therapeutic itself.

Preliminary transplantation experiments have been performed using autologous pig epidermal stem cells with the fluorescent marker. A single holoclone was isolated, expanded and transplanted onto a pig. Survival of the autologous grafts was observed for several weeks, which has demonstrated the feasibility of a single stem cell strategy in a large animal model. This result is very important for the development of many future cell therapies as isolation of as few stem cells as possible while retaining maximum therapeutic potential means as little discomfort as possible to the patient.

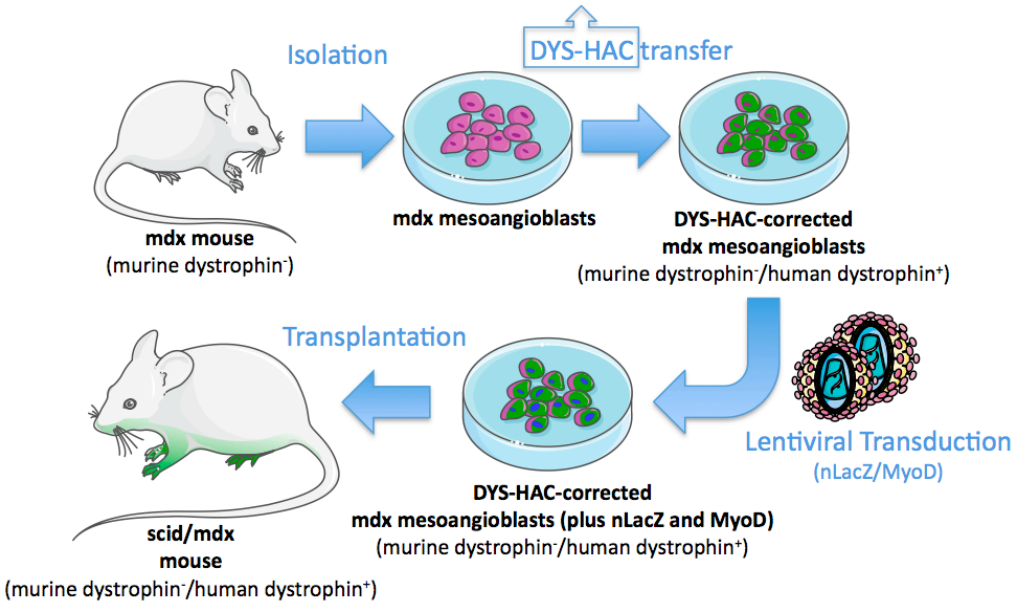
C) Modelling and optimising cell therapy in animals

To optimise innovative new therapies, and particularly regenerative approaches the work requires, whenever possible, a real time feedback loop between the data obtained via clinical trials and basic research so that this

research can be tailored based on patient need. results in animal models of diseases, either mutant mice (possibly immune deficient to accept human donor cells) or, when available, large animal models, whose pathology more closely resembles the human condition.

In the context of the muscular dystrophies, as this is a genetic defect, outcomes pointed towards developing a combination therapy involving the cell with genetic modification. The problem with such an approach for correcting the loss of dystrophin is the size of the gene, which is rather large. To address this, we assessed the use of a noval strategy involving artificial chromosomes.

In contrast to conventional gene therapy vectors, human artificial chromosomes (HACs) are episomal vectors that can carry large regions of the genome containing regulatory elements. So far, HACs have not been used as vectors in gene therapy for treating genetic disorders. Therefore we tried to ameliorate the dystrophic phenotype in the mdx mouse model of DMD using a combination of HAC-mediated gene replacement and transplantation with MABs. The strategy is summarized in following scheme:



We first genetically-corrected mesoangioblasts from dystrophic mdx mice with a HAC vector containing the entire (2.4 Mb) human dystrophin genetic locus. Genetically corrected mesoangioblasts engrafted robustly and gave rise to many dystrophin-positive muscle fibers and muscle satellite cells in dystrophic mice, leading to morphological and functional amelioration of the phenotype that lasted for up to 8 months after transplantation.

Success in this field led us to address potential other therapeutic avenues. MABs are derived from a subset of cells found in muscle and have been shown to ameliorate the disease phenotypes of different animal models of muscular dystrophy which gave rise to the clinical trial that was performed as part of this project. To extend the potential application we demonstrated that patients with a related disease, limb-girdle muscular dystrophy 2D (LGMD2D), which is caused by mutations in the gene encoding a-sarcoglycan, produce too few MABs for use in autologous cell therapy.

Therefore, we reprogrammed fibroblasts and myoblasts from LGMD2D patients to generate human induced pluripotent stem cells (iPSCs) and developed a protocol for the derivation of MAB-like cells from these iPSCs (named HIDEMs). The iPSC-derived MABs were expanded and genetically corrected with the gene encoding human a-sarcoglycan (which is missing in these patients). When these genetically corrected HIDEMs were transplanted into the LGMD2D mouse model, they generated muscle fibers that expressed a-sarcoglycan. Transplantation of mouse iPSC-derived mesoangioblasts into a-sarcoglycan–null immunodeficient mice resulted in functional amelioration of the dystrophic phenotype and restoration of the depleted progenitors as shown in Figure 8. These findings suggest that transplantation of genetically corrected mesoangioblast-like cells generated from iPSCs from LGMD2D patients may be useful for treating this type of muscular dystrophy.

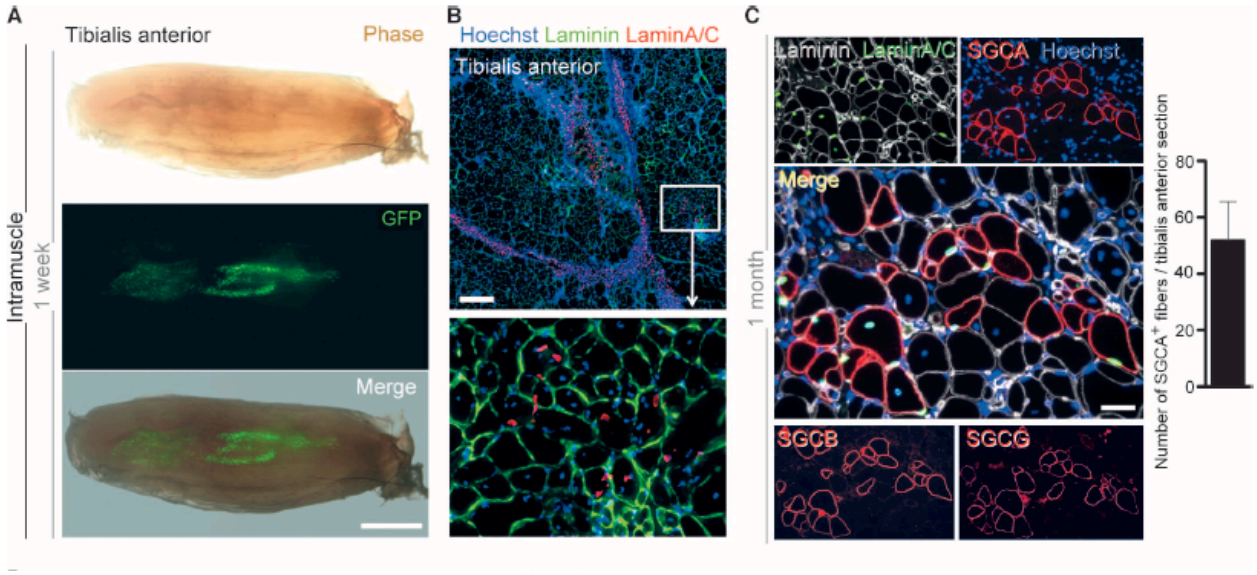


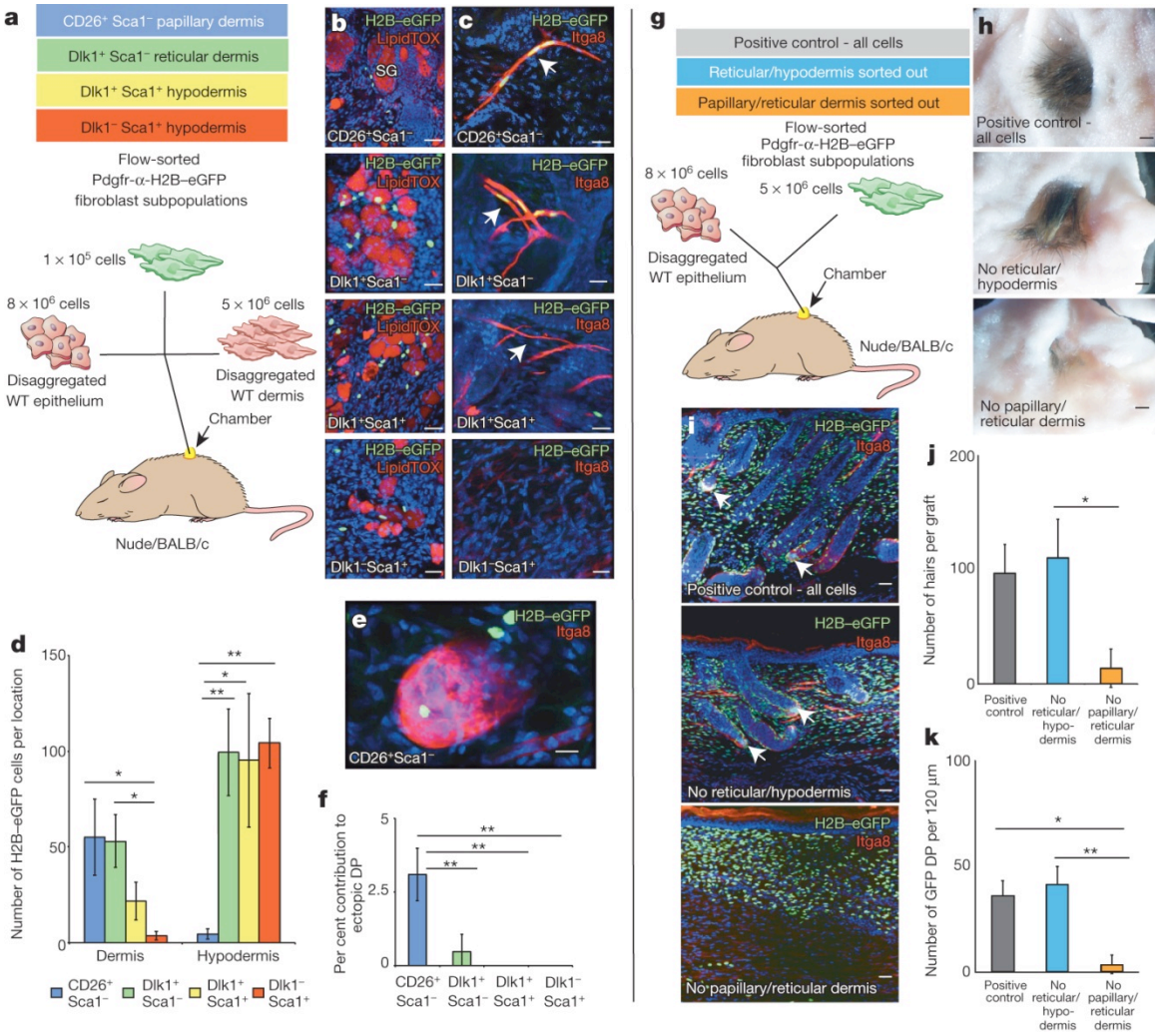
Figure 8. Transplantation of HIDEMs into Sgca-null/scid/beige mice. (A) GFP fluorescence 7 days after intramuscular injection of 106 genetically corrected LGMD2D HIDEMs into the tibialis anterior muscle of Sgca-null/scid/beige mice. Scale bar, 1 mm. (B) (Top) Immunofluorescence staining of a section from the muscle shown in (A) demonstrating engraftment of genetically corrected LGMD2D HIDEMs as revealed by lamin A/C+ nuclei (lamin A/C marks the human nuclear lamina). (Bottom) Magnification of the area inside the white box in top image showing a cluster of myofibers containing donor human cell nuclei. Scale bar, 500 mm. (C) Immunofluorescence showing a cluster of SGCA+ myofibers containing human nuclei 1 month after intramuscular transplantation of genetically corrected LGMD2D HIDEMs (quantified in the bar graph; error bars show SD and the number corresponds to an average of 2% of tibialis anterior myofibers). Scale bar, 60 um. Bottom images show the same cluster in serial section stained for b- and g-sarcoglycan (SGCB and SGCG).

Identical to linking the human clinical application and fundamental development of therapeutics for muscular dystrophies, there was also significant effort dedicated to better understanding the development of epithelial based cell therapies, with a specific focus on diseases of the skin. It is important to keep in mind that the validation of a therapeutic approach with a cell type for one disease successfully precipitates the application of the same cell type for other diseases. As skin trauma linked to burns or ulceration or damage linked to diseases such as diabetes has such a high socioeconomic impact, it made sense to assess the application in this pathological area. The major outcome that has been witnessed from treating such diseases with cell therapy is that the therapy only results in the generation of a few of the different layers involved in the skin; the classic one being absent is the hair follicle, which is the best indication that the skin has completely regenerated as a tissue if one is generated (note that the skin is the body's largest tissue with a critical role in metabolism and defence).

We therefore originally set out to determine the genes that regulate human hair follicle growth in a mouse xenograft model. Our initial results indicated that hair follicle forming signals in the epidermis alone were not sufficient for hair follicle morphogenesis to occur in the grafts. We subsequently determined that epithelial cells from hair follicles require the association of the upper-papillary fibroblast populations to support hair growth and that activation of beta-catenin in the epidermis was sufficient to expand this population in vivo.

We found that the type of fibroblasts implanted into xenograft conditions influences the hair forming capacity of the epithelial cell types. To establish whether the lower fibroblast lineage cells were less effective in supporting hair follicle formation we compared control grafts containing unfractionated cells with grafts in which we excluded upper-papillary/reticular cells or reticular/hypodermal cells (Figure 9 g–k) All grafts contained dermal papilla cells. Hair follicle formation was similar in the grafts of unfractionated dermal cells and those depleted of reticular and hypo-dermal cells. By contrast, when the papillary and reticular dermal cells were excluded very few hair follicles formed (Figure 9 h–k). Thus fresh isolated skin cells contains cells that are restricted to forming either the upper or lower dermal lineages on skin reconstitution, the upper dermal cells being required for hair follicle formation.

Figure 9



This novel work has highlighted the importance of the role played by dermal fibroblast populations in hair formation using an adult mouse model system. We are currently examining the cellular composition of human dermis with the aim of using the preliminary evidence gained so far to create a fully humanised system for hair follice growth.

D) Molecular and Cell Biology of Stem Cells

To complement application in humans and innovative approach development molecular understanding and manipulation of the stem cell population can provide both clinical and fundamental insights. This detailed knowledge of the factors that control the choice a stem cell makes between undergoing differentiation or self-renewal is crucial. Sustaining a viable stem cell pool will ensure ongoing tissue regeneration that will create the conditions for donor cell integration into newly regenerated complex structures. The role of the stem cell environment and of signaling pathways that regulate stem cell behavior were therefore investigated.

i) Muscle Stem cells

We had previously found that muscle progenitor cell activation and entry in the cell cycle was partially controlled by sphingosine-1-phosphate (S1P), and that S1P biosynthesis is required for muscle regeneration. It was therefore necessary to determine the role of S1P receptors (S1PR) in muscle satellite cell function. The three principle receptors, S1PR1, S1PR2 and S1PR3 were chosen for study and it was found that over expression of S1PR3 suppressed cell cycle progression but did not overtly affect the muscle development

program. Consistent with this, S1PR3 levels are high in quiescent myogenic (has muscle potential) cells, but fall during entry into cell cycle. Conversely, muscle progenitor cells isolated from *S1PR3*-null mice exhibited enhanced proliferation ex-vivo. In vivo, acute muscle regeneration was enhanced in *S1PR3*-null mice, with bigger muscle fibres compared to control mice. Importantly, genetically deleting *S1PR3* in the *mdx* mouse model of DMD produced a less severe muscle dystrophy, than when signalling though S1PR3 was operational.

Publication of this work elicited interest from industry and there has been communication and an initial meeting with a leading Pharma company about the potential use of their agonists/antagonists for treating muscular dystrophy. It is hoped that this will progress into an active collaboration.

Along similar lines we previously showed that $p38a^{\Delta Pax7}$ mice lacking the key muscle factor Pax7, and p38 (a signalling molecule linked to cell stress and differentiation) displayed normal behaviour and lifespan as compared to their littermate controls. However, the p38a conditional mutation resulted in a significantly reduced body weight that became evident at about one week after birth and became more pronounced with age. Analysis of the tibialis anterior (TA) and soleus (Sol) muscles indicated that there was a reduction in muscle cross-sectional area (CSA) but no change in total fibre number, indicating that the $p38a^{\Delta Pax7}$ mice had significantly smaller myofibres as compared to the $p38a^{WT}$ mice. Overall, these experiments demonstrated that p38a is necessary for skeletal muscle formation and homeostasis, and that its absence in skeletal muscle precursor cells leads to postnatal muscle growth defects and muscle weakness and instability.

The data revealed that the mechanisms of action of p38α differ between embryonic muscle progenitors and adult satellite cells. During postnatal myogenesis, deletion of p38α lead to an increase in proliferation and a delay in differentiation, as expected based on results obtained with primary myoblasts. In contrast, deletion of p38α in adult muscle progenitor cells did not affect proliferation although it was important for proper adult muscle progenitor cell activation. The data demonstrating that p38α has distinct functions in these two periods of myogenesis indicates that p38a is one of the molecules important for defining the age-dependent behaviour of muscle progenitor cells vastly expands our current knowledge of muscle cell biology and opens new perspectives for therapy development in muscle diseases.

At the transcriptional level we demonstrated that silencing of the transcription factor PW1 in murine MABs strongly interferes with the main hallmarks of MABs: they failed to differentiate in skeletal muscle and when systemically injected in a dystrophic mouse model, they were unable to cross the vessel wall and migrate towards the inflamed, damaged muscle. We demonstrated that the absence of PW1 causes a block in MAB myogenic differentiation while the robust increase of JAM-A expression (see section F on blood vessel development later in this report) in absence of PW1 is the cause for MAB loss of trans-vessel migration capacity that could be rescued following JAM-A interference.

In the perspective of a potential future clinical use of this marker, we explored whether PW1 may represent a marker of myogenic potency also in human MABs (hMABs). The idea was to use PW1 as a screening molecule in order to identify the best donors (all the remaining inclusion criteria matched) of hMABs in terms of myogenic competence and ability to cross blood vessels. Therefore, we surveyed PW1 expression levels in different hMAB cell lines which revealed that PW1 was strongly expressed in the competent populations of hMABs (Figure 10 B). In addition, the transmigration assay on human umbilical vein endothelial cells (HUVECs) performed on competent (02XY and 27XY) versus non-competent (32XY and 03XY) differentiating hMABs confirmed that the ability to cross the vessel wall correlated strongly with levels of human JAM-A (Figure 10 C,D). Taken together, these data confirmed the utility of PW1 as a screening molecule/biomarker in future cell therapy protocols based upon MAB transplantation for muscle disorders.

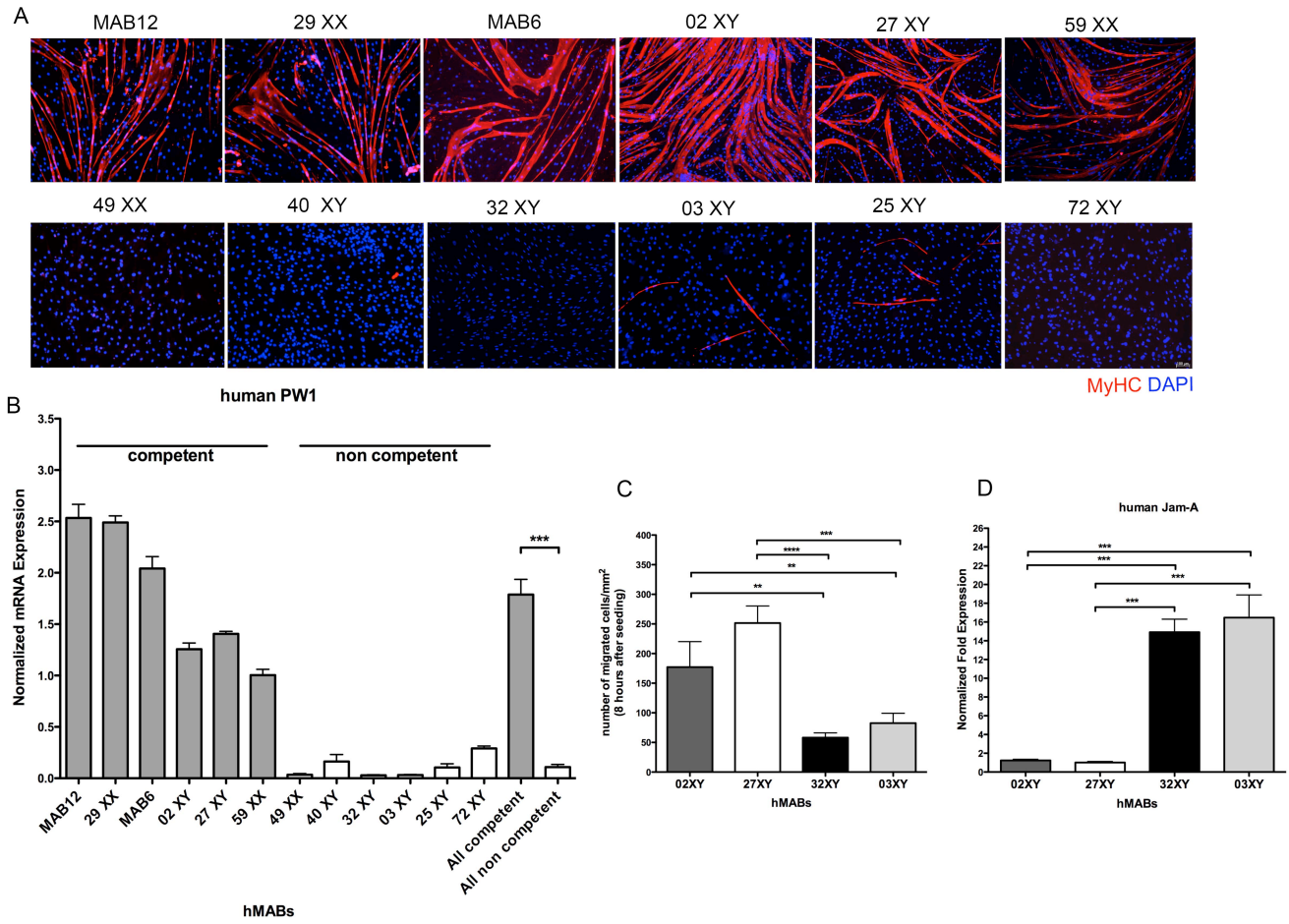


Figure 10. PW1 levels strongly correlate with the myogenic and transmigration ability of human MABs. (A) Immunofluorescence analysis for MyHC (red) in 12 different populations of humanMABs. DAPI was used to stain the nuclei. Scale bar represents 100 mM. ***(B)*** *human PW1 expression by qRT-PCR on 12 different populations of human MABs (hMABs) divided in competent and non competent on the basis of the myogenic property shown in* ***(A).*** *Values are plotted as relative expression and normalized to GAPDH expression. Each assay was performed in triplicate. ****P < 0.0001, unpaired t Test.* ***(C)*** *HUVECs Endothelial cells were seeded on gelatin-coated filters. 4 different polyclonal hMABs, previously labelled with fluorescent 3,33 μM 6-carboxyfluorescin diacetate (6-CFDA), were added to the upper chamber and allowed to migrate for 8 hs. Migrated hMABs on the lower side of the filters (FITC positive cells) were fixed and counted. Quantification of migrated hMABs per area is shown. Data are means (±SD) from five independent experiments, each of these was run in triplicate. **P < 0.005, ****P < 0.0001, unpaired t Test.* ***(D)*** *Human JAM-A expression by qRT-PCR on hMABs. Values are plotted as relative fold expression and normalized to GAPDH expression. Each assay was performed in triplicate. ***P < 0.0005; unpaired t Test.*

This work identified PW1 as a key molecule in regulating mesoangioblast competence and proposed the use of PW1 as a tool to isolate and identify the best donor cell to use in the cell based Therapy of the Muscular Dystrophies. Moreover, this work should lead to a more wide spread use: since it has been demonstrated that PW1 identifies multiple adult stem and progenitor cell populations, we can postulate that PW1 may regulate other stem cell competence, such as the neural stem cells, and therefore may be useful for other diseases.

ii) Epithelial Stem cells

Stem cells are defined by their ability to self-renew and to generate all the differentiated cell types in the tissue. Keratinocytes stem cells (KSC) are instrumental in maintaining and repairing the epidermis and other squamous epithelia. Each KSC generates columns of epidermal proliferative units composed by Transient Amplifying Cells (TACs) that reside in the basal layer; TACs undergo few rounds of cell divisions before

terminal differentiation. Even if KSC are widely used in the clinic, the biology of their stemness is not completely defined and the lack of univocal KSC markers limits their use in regenerative medicine.

In light of functional data demonstrating the role of the integrin (ITG) α6β4 in mediating adhesion of basal keratinocytes to the basement membrane via hemidesmosomes, integrin-α6 may provide a suitable marker for the isolation of basal keratinocytes endowed with proliferative potential. We thus designed a method based on magnetic micro-beads integrin selection to achieve three cell populations: ITGA6-bright cells (presumably enriched-Stem Cell population, EnSC), ITG6-dim cells (Transient Amplyfied Cell population, TAC) and post-mitotic differentiating cells (PMDCs). We performed a gene expression profile in order to obtain genes differentially expressed in these cell populations which revealed that at both the mRNA and protein level, ITGA6-bright cells expressed high levels of stem cell-related molecular markers (p63, Bmi1 and CEBPδ) and low level of terminal differentiation markers (involucrin and K10). 804 genes were differentially expressed in EnSC and PDMC. However, EnSC ITGA6-bright cells ITG6-dim cells (TAC) had a similar gene expression profile. Thus, thee level of expression of integrin α6β4 could not be used to distinguish epidermal stem from the TA progenitors at a molecular level.

This prompted us to analyse the gene expression profile of stem and TA progenitors by microarray analyses performed on cultures established from single cells obtained by clonal analysis of sub-confluent primary cultures. The proliferative compartment of human squamous epithelia contains three types of clonogenic keratinocytes, referred to as holoclone-, meroclone- and paraclone-forming cells. The holoclone- forming cell is the stem cell of all squamous epithelia. Holoclones produce meroclones and paraclones, which have properties expected of transient amplifying progenitors. Epithelial cultures containing an appropriate number of holoclones can permanently restore massive epithelial defects, such as skin and ocular burns and can be used to produce a transgenic cultured epidermis able to generate a functional epidermis in Junctional Epidermolysis Bullosa, a devastating genetic skin disease.

Starting from a skin biopsy, we set up a clonal analysis and collected keratinocytes, deprived of feeder cells, from a culture established from single KSCs or TACs (defined by a Colony Forming Efficiency assay). While Holoclones represent a precise class of clones that give rise to less than 5% of aborted colonies, meroclones are defined by a broad percentage (from 6% to 95%) of aborted colonies. We thus divided meroclones in two groups, Early Meroclones, containing 20 to 40% of aborted colonies and Late Meroclone, containing 40 to 60% of aborted colonies (Figure 11 a). We demonstrated that p63alpha was highly expresses in Holoclones, while its expression progressively decreased in Early and Late Meroclones (Figure 11 b).

RNA was extracted and processed on Affymetrix platform. The Principal Component Analysis confirmed that transcriptomes derived from Holoclones (red dots) and Late Meroclones (green dots) are quite different, even though both cell types are clonogenic and able to proliferate. Interestingly, Early Meroclones (blue dots) can either cluster close to Late Meroclones or very close to Holoclones (Figure 12). This suggested that Holoclones and Late Meroclones are quite different, and all samples correctly clustered in their own group (Figure 12). Of note, we found 1038 genes that were differentially expressed in Holoclones and Late Meroclones. Early Meroclones clustered between Holoclones and Late Meroclones, with some samples more similar to Holoclones and others to Late Meroclones.

In the ANOVA analysis (Figure 13) we found that genes related to keratinocyte differentiation (involucrin, filaggrin and loricrin) were highly expressed in Late Meroclones as compared to Holoclones. Differentially expressed genes were also analyzed by the network-based Ingenuity Pathways Analysis tool (Ingenuity Systems, www.ingenuity.com), to search for the most relevant molecular interactions, functions and pathways differentially expressed in the different clonogenic populations. Amongst the 1038 gene differentially expressed in Holoclones and Late Meroclones some were related to the Cell cycle: G1/S checkpoint Regulation, others to p53 signalling or Tight junction Signalling.

One of the most interersting genes differentially expressed is ING5, which is upregulated in Holoclones as compared to Late Meroclones. ING5 belongs to a large family group of tissue homeostasis regulators, the MORF complex. Through WB analysis we confirmed that ING5 is highly expressed in Holoclones as compared to Early and Late Meroclones were it is barely detected (Figure 14). Recently, other teams from outside the project revealed a strong correlation between keratinocyte stemness and this complex. Indeed, ING5 interacts directly with EZH2 and UHRF1, two main regulators of epidermal self-renewal.

In summary we established that stem cells and TAC have a quite different gene expression profile and it is possible to define a specific gene signature of human KSC. Holoclones are generated by keratinocyte stem

cells, but probably some early meroclones can be endowed with stem cell properties. These experiments pave the way for a deeper understanding of the molecular basis that underlies the biology of skin stem cells and epidermal homeostasis.

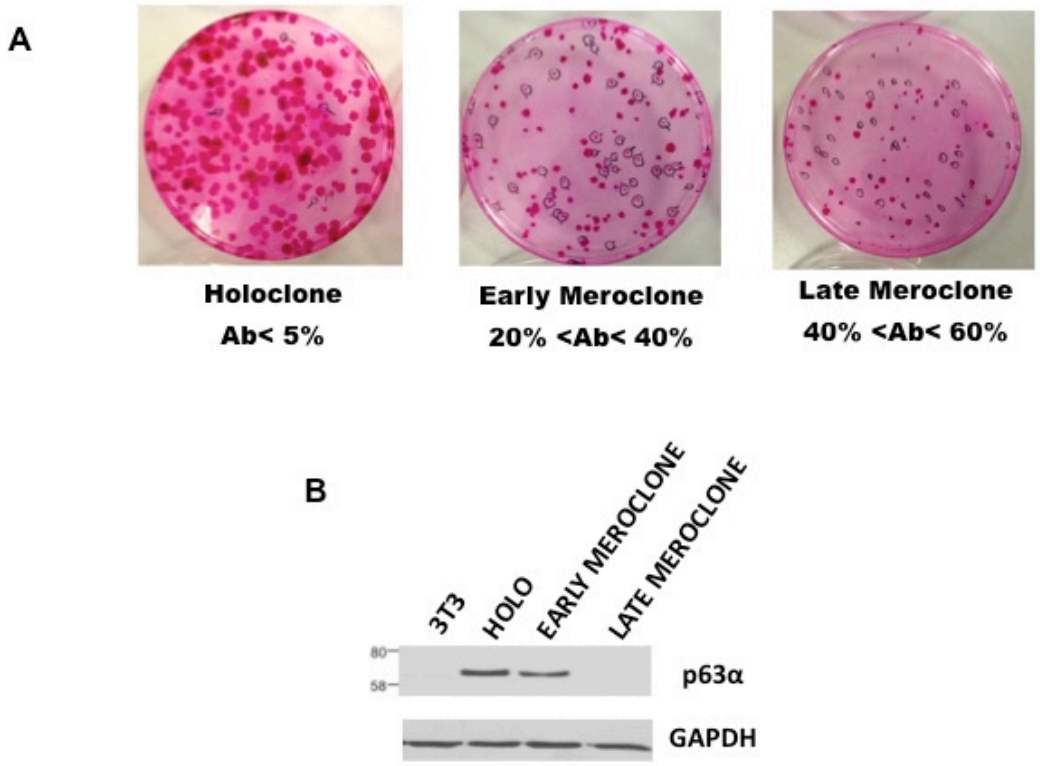


Figure 11: A) CFE assay representing a Holoclone, an Early Meroclone and a Late Meroclone used for microarray analysis. B) p63alpha is highly expressed in holoclones, its expression decreases in early meroclone and in undetectable in late meroclones.

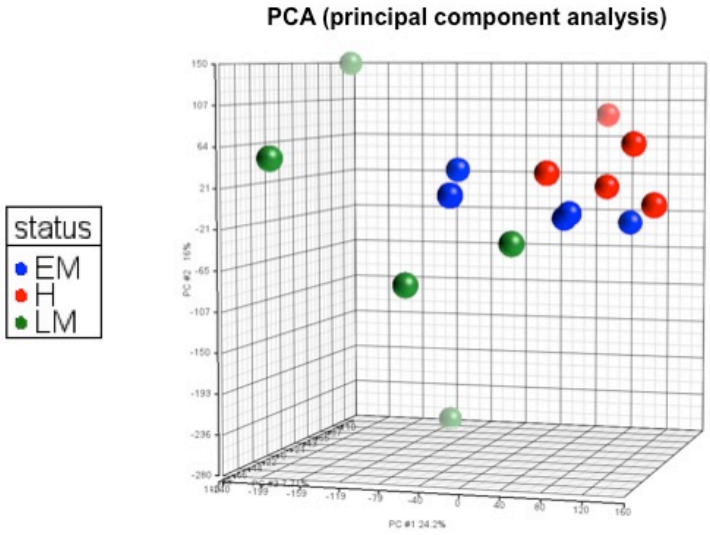


Figure 12: Principal Component Analysis (PCA) shows that Holoclone (H, red dots) and Early Meroclone (EM, blue dots) are close to each other sharing some properties. Late Meroclone (green dots) seems to be very different when compared to the Holoclone.

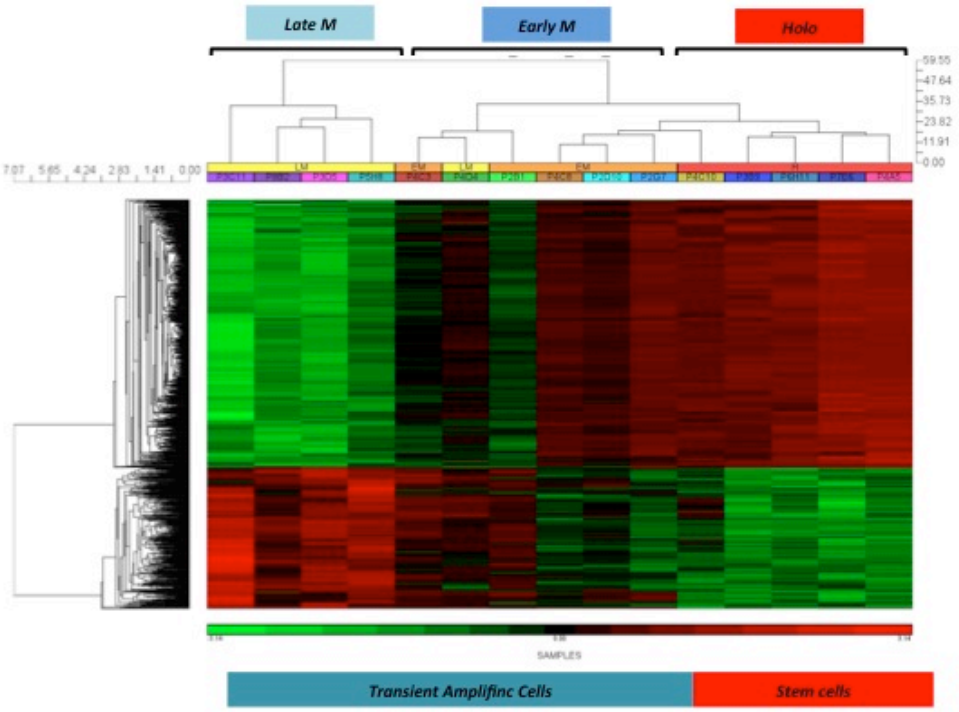


Figure 13: Unsupervised hierarchical clustering analysis performed on arrays of Holoclone, Early Meroclone and Late Meroclone. Red patches represent up-regulated genes, while green patches represent down-regulated genes.

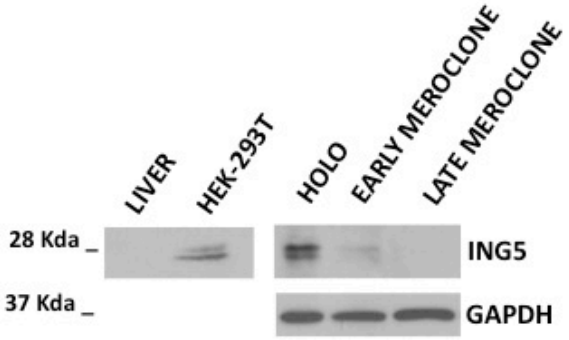


Figure 4

Figure 14: Ing5 protein is expressed in Holoclone but is almost undetectable in Early and Late Meroclone.

E) Preventing fibrosis and scar formation

Tissue fibrosis plays a prominent role in the clinical decline in many degenerative diseases and also represents a major obstacle for successful engraftment of donor cells. To reduce fibrosis an attractive approach is to increase proteolysis by modulating the activity of specific proteases. More specifically, extracellular proteases of the plasminogen activation system (fibrinolytic system) are major regulators of extracellular matrix (ECM) turnover. Using dystrophic muscle as the model system which is known to have excessive amounts of fibrotic deposit during the disease progression we investigated new ways to reduce the scar forming effect. By generating a transgenic mouse that was deficient for the fibrinolytic system and crossing it with a mouse manifesting muscular dystrophy we could generate a disease state in which excessive fibrotic activity could be obtained as part of disease progression. Then, analysis of this system to identify what were the key molecules involved in the significant increase in fibrotic deposition in the muscle

revealed a new molecular target, miR21. This new target falls into the miRNA class of molecules that are receiving high levels of interest by industry worldwide as they appear to be the fine control of cell function and any changes in their activity can represent real therapeutic targets.

With this important work, we have characterized a new age-associated fibrogenic regulatory axis in dystrophic and injury-induced skeletal muscle fibrosis in mouse models. In both cases, the expression of miR-21, which is barely detected in normal muscle, increased concomitantly with age-dependent fibrogenesis. After demonstrating its role, we could also prove that inhibiting miR-21 reduced fibrosis, which is classically considered irreversible at advanced ages, and improved muscle homeostasis (Figure 15)

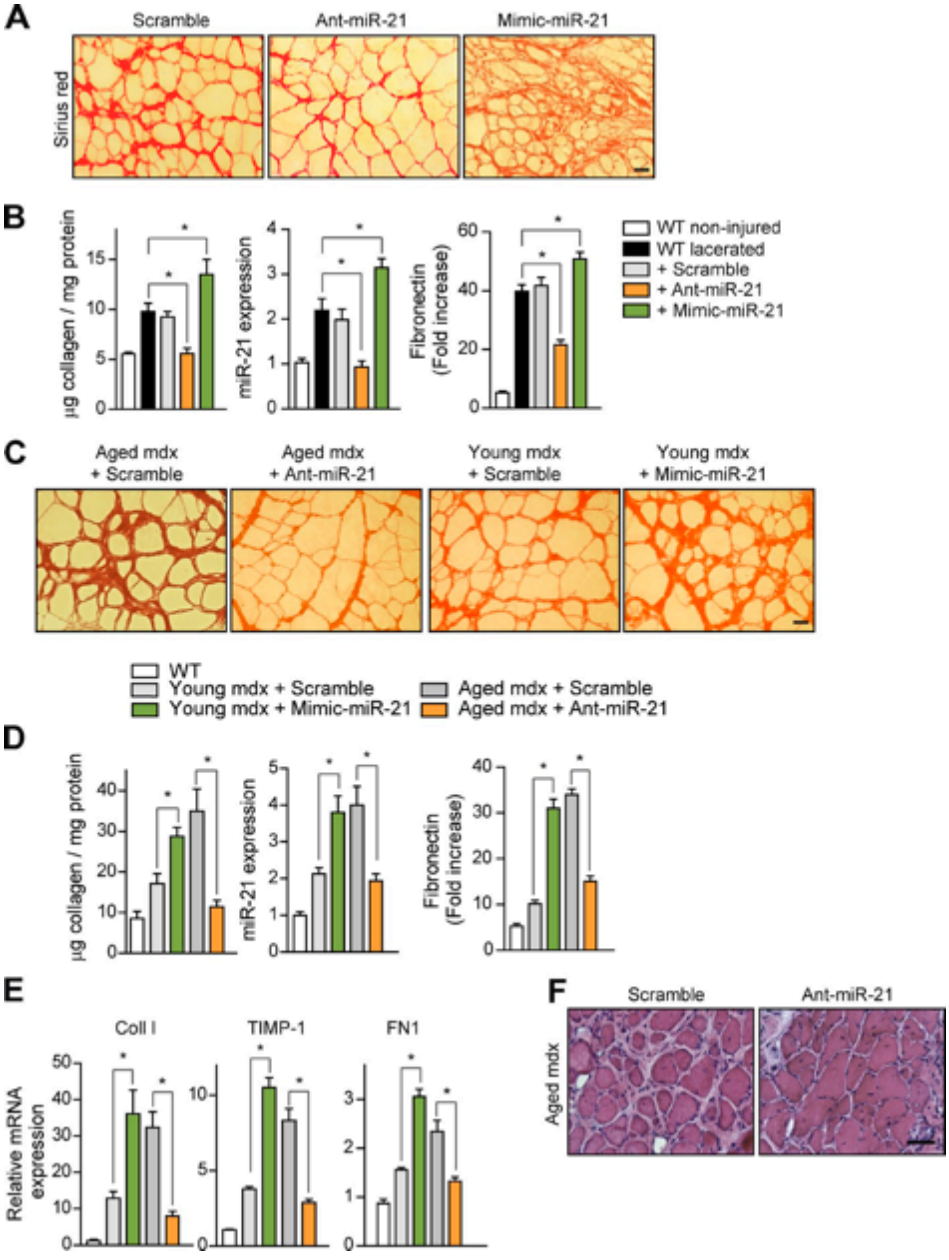


Figure 15. Efficacy of miR-21 silencing in preventing collagen accumulation after injury and treating muscular dystrophy by reversing fibrosis. (A) Prevention of fibrosis. TA muscles of WT mice were lacerated, and an antagomir for miR-21 (Ant-miR-21), a mimic for miR-21 (Mimic-miR-21), or Scramble oligomiR (Scramble; used as a negative control) was injected daily for 3 d starting at day 16 after laceration (before significant collagen deposition, which peaks at 21 d), and muscles were collected at day 21 after laceration. Sirius red staining on muscles of treated mice is shown. (B) Collagen protein accumulation and miR-21 expression were quantified in non injured and 21-d lacerated muscles of WT mice; similarly, fibronectin was analysed by immunofluorescence with a specific antibody, and the positively stained areas were quantified by image analysis. (C) Therapy for fibrosis. Sirius red staining of gastrocnemius muscle from 24-mo-old mdx mice (aged mdx) after administration of anti–mir-21 (or Scramble) every other day for 1 mo before collection of the

muscles (left) and from 3-mo-old mice (young mdx) after administration of Mimic-miR-21 or Scramble with the same protocol (right) is shown. (D) As in B, collagen accumulation and miR-21 expression were quantified; similarly, fibronectin was analysed by immunohistochemistry and quantified. (E) The mRNA expression levels of Coll I, TIMP-1, and FN1 in the muscles of mice described in D are shown. (F) H/E staining of gastrocnemius muscle sections from 24-mo-old mdx mice treated with Ant-miR-21 (or Scramble) for 1 mo. Bar, 50 μm.

F) Inducing blood vessel growth and migration of stem cells into the damaged tissue

Stimulation of new blood vessel growth and enabling the entrance of the stem cells into the damaged tissue are essential for effective repair of damaged tissue. Proliferating vessels create a favourable niche for donor cell implant and proliferation and further reduce the extent of fibrosis. Furthermore, the constituent cells of the blood vessels, endothelial cells, produce growth factors able to improve cell division and resistance to cell death while simultaneously allowing a correct delivery of oxygen and nutrients to the damaged area. To address this we implemented two complimentary strategies: targeting the blood vessel cell junctions so that they would be more permissive to stem cell migration and treatment of the tissue with new medicines that would potentially induce better blood vessel development and migration.

Junctional proteins are responsible for connecting adjacent cells to form a contiguous structure, and preliminary data strongly suggested that increasing vascular permeability by loosening these connections in blood vessels could effectively increase homing of circulating stem cells to tissue. Two candidate junctional proteins, named PECAM and JAM-A are known to be key proteins in this system which we first confirmed could indeed be disrupted using antibodies that would block their binding, with JAM-A being the better target. Quite importantly we revealed that by blocking their activity we could indeed induce and increase muscle stem cell migration, while not influencing the migration of other cell types such as immune cells. This is important as it indicates a specific and targetted effect on the stem cell behaviour, not on the total integrity of the vessel itself which retained its normal function. To validate this two key experiments were performed.

The first involved using a human system based on Human Umbilical Vascular Endothelial cells, blocking the JAM-A with the antibodies which prevented their normal behaviour while simultaneously increasing human muscle stem cell migration. As the use of high concentration of antibodies can be both expensive and potentially detrimental we moved to blocking JAM-A activity using a pharmacological agent (GGTI-298) that also targets it. Using a mouse animal model that replicates muscle wasting disease, by pretreating it with the pharmacological agent we could first demonstrate that JAM-A continued to be disrupted in an *in vivo* setting, and more importantly that after treating mice with the agent and then administering muscle stem cells, the stem cells engrafted more efficiently and more importantly, functional experiments performed on these mice showed that treating them with pharmacological agent prior to stem cell intra-arterial injection significantly improved exercise tolerance (Figure 16).

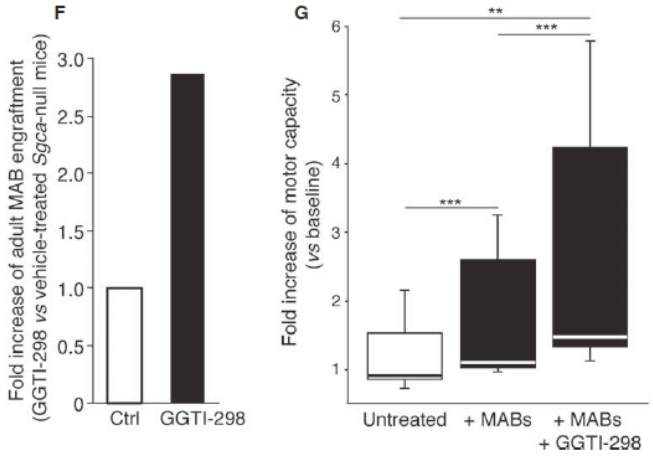


Figure 16: Treatment of dystrophic mice with GGTI-298 prior to stem cell transplant increase both the number of muscle stem cells engrafting and the functional capacity of the muscle itself.

In the same context and as an extension of using pharmacological agents, NCX320 is a new medicine that combines the activities of ibuprofen (anti-inflammatory/analgesic) with Nitric Oxide. The anti-inflammatory activity can reduce the influence of the body's immune cells in interfering with stem cell activity, while the Nitric Oxide component is known to regulate expression of matrix associated enzymes and adhesion molecules

relevant to the architecture of the vascular bed that help to create a favorable niche for donor cell implantation and proliferation, by relaxing the vessels. We therefore assessed whether such a molecule could have an influence on the vessels in animal models. Treating mice that manifest dystrophic muscle degeneration similar to humans, with the new medicine as compared to other medicines did indicate that blood vessels in the disease state do relax more when exposed to it as indicated in Figure 17, strongly suggesting that this drug may have an adjuvant role in future stem cell based therapies.

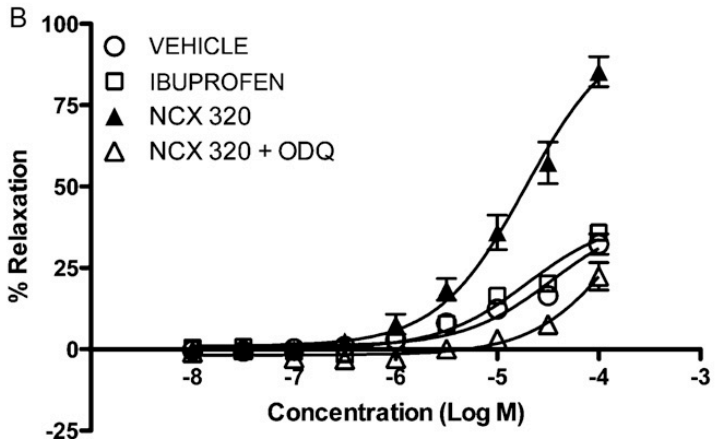


*Figure 17. Cumulative-concentration curve to NCX 320 (filled triangles) and ibuprofen (open squares) on aortic rings. Data are expressed as mean±SEM (n = 3). *p < 0.05 vs ibuprofen.*

NCX 320 was also studied as a novel therapeutic tool for muscular dystrophy by administering it to mice that manifest muscular dystrophies. Results indicated a significant and persistent therapeutic effect that preserved muscle function, reduced necrosis of fibres and inflammatory reaction and maintained the regeneration capacity of muscle. A striking aspect of this compound was its ability to stimulate proliferation and engraftment of stem cells. These data confirmed further the validity of NO-based approaches to sustain cell therapies of dystrophy and given the well-established and wide use of ibuprofen, these findings indicate a path forward for the development of new potential effective agent for treatment of muscular dystrophy, which are presently being explored.

Combined this work points towards future stem cells therapies which are used in conjunction with known pharmaceuticals to enhance the restorative action and functional repair of damaged tissues.

G) Regulation of the immune response

Stem cells have been reported to modulate the bodies immune system and render it 'quiet' thereby permitting the stem cells to engraft and facilitate tissue growth. It was therefore essential to determine if this effect could be elicited by the cells being used in the Optistem project, and thereby circumvent one of the key barriers that prevents the effective application as a therapy.

As the immune system is a highly complex series of diverse cells that plays a critical role in the maintenance of a healthy organism, both mechanism and effect were essential to be determined, which predisposes passing the cells to be transplanted through a series of experiments that start in the test tube and move to the animal model (the *in vitro* to *in vivo* transition).

For both the present work and for future studies it was necessary to assay all potential sources of cells; these included the patient mesangioblasts that were being used in clinical trials and mesangioblasts that had been created by engineering the patients cells to become mesangioblasts (named: HIDEM's).

The first series of experiments performed were done in the in vitro setting, following immune reaction tests that are performed by experimental immunologists the world over. The test involves stimulating the cell to be transplanted with a cocktail of pro-inflammatory factors similar to those they would be exposed to in a damaged tissue and assess if following this treatment the cells induce the expansion of the T-cell; a standard immune cell which expands in response to tissue damage or infiltration of foreign bodies or molecules.

It was found that both patient derived mesangioblasts and HIDEM's induced no T-cell expansion or response, while simultaneously not preventing the T-cells from behaving as they normally would to other foreign stimuli. Specifically:

Human mesoangioblasts

- do not induce T cell proliferation *in vitro*.
- express HLA-ABC and low levels of HLA-DR under resting conditions, and that HLA-DR expression was upregulated after stimulation with IFN-γ.
- suppress T cell proliferation in a dose and time dependent manner.
- do not inhibit the upregulation of the activation markers CD25 and CD69 or the production of IL-2 and IFN-γ

HIDEMS

- do not induce T cell proliferation *in vitro* under basal culture conditions or pre-stimulated with the pro-inflammatory cytokines IFN-γ, TNF-α or IL-1β. Like human mesoangioblasts,
- can exert immune suppressive effects. HIDEMs suppressed $CD3^+$ T cell proliferation in a dose-dependent manner
- do not interfere with T cell activation
- from both healthy donors and patients showed greater suppressive potency compared to mesoangioblasts at the same cell:PBMC ratio.
- an activation step involving IFN-γ or TNF-α was a prerequisite for HIDEM suppression of T cell proliferation; neutralisation of either cytokine significantly abolished the inhibitory effect
- IDO and PGE-2 were identified as key mechanisms of action involved in HIDEM suppression of T cell proliferation.

These very encouraging results prompted moving the tests into a more physiologically relevant in vivo setting, in which the same cells would be injected into living mice and the same assays performed to assess any immuno-modulatory effect.

It was essential that these animal studies were performed under conditions mimicking as close as possible the scenario that would happen when treating a human patient. Specifically that the stem cells would be expanded first in culture prior to transplant into mice; this approach is based on the documented effect observed by all cell biologists that culturing cells in the test tube results in the reduction of certain characteristics that are observed in the living animal.

To achieve this a complex system involving transplanting the human stem cells along with human T-cells into an immune incompetent mouse and then assessing the duration of the stem cells in the mice to see if the T-cells attacked them. Unsurprisingly, they did:

- Mesoangioblasts/HIDEMs could be detected 7 days after transplantation.
- Human T cells infiltrated the transplanted mesoangioblasts/HIDEMs, indicating the targeting of these cells by the T cells.
- The number of transplanted mesoangioblasts/HIDEMS was reduced in T-cell pre-reconstituted mice, suggesting the killing of those cells by them.
- Growth factor analysis revealed a specific and known immune response to the transplanted mesoangioblasts and HIDEMS.

The reason this is unsurprising, is that this is an effect witnessed in many other types of stem cell transplantation, including hematopoietic ones, and as such there are also solutions. The first solution, and one that is not favoured, is to destroy the T-cells in the body prior to transplantation of the stem cells; this permits the stem cells to take up residence while the body regrows its immune cells and a happy medium is found in which all cells cohabitate. Experiments performed by us, also revealed the same thing, and by destroying the T-cells the mesangioblast/HIDEM stem cells were not killed.

Another approach, which is much gentler on the patient, is to expand a sub-population of their T-cells, named Treg (short for T-regulatory cells). These unique cells can be isolated from the patient, expanded and transplanted back into the host which then modulate the immune response permitting stem cells to reside in greater numbers and better repair the tissue. Performing a similar experiment as before, co-transplanting Treg cells with the mesangioblasts/HIDEMs a prolongation of the survival and increase in number of the stem cells could be achieved.

Major outcomes from Optistem

In summary there have been significant data with high impact obtained from the Optistem project, including, but not exclusively:

- **Clinical demonstration of the successful applicability of stem cells as therapeutics being used to correct a localised disease**

- **Amelioration of a whole body tissue dehabilitating disease affecting children through the application of stem cells**

- **Development of scale up procedures and culture systems which enable the reproducible production of high quality and regulatory approved stem cells**

- **Interpretation of the clinical scenario and patient need to create a pipeline of new therapeutics which can be used as new stand alone cell therapies or as adjuvants to compliment the cell therapy and enhance its impact**

- **Validated concepts that can be used to increase blood supply and decrease fibrosis during a regenerative therapy; concepts that can be applied to almost every regenerating tissue that has been damaged**

- **Identified new stem cell sub-populations that can be explored to further enhance the future potential of this exciting field of endeavour**

- **Opened avenues to further develop approaches to circumvent the immune system so that stem cell therapeutics perform their intended objective of restoring function.**

Impact of the project outcomes

Impact

To create high impact the outcomes must be clearly applicable in the present market as standardised products. For the pharma market, the final customers are to the greater extent the national governments via the health services: as these bodies are routinely now applying Cost:Benefit ratio analysis, matched with QALY in the reimbursement of all therapies, it is critical that the costs be kept as low as possible and benefits clearly demonstrated. This clearly has relevance for addressing the economic burden indicated above related to the ageing population who contribute little in taxes but cost a lot to maintain; the recession compounds the problem as the 'final customer' cannot afford expensive therapies.

This problem is exacerbated by the patent cliff in which 150bn€ of protected pharmaceuticals will be available for generic competition while the development of novel biopharmaceuticals has an industry average of -7% return on investment. To be explicit, this means it costs companies to produce novel therapeutics; by performing R&D they are actually destroying their own value, despite their pivotal role in the industry as clinical translation costs are stifling, which only they can afford. There is therefore the necessity to extract as much value as possible from all innovations; To quote E. Tobinick from UCL*,*

" De novo drug discovery has failed to efficiently supply pharmaceutical company pipelines. A rational approach to drug repositioning may include a cross-disciplinary focus on the elucidation of the mechanisms of disease, allowing matching of disease pathways with appropriately targeted therapeutic agents. Repurposed drugs or biologics have the advantage of decreased development costs and decreased time to launch due to previously collected pharmacokinetic, toxicology and safety data. For these reasons, repurposing should be a primary strategy in drug discovery for every broadly focused, research-based pharmaceutical company.'

While approvals of drugs maybe holding steady or indeed increasing according to some reports, and this does bode well for confidence in the R&D and approval process, there does seem to a worrying increase in the reimbursement agencies refusing to buy the therapies e.g. BMS and Orencia, GSK and Benlysta and more recently Genomic Health Inc and Oncotype DX, because they were considered not cost effective with regard to QALY; interestingly level of innovation was not a criteria used for reimbursement. While in two instances the decision was reversed after two years, in the other it was not; irrespective this has a large impact on the return on investment (ROI) and bottom line of any business and the industry overall. Shareholder confidence can be eroded, followed by the investment market, then specialist investors. If published figures are to be believed the industry average ROI on R&D for new therapeutics is between minus 2 to minus 7%, which is fundamentally value destruction.

Even though at present many companies are presently doing well on their existing pipeline, the future does not seem so bright; indeed it seems that single class blockbusters are quite possibly now market anomalies. Common sense and addressing how most agencies are presently operating would indicate that if a company is making that much money from one drug, the reimbursement agencies or HMOs are logically going to demand buying it cheaper.

In such an environment there is a unique opportunity to pull together opposing cultures in the life science sector, specifically the regenerative medicine sector, from academia and industry, and generate real value, which leverages the issues that are facing the industry and turn these into market drivers and enablers. In the context of the ageing demographic, the fact that academic research focuses generally on specific issues related to rare or less common diseases (as those with high commercial value are already extensively addressed), there is real strength and opportunity. In rarer degenerative diseases, to generate any significant impact of the research, not only do the key molecular and cellular triggers for the disease have to be detailed, but also their impact on tissue function and the potential underlying regenerative mechanisms should be defined. Amongst many diseases, and across tissues, some of the major obstacles to restoring function are shared; therefore insights can generate therapies for rare diseases with an impact on more common disorders. Critically, the outcome should be a limitation of the degenerative process, matched with a restoration of tissue function, as opposed to a palliative treatment. If extended to the global issue of keeping the ageing population operational and contributing to society, will represent the real impact of regenerative medicine research. Optistem's outcomes therefore remain highly beneficial and lucrative, and low costs should be achievable.

It is in such an market that the outcomes of Optistem have had and continue to have the most impact. The project was able to demonstrate the feasibility of using stem cells as therapeutics and effectively correct an illness for which there is no other available treatment. By doing so, the project has managed to shift the nature of therapeutic development and provide the basis for the continued importance of developing cell based therapies as affordable products.

The prevailing conundrum of high research expenditure by industrial players but with low product approvals and elimination of patent protection on the products they do own and are marketed, is forcing a structured paradigm shift on the way the these players obtain new knowledge and which therapies offer the bigger future benefit. Mimicking the ICT field, bio/pharmaceutical companies of all sizes are moving towards the laboratory equivalent of open collaborations in which normally non accessible knowledge is integrated due to ongoing collaborations with public entities. Large collaborative projects in the FP7 are therefore becoming useful catalysts to facilitate these plans: Optistem represented another example of such a success. Inversely one large company collaborated with one SME specialized in the cell and gene therapy and a select group of academically leading and well infrastructued laboratories whose insight, knowledge and expertise the industrial partners would never be able to afford to financially create and support on their own. These industrial partners inside the project, via the grant agreement and consortium agreement have worked in a contractually controlled and legally defined collaborative environment. Knowledge on fundamental aspects of stem control and manipulation, developmental biology and molecular control, matched with real clinical translation and application have been integrated into the decision making processes which has permitted an open collaboration strategy that deliberately fostered information sharing even amongst what are typical competitors to generate benefit and hope for patients.

Integration of real time clinical application into fundamental knowledge generation

One of the major caveats of all regenerative medicine is due to the time it takes to validate a product (typically 14 years from concept) which precipitates a speculative approach to what is required or applicable. It is very easy to create experimental approaches which while interesting scientifically have no relevance for humans due to an inability to be applied in a clinical setting. These innovations therefore lack a critical component in their design to correlate between the disease pathology and the therapeutic need.

Optistem, from its original conception, surpassed this bottleneck by having real clinical application as its foundation. No lag phase based on publication of results was experienced as patient treatment and outcomes was systematically fed into the partnership, enpowering and enabling all partners to position their fundamental or pre-clinical work in the context of patient need and, most importantly, regulatory requirement.

Through the participation of several leading PIs who performed (and had previously performed) clinical trials in the project (e.g. Giulio Cossu, Michele de Luca, Claudio Bordignon, Yann Barrandon) we ensured that from the start a real understanding of the patient was included in the design and that there was a clear route to value addition and patient benefit. A conceptual reverse engineering was integrated into the fundamental research teams of the project, who while generating data that maybe years from potential application, could obtain insights not previously obtainable, that would direct their research and inform their decision making to prioritise areas which they may have previously not considered. The clear outcome is that there are now more relevant molecular targets, potential new cell therapies and methods to generate a complete and functional tissue that were not previously known.

Through this we have also created significant value for all the participants; the volume of high quality and high impact publications has the beneficial result of creating more grant opportunities for the teams. It has also generated a more direct financial value as the methods to make the final products are applicable in both the local and foreign markets. In the regenerative medicine market, the final customers are to the greater extent the national governments via the health services and therefore it is critical that the costs be kept as low as possible and benefits clearly demonstrated. This was achieved and clearly has relevance in addressing the economic burden related to the ageing population; the ongoing economic recession compounds this problem since the ‘final customer’ cannot afford expensive therapies. Through demonstration of effective treatment, further scale up and technological development we anticipate that we will have facilitated the generation of low cost, high quality and hi-tech cell therapy products that can be eventually developed cheaply for a broad range of applications.

Ensuring broad application of the generated inventions

The broad applicability of Optistem's outcomes are a major benefit: while focused on skeletal muscle and skin, the outcomes impact the whole organ market. The direct healthcare costs of organ replacement are about € 240 billion globally (about 8 percent of global healthcare spending) arising from therapies that keep people alive (such as kidney dialysis), implanted replacement devices, and organ transplants. With a € 240 billion global industry already built on first generation tissue and organ therapy products and substitutes, regenerative medicine has a potential to exceed € 600 billion by 2030.

As indicated in the objectives, one of Optistem's primary scientific objectives was the understanding of angiogenesis within a damaged and regenerating tissue, following cell therapy, which if combined with targeting fibrotic events represent the pivotal components that are at the start of the physiological response to alleviating all types of tissue damage. Through this approach to stimulate complete tissue regeneration and the local control the potential wider impact of the results obtained stretches to a larger number of potential tissue targets, as indicated below:

I) Cardiac diseases: The prevention and amelioration of pathological cardiac remodelling as a consequence of acute or chronic ischemic heart, myocardiopathies due to inflammatory, infectious or genetic causes is an immediate requirement. Just in the Western world presently there are over 9 million people suffering of heart failure where the treatment available (with the exception of heart transplant) are only palliative. Yearly, more than a million new patients join this group. The average life-span after the first episode of heart failure for these patients is of ~5 years. By 2016 the annual global market for all cardiovascular products is estimated to reach €160bn, with the top ten companies estimated to possess half of that market. Critically, cardiac disease and myopathies are global issues.

There are over 800 different chemical and biopharma type drugs that are in development around the world that are targeting cardiac diseases, while there are over 10000 cardiovascular and circulatory drugs on the market, therefore it is fair to indicate that existing therapies are not restoring function in a physiological way and it is unlikely that non regenerative based therapies will ever fully repair the damaged tissue of an infarct or myopathy.

To quote, Frost and Sullivan report ' Advances in cardiovascular therapy':

"A fundamental driving force in the cardiovascular therapeutic field is the realization that pharmaceuticals can only go so far in the treatment of cardiovascular diseases. With increasing complexity of the genetic makeup and the role of multiple factors in the etiology of cardiovascular diseases, there has been a drive to think beyond the ordinary and consider the use of technologies such as tissue engineering and stem cells in managing cardiovascular diseases and leading to treatment of the disease."

II) Urinary incontinence: Although UI is an extremely common problem, and has a significant impact on quality of life, the vast majority of those who experience the condition do not undergo treatment, in part due to cost, embarrassment, or fear of risky surgical procedures. There is therefore a very strong demand for less costly, less invasive and more tolerable, discreet, nonsurgical UI therapies. UI is due to both age-related muscle de generation and to iatrogen lesions in young women due to ephysectomy during delivery. Trials with myoblasts injections are ongoing but the low engraftment of cells injected in saline solutions lowers efficacy. The global Urinary Incontinence (UI) therapeutics market was worth approximately €2.5 billion in 2009. In 2001 the market was valued at €1.4 billion and it grew at an approximate CAGR of 7.8% from 2001 to 2009. The global UI therapeutics market is expected to reach €3.4 billion by 2017 after growing at a CAGR of 3.5%. Existing therapies do not cure the non or dys functional tissue.

III) Surgical management of malignant lesions of the oro-facial region: Tumors of the spanchonocranium often require demolitive surgery. Patients survive but with mutilations that severely limit their normal life functions and usually abolish their social life. Plastic reconstruction is a major challenge and could be enormously helped by the possibility of developing in situ, muscles, as the proponents have demonstrated to be possible by using stem cells.

IV) Soft tissue: Progress has been made in the discovery of growth factors that can regulate skin repair (for burns, genetic diseases), but there remain important challenges. Johnson & Johnson has introduced PDGF-

BB to the clinic and marketplace, which costs several k€ and is plagued by very inconvenient dosing. Adoption in both Europe and the USA has been very limited due to these considerations, penetrating less than €150 million/year into a market that is thought to be approximately €5 billion/year deep. Treating superficial local soft tissue is unlikely to be attractive as the tissue is likely to repair itself, however in the context of large scale or profound soft tissue damage the concept of our approach should be easily translatable. *Global Market size: > € 912 million 2016*

V) Bone disease and repair: The cells used in Optistem share characteristics with those that can be used for bone repair. The dominant clinical and market needs are in spinal fusion and healing of non-union fractures; accelerated repair of traumatic fractures, and accelerated healing after dental surgery. Good progress has already been made in the marketplace in spinal surgery with BMP-2 delivered in a collagen matrix, developed by Wyeth and marketed by Medtronic Sofamor-Danek; adoption in spinal fusion in the USA has been very high (selling more than $2 million/day); however, its very high cost has led to little use in spinal surgery in Europe. This cost is driven by the extraordinarily high amount of therapeutic BMP-2 protein that is included in the formulation – equivalent to the amount present in ~1000 humans per spinal level – pointing to inadequacies in localized release on an optimal time course. It is estimated that annually more than one million patients need treatment for skeletal problems worldwide. As the European population is aging considerably, orthopedic diseases are a major concern for the forthcoming decades. Thus, even in cases where products have been successfully developed, cell based therapies may offer longer term benefit.

Main dissemination and exploitation outcomes

Stem cell research continues to be contentious and it was therefore important to engage with the public in a structured and informative way to stimulate informed debate.

The project website (www.optistem.org) was set up as the public face of the research consortium. During the course of the project (time period 1st September 2009 – 30th November 2013) the website had 9,722 unique visitors (13,776 visits, therefore ~30% repeat visitors). The top countries of origin of visitors to the website (in order of frequency) are: United States of America, United Kingdom, Italy, France, China, Germany and Spain.

The website provided information about the consortium members and their research aims for scientific communities. Publications, current news and publishable reports were all available on the site. The most popular pages that vsitors read on the website were the pages about the ongoing research (including the lay summaries), the 'about us' pages and the pages that list the OptiStem principal investigators.

OptiStem also collaborated closely with the FP7 funded project EuroStemCell. The main output of this project was the EuroStemCell website (see below www.eurostemcell.org). OptiStem continued to work synergistically with EuroStemCell in all its public engagement work and rather than using the OptiStem website for dissemination to publics, the consortium used the EuroStemCell website to disseminate all educational resources, news, events and information which was produced as part of the public engagement effort of OptiStem. This maximised the impact and longevity of our public engagement and outreach work, whilst also avoiding duplication of effort and resources.

Outreach

Our public engagement strategy at OptiStem, one of the first EC-funded consortia to take new stem cell therapies into phase I/II clinical trials, was to focus on informing and engaging European citizens with the process of research and, in particular, the often misunderstood concept of clinical trials. It was important that a clinical trial was viewed as research, not a therapy, and seen as part of an ongoing research process, not an end in itself. Our engagement was a two way process providing opportunity for OptiStem members to listen to, and discuss issues with, non-specialists as well as disseminating information to citizens.

Production of Multi-lingual Hope Beyond Hype Graphic Story with Online Interactive Version

Hope Beyond Hype was a 10 page graphic short story designed to engage publics around the story of stem cells - from discovery to therapy. Written by Ken McLeod, with Jamie Hall, Edward Ross, Cathy Southworth, and illustrated by graphic artist Edward Ross, Hope beyond Hype was produced as part of the public engagement activities of OptiStem, and featured OptiStem science and researchers. The aim of the graphic story was to produce an accessible resource that portrayed the length and complexity of the 'bench to

bedside' process to a wide variety of adult stakeholders – including hard-to-reach audiences - in an engaging way.

The story is available online (www.eurostemcell.org/hopebeyondhype) in six European languages and as hard copy. It will continue to serve as one of OptiStem's main and lasting public engagement outputs.

Since its launch in May 2012 on International Clinical Trials Day, this extremely successful resource has reached a very large number of European Citizens:

- Between January 2013 and November 2013 the number of views of the resource via the online magazine Issue increased to 265,000 views.
- OptiStem was contacted by the National Library of Scotland who requested a copy so they could add it to their collection in the national archives;
- Following the launch of the online interactive resource (containing videos, feedback walls, activities and deeper content) Hope Beyond Hype has continued to attract positive online reviews "*EuroStemCell and OptiStem… have now turned this "made for print" form of communication into something that resonates with an online audience. Earlier this year, Hope Beyond Hype was re-released in an interactive format, complete with videos, links to additional information and a feedback wall. One year later, I'm amazed all over again*. " http://www.signalsblog.ca/right-turn-bench-to-bedside-goes-interactive/;
- To date approximately 11,000 hard copies of the English version, 2000 copies of the Italian Version, 1000 copies of the German version, 1750 copies of the French version, 50 copies of the Polish version and 250 copies of the Spanish version have been disseminated via open days, science festivals, music festivals, patient support events, European research institutes, scientific meetings, social science festivals, scientific meetings, parliamentary briefings, teacher training days, schools and community family events predominantly across Europe but also in USA, Canada and Australia**.**

- The Research Councils UK requested copied of Hope Beyond Hype to use at an event on regenerative medicine in the UK Parliament, Westminster;

Following feedback from teachers and science centres that they would interested in using a stand-alone offline version of the interactive Hope Beyond Hype resource, significant effort was used to create a fresh, copyright appropriate, interactive version of the comic. OptiStem again worked closely with EuroStemCell to develop this resource, which required substantial user research and development of practical know-how around the complex technology associated with creating an interactive comic suitable for multiple platforms and devices. In the new offline version of the interactive, each page had a video and a mini-task associated with it. Six of these mini-tasks were inventive, newly designed interactive flash animations.

Communication of 'bench to bedside' process through production of an interactive game called "The Road to the Clinic".

In 2012 OptiStem collaborated with the Scottish-based MRC Centre for Regenerative Medicine to produce a table-top game called 'Road to the Clinic'. Users of the game had to negotiate balls painted to look like researchers, around an obstacle race. This fun game showed the stages of the 'bench to beside' process and what must be achieved at each stage before the research can move towards the clinic. A series of card games were also developed to foster deeper discussion about the people involved in the process (e.g. clinicians, scientists, regulators, patients, ethicists), their roles and how they must work together to get a therapy into clinics.

The game formed part of the Edinburgh International Science Festival in 2013, where approximately 3000 people interacted with it. It has now been taken to remote Scottish communities and patient groups as part of a new Scottish Government Funded initiative by the Centre for Regenerative Medicine, University of Edinburgh.

Continuing core collaboration with EuroStemCell

OptiStem made a commitment to be a major contributor to, and supporter of, EuroStemCell. This close partnership was designed to maximise the impact and minimize duplication of stem cell-related public engagement effort. EuroStemCell's mission is to provide a trusted, multilingual, accurate and up-to-date information source on stem cells and regenerative medicine for European citizens. EuroStemCell partners

with significant key players, consortia and research institutes across Europe in order to provide a co-ordinated effort in this arena. Throughout this report effort has been made to specifically highlight the projects in which OptiStem and EuroStemCell collaborated.

More than 150 scientists, ethicists, legal experts, science communicators and social scientists from all over Europe contributed content to the EuroStemCell, with many more participating in the translation effort. The use of social media channels such as Twitter, Facebook and YouTube is integrated into all the communications efforts. These channels provide supplementary platforms for dissemination, supporting and extending the reach of the website, resources and direct public engagement for general audiences. To serve the needs of science communicators and educators, EuroStemCell established a resource directory of educational materials, tools and activities for public engagement, which now comprises more than 100 resources.

OptiStem significantly contributed to translation of the public-facing elements of the EuroStemCell website into six European languages – ensuring that the outputs of EC-funded research projects, together with EuroStemCell tools and resources on stem cells and regenerative medicine, are available to 80% of European citizens in their native language. Another key area of collaboration has been the development, production and translation of a high quality film about how the identity of specialised cells is regulated in the body - a subject particularly appropriate for OptiStem's scientific area. OptiStem also worked with EuroStemCell to disseminate copies of the award-winning film Stem Cell Revolutions to OptiStem members and our stakeholder engagement partners.

Some notable achievements of EuroStemCell from 2013:

- The website is reaching more European citizens every month: website traffic continues to grow. Latest statistics (1st January 2013 – 30th November 2013): 472,995 visits (288,000 last year; 64% increase) of which 379,038 unique visits (217,500 last year; 74% increase) with a total of 947,512 page views (557,947 last year; 70% increase) from 214 different countries worldwide (190 last year). 41% of the site visitors are from Europe.

- Up until end November 2013 the films page received 127,714 views, the YouTube channel 141,185 lifetime views and 526 subscribers and the Twitter account 3,352 followers (2007 tweets so far).

- EuroStemCell began to add multilingual functionality from 2011 – the interface and key content are now available in English, French, German, Italian, Spanish, with Polish to be added in 2012/13; these 6 languages make the site accessible to >80% of Europeans.

- The 3350 followers on Twitter (109% increase from 2012, 2007 tweets so far) included influential patient organizations such as @ParkinsonsUK, @patientslikeme and @MichaelJFoxOrg; individual patients and patient advocates; scientific journals including @CellStemCell, @CellCellPress, @PLoSBiology and @NatureCellBio; scientists, bioethicists, journalists, stem cell companies, teachers, educational organisations and more.

- The Facebook page (facebook.com/eurostemcell) has 1052 likes (78% increase from 2012).

- The newsletter has 2383 subscribers.

Production of Lay Summaries of OptiStem Publications

OptiStem also committed to producing lay summaries of its research papers producing 12 lay summaries, that can be found on the OptiStem and EuroStemCell websites (http://www.optistem.org/research-spotlights and http://www.eurostemcell.org/stem-cell-research-updates). These lay summaries have attracted 2304 page views on EuroStemCell.org.

Engagement with European patients, carers and patient advocacy groups

Due to the translational aspect of OptiStem's research, patients, carers and patient advocacy groups were a key stakeholder for the public engagement and outreach activities. Much of this happened via direct relationships between principal investigators and their local/national networks.

OptiStem also collaborated with EuroStemCell to initiate a small Europe-wide research project about patient engagement. The research aimed to firstly elucidate the type of information patients and carers from across Europe could access about stem cell related clinical trials and the benefits/downfalls of that.

A major achievement for OptiStem was the dissemination of an extensive toolkit of stem cell dissemination and outreach resources to nearly all OptiStem related research institutions. Discussions were held with each of the principal investigators to determine first, whether they would use such a kit and second, who would be the designated 'keeper'. The kit contained the "Start as a Stem Cell" resources and an additional selection of activities, card games and films (http://www.eurostemcell.org/toolkititem/start-stem-cell-outreach-kit-scientists). Where possible language specific content was included e.g. DVDs with subtitles, comics and card games in European languages. Importantly, this action meant that as the OptiStem research projectcomes to a close in its present iteration, the materials produced by OptiStem (either alone or in partnership) will continue to be available to European researcher for use in all their future dissemination, engagement and outreach activities.

Fostering engagement and dialogue between scientists, parliamentarians and regulators

Another key stakeholder identified for OptiStem engagement work was parliamentarians and regulators. OptiStem collaborated with the UK Office of the European Parliament and EuroStemCell to run a panel and discussion event in February 2013 at Europe House, London. We secured the participation of the Rapporteur for the amendment of the Clinical Trials Directive, MEP Glenis Wilmott, and due to the timely nature of our collaboration the event was fully booked, with 65 high level delegates attending. The audience consisted of industry, funders, national regulatory bodies, journalists and other policy makers. OptiStem principal investigator, Professor Michele De Luca was one of the four panel speakers (along with MEP Wilmott, Stefano Soro, Head of European Commission's Directorate-General Health and Dr Liz Philpots Head of Research, Association of Medical Research Charities).