# OptiStem Year 2 Annual Report: Public summary

### 1.1. Project Objective

The **overall objective** of OptiStem is to leverage European scientific excellence through a collaborative project in order to develop new strategies that will enhance the efficacy of clinical trials with adult, tissue stem cells for degenerative deseases of epithelia and skeletal muscle. This synergistic approach will ensure that ongoing and future clinical trials have a greater chance of success. To this end, key actions have been identified :

1. Phase I and potentially Phase IIa cell therapy trials for patients with muscular, skin or ocular disorders
2. In-depth optimisation of cell therapy studies in large animal models
3. Systematic analysis of stem cells fate in these large animals to address *(i)* differentiation, *(ii)* molecular control and *(iii)* transplantation efficacy
4. Identification of novel therapeutic strategies based on clinical trials results and small animal modeling
5. Characterisation of *(i)* regulatory, *(ii)* transcriptional and *(iii)* signalling pathways that control stem cell activity *in vivo*
6. Stimulation of tissue regeneration through activation of angiogenesis, a key success factor in efficient cell therapy
7. Immunological studies both *in vitro* and *in vivo* in order to determine *(i)* any adverse or beneficial effect and *(ii)* factors that can modulate the immune response.

To achieve these stated goals, the programme of work focuses on six interrelated projects in which the partners bring in specific expertise and resources which are collectively leveraged.

The primary focus is on clinical trials (CT) with epithelial and muscle stem cells. CT represent the core of the OPTISTEM project : they will benefit from discoveries during preclinical trials in large animals and their results will lead to the identification of new therapeutic approaches through further studies in small animals. These clinical trials will 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 is on preclinical large animal models. It relies on results produced in small animals and constitutes a key translational interface between stem cell biology and the implementation of clinical protocols. In this regard, dystrophic dogs will 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 will 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 is on developing mouse models for cell therapy including immune-deficient animals for human cell transplantation. The injection of human keratinocytes into immune-deficient mice will provide a quantitative assay for the effects of different signaling pathways on epidermal lineage selection. These xenografts of modified human epidermal cells will 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 is on the factors that control stem cell activation and renewal at a mechanistic level. Using cutting-edge molecular and cellular approaches we are studying the different signalling pathways and transcription factors that control *(i)* satellite cell behaviour and repair of skeletal cell muscle, *(ii)* muscle interstitial cells and *(iii)* mesoangioblasts.

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

Finally, we also address the immunological aspects that result from stem cell engraftment. The regulation of the immune response is a key success factor for stem cell therapy. Here we shall determine *(i)* to what extent mouse and human stem cells, prior or after manipulation, elicit or not an immune response, *(ii)* how this response is modulated and *(iii)* how we can induce tolerance.

### 1.2 Work performed since beginning of the project

The work performed since the beginning of the project covers all the areas mentioned above. The first clinical trial for children affected by Duchenne muscular dystrophy has been completed. It enrolled 28 patients and demonstrated the feasibility of monitoring the evolution of the disease in DMD individuals undergoing experimental treatments. A phase I/II clinical trial (first in man) is now scheduled to start in March 2011, based on transplanting mesoangioblasts from HLA-identical sibling into the arterial ciculation of three DMD patients. Three more patients are eligible because of an HLA-identical sibling and we are seeking funds to treat also them in year 2012. For the other trial based upon transplantation of autologous engineered muscle-derived CD133+ stem cells, pre-clinical work in dystrophic dogs in being completed and recuitment of patients should start next year. Additionally we

have also completed the first effective autologous cell therapy clinical trial of stem cell-derived cornea transplantation finally demonstrating the real potential of stem cell therapeutics in treating a common disorder.

Preclinical studies using large animal models are well on their way, demonstrating for the first time a clinical effect in old dystrophic dogs that regained walking ability after autologous transplantation of engineered stem cells. Stimulated regeneration capacity of myogenic precursor cells, reduced muscle necrotic damage and inflammation have been achieved through a combination of drugs already approved for humans hence improving stem cell homing. A major step forward has also been performed with an innovative device that successfully permits the transplantation of single stem cell on the ocular surface of the pig. Stem cells including cells from the ocular surface, the thymus, the tooth and the hair follicle have been successfully transplanted and imaged.

A number of tools including about ten mouse lines and six cell lines have been generated to study the mechanisms that control stem cell activation and differentiation. First results have already been produced and will lead to promising new developments that will, after regulatory review, help the optimisation of stem cell therapy. For example, using such mice, we found that the Notch pathway is implicated in atopic dermatitis and in maintaining corneal integrity. We have also identified a new bipotent stem cell population that can be purified from any tissue which opens perspectives that go even beyond the scope of the project since they might benefit to the entire regenerative medicine field.

### 1.3 Main results achieved so far

The preliminary study “Validation Outcome” for children affected by Duchenne muscular dystrophy has been completed. This study was necessary in order to evaluate efficacy of subsequent cell transplantation. The study showed that it is possible to establish tools to monitor the evolution of the disease in DMD individuals undergoing experimental treatments.

In order to carry out a phase I/II clinical trial based on transplanting mesoangioblasts from HLA-identical sibling into DMD patients, cells from the three selected donors have been expanded, characterized and frozen as Intermediate product in December 2010. This clinical trial is now scheduled to start in March 2011.

The preclinical work towards a phase I/II clinical trial based on intra-arterial transplantation of autologous engineered muscle-derived CD133+ stem cells from DMD patients has been initiated. The design of the clinical study was discussed with the regulatory agency.

In parallel, the cGMP certificate of the Centre for Regenerative Medicine in Modena has been obtained; this certificate is necessary to extend clinical trials on epithelial reconstruction and advance their ongoing cell therapies.

As part of the work package involving large animal models, five dystrophic dogs were treated with their own transduced CD133+ stem cells. All treated dogs had a clinical performance improvement. Muscles biopsies confirmed the presence of different amount of dystrophin. Two dogs, received their own muscle-derived CD133+ stem cells without lentiviral transduction. No clusters of dystrophin positive myofibers and clinical modifications of the performance were seen in these two dogs. This is the first demonstration of a clinical effect in old GRMD dogs that regained walking ability after autologous transplantation of engineered stem cells.

We found that circulating CD133+ were always positive for the Pax7 expression and never for Pax3 indicating that physical contact between myofibre and stem cells is required to maintain subtype identity.

One objective was to increase the long term survival of transplanted genetically corrected mesoangioblasts. Engraftment has been increased when transplantation was carried out in neonatal mice. Specifically, three weeks after the injection of human mesoangioblast in the gastrocnemius muscle of P10-P14 mice, we were able to identify almost 10 times more cells than in the adult dystrophic mice.

We also tested, in $\alpha$-Sarcoglycan null mice, whether a combination of drugs approved for human use, isosorbide dinitrate (ISDN) and the NSAID ibuprofen was efficacious in slowing disease progression. The combination of ibuprofen and ISDN stimulated regeneration capacity of myogenic precursor cells, reduced muscle necrotic damage and inflammation hence improving stem cell homing.

To increase the number of mobilized progenitor cells and their localization in the damaged muscle, we set up a new protocol using a combination of VEGF or G-CSF with a CXCR4 antagonist (AMD-3100), which appeared to be more effective than the previously reported in vivo treatment by Adeno-associated vector expressing VEGF.

In addition, we showed enhanced collagen deposition (fibrosis) in PAI-$1^{-/-}$mdx early after disease onset (i.e. 2.5 and 3.5 months of age) with a marked increase in the expression levels of ECM genes. However, similar plateau levels of fibrosis at 12 months of age were seen compared to *mdx* mice. To study the implication of plasminogen activation system (fibrinolytic) in the inflammation/fibrosis development in degenerating muscle, we also showed an increased number of macrophages in PAI-$1^{-/-}$mdx coinciding with disease appearance. Furthermore, less muscle degeneration and less fibrin/ogen deposition in damaged muscle areas was seen in Fib-/-mdx mice compared to Fib+/+mdx mice at one month of age.

Further to our data showing that mesoangioblasts could diapedese much more efficiently through JAM-/- endothelial monolayers than through control cells, embryonic mouse mesoangioblasts and adult mouse mesoangioblast also migrate more efficiently *in vivo* to leg muscles of JAM-A-null mice than to leg muscles of the PECAM-null mice and VE-Cadherin-null mice. Our results also suggest that the absence of JAM-A which in turn affects claudin-5 expression favour mesoangioblast homing.

We initiated the characterisation of the immune response to human mesoangioblasts *in vitro.* She showed that they are hypoimmunogenic and do not evoke an immune response *in vitro.* In addition, stimulation of human mesoangioblasts with IFN-γ, but not TNF-α or IL-1β induced expression of HLA-DR and upregulated expression of HLA-ABC. Importantly mesoangioblasts were found to inhibit proliferation of both CD4 and CD8 T cells.

Moreover, we have established that NO generation and the consequent elevation of cGMP are required for myogenesis through the inhibition of mitochondrial fission. Such results highlight the role of bioenergetic functions in regulating myogenic differentiation.

We have demonstrated that sphingosine-1-phosphate signalling via the S1PR3 is a crucial regulator of satellite cell function. Indeed, *ex vivo* experiments using *S1PR3*-null mice showed that satellite cells proliferate more and also have a greater capacity for myogenic differentiation. *In vivo* experiments showed that S1PR3-*null* mice had more myotubes with centrally located nuclei: indicative of an enhanced regenerative response

Using the muscle specific p38α deficient mice, we found different gene networks regulated by p38α. Among which, many transcription factors that had not been previously related to skeletal myogenesis were validated. Interestingly, some genes implicated in muscle growth, were also regulated by p38α.

We have identified interstitial PW1+ cells (PICs), which constitute a novel population of resident myogenic cells that participate with satellite cells in generating new muscle *in vivo*. The PW1 reporter mice was also used to obtain a pure population of PW1+ cells that can be separated into satellite cells and PICs. In addition, we showed that cell expressing reporter activity are fully competent stem cells capable of giving rise to differentiated epidermal cell fates as well as reconstituting their niche and show pronounced self-renewal. Furthermore, silencing PW1 dramatically inhibits, *in vitro*, the ability of mesoangioblasts to differentiate in muscle due to lack of MyoD expression. In parallel, *in vivo* experiments showed that lack of PW1 in mesoangioblasts led to a defective ability to rescue muscle in dystrophic mice.

The transcriptome analysis of satellite cells identified a potential role for Pitx2/3 and MicroRNA31, which targets Myf5 mRNA and is present in quiescent satellite cells. Results to date, suggest that miR31 is important in preventing Myf5 protein accummulation and hence premature myogenesis in these cells.

The *Pax7* conditional transgenic mice generated during the first year of the project was used in different mutant backgrounds to determine the role of Notch signalling in satellite cells. Ongoing experiments indicate that loss of RBPJk activity results in exit of quiescence of the satellite cells and myogenic differentiation.

We have shown that Notch mutant mice lacking the TSLP receptor developed invasive skin tumours. We propose a model in which TSLP mediated inflammation in the Notch mutant mice protects against tumor development. Interfering with TSLP receptor signalling in Notch mutant mice results in the loss of CD8 T cells, which is accompanied by the accumulation of myeloid cells within the stroma. These myeloid

cells together with stromal cells are able to drive β-catenin mediated tumor growth in neighbouring Notch deficient epithelial cells.

Using a mouse model, we found that the Notch pathway in implicated in atopic dermatitis and in maintaining corneal integrity. Indeed, conditional deletion of Notch1 in the corneal epithelium, results in the formation of corneal plaques in which the epithelium undergoes a fate conversion to epidermis. We now have evidence that chronic inflammation is sufficient to induce conversion to epidermis.

Together with a new generation of mini-incubators that allows the long-term maintenance of isolated pig eyes in organ culture, we have constructed a device that successfully permits the transplantation of single stem cell on the ocular surface. This represents a major step forward. An array of EGFP labeled stem cells including cells from the ocular surface, the thymus, the tooth and the hair follicle have been transplanted and imaged.

We also demonstrated the feasibility of single stem cell transplantation onto pig skin, a large animal model.

We have identified a new role for a transcription factor, Sox2, in a hair reconstitution assay, leading to a new investigation route towards the understanding of stem cell mobilisation. Sox2-positive and negative dermal papilla cells have different gene expression signatures that could in turn impact on signalling to the epidermis and thereby influence hair follicle type. We also developed an hydrogel culture system to support dermal papilla growth and maintains alkaline phosphatase activity, which may correlate with hair follicle inducing ability.

We have identified clonogenic, multipotent stem cells in the stratified squamous epithelia of the rat. Our genome-wide analysis supports that, similarly to the haematopoietic system, those cells follow a multilineage priming mechanism. In addition, we also have analysed thymic epithelial cells and discovered that, when exposed to an inductive skin microenvironment, they could acquire new properties and behave like hair follicle multipotent stem cells.

As an indication of the volume and quality of the output, partners of the consortium have published 33 peer-reviewed articles in international journals such as *Cell, Nature* and *New England Journal of Medicine.*

### 1.4 Expected final results & their potential impact and use

The proposed projects capitalize on combined expertise in different areas of regenerative medicine. The proposal involves collaborative interactions that allows us to merge our unique and complementary expertise in the field.

**Impact on science**

Our expertise in stem cell biology is already being translated into realistic strategies that will increase the efficacy of stem cell therapies. This project aims to develop and implement clinical trials for genetic and acquired diseases of epithelia and skeletal

muscle, by transplantation of donor or genetically corrected, autologous adult stem cells. While transplantation of epithelial stem cells for skin and cornea are now routine clinical procedures, none of them is yet optimal as skin appendages are not restored and bilateral corneal damages cannot be treated. At the same time, a clinical trial with autologoous genetically modified stem cells has produced excellent results for a severe genetic disease (epidermolysis bullosa). In the case of muscle diseases, donor cell transplantation has produced encouraging results in a large animal, pre-clinical model of Duchenne muscular dystrophy (DMD); based on this result a first clinical trial is planned. The proponents of this proposal have pioneered this work and are world leaders in the field.

It is widely recognized that the efficacy of these trials relies on continuous optimization, based on in depth understanding of the biology of the stem cells used, of the pathology of the host tissue as well as the immune response against donor cells, viral vectors and transgenes. This project addresses coordinately all these issues, ranging from clinical experimentation, pre-clinical tests in large animals and in immune deficient mouse models, to cell biology of stem cells and targeted use of pharmacological tools. The project also addresses the biology of epithelial and skeletal muscle regeneration, as well as the control of angiogenesis, inflammation and immune response in the host. The expertise of the participant groups, the documented long lasting collaboration among most of them and their prominent position in the field is already focusing toward a success for this project.

**Economic benefits**

The musculo-skeletal market globally is anticipated to be greater than € 262 million, while soft tissue and wound repair, for which epithelial stem cells are used is anticipated to reach greater than € 912 million.

OptiStem and its European partners will demonstrate a proof of principle through the interaction and integration of the fundamental, pre-clinical and clinical partners which will boost the European biotechnology industry, especially the SME sector, and contribute to developing standards and regulations in the area. Industry, particularly SMEs, will be kept routinely informed of progress and therefore play a significant part: this is specifically pertinent in paving the way for the application of the diversity if cellular based products in development in the diverse degenerative diseases that can be targeted via cell therapy.

Degenerative diseases create a life-altering experience for the person with injury, for their partner, parents, siblings, and children. The subsequent diminishment of body functions associated with the diseases can cause depression and loss of self-esteem. It has been considered essential, based on European policy consistent with human rights principles, that people with disabilities should be treated with dignity, encouraged to have independence, be given equality of opportunity, encouraged to have an active participation, a full citizenship and a high quality of life. Given the diversity of degenerative diseases indicated above, pathological manifestation can occur at any age: either as a child, during an individual's most productive years, or as

an aged person. The trauma frequently results in morbidity, and as a result, patients typically require continuous physical and medical care depending on the disease, severity of manifestation, degree of disability, and location of injury.

**The prevalence of degenerative diseases is on the rise because aging population is increasing and this has created the need for novel regenerative therapeutics.** 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. While degenerative diseases are not the exclusive domain of the aged, they do impact this sector of society the highest with subsequent increased social and economic burdens on the health care systems on which they depend.

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.

## Impact on society

Degenerative diseases create a life-altering experience for the person with injury, for their partner, parents, siblings, and children. The impact on and subsequent dimishment of body functions associated with the diseases can cause depression and loss of self-esteem. It has been considered essential, based on European policy consistent with human rights principles that people with disabilities should be treated with dignity, encouraged to have independence, be given equality of opportunity, encouraged to have an active participation, a full citizenship and a high quality of life.

Given the diversity of degenerative diseases indicated above, pathological manifestation can occur at any age: either as a child, during an individual's most productive years, or as an aged person. In most cases, patients require continuous physical and medical care depending on the disease, severity of manifesation, degree of disability, and location of injury. The burden of caregiving most frequently falls on the partner. Care giving partners are often severely stressed, particularly due to health issues that arise after tissue degeneration initiates and suffer emotional stress that is comparable to or greater than those of the injured partner. Caregivers have a higher incidence of physical stress, emotional stress, burnout, fatigue, anger, and resentment.

Hope is considered an important coping strategy for both the person and family with degenerative diseases. Goal-directed hope based on realistic perceptions of life, focusing on progress, positive interpretation of events, are important in helping people and families cope with the disease. Hope is also focused towards the society at large, that new therapeutics are developed.

In addition to imposing direct medical costs on society, degenerative diseases also result in indirect costs, primarily related to reduced productivity due to disability with a further loss of self-esteem of the sufferer and diminished integration into society.

OptiStem aims to provide more than hope: we aim to provide validated cell therapies.

### 1.5 Project Contact Details and Logo

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