Speaker abstracts Spring School

The origin of CRISPR-Cas9 technology

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Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are direct repeats of DNA separated at regular intervals by unique, spacer sequences. CRISPR have been identified in many prokaryotes pertaining to the bacteria and archaea domains, where they play biological roles related to the regulation of gene expression or form part of a genetic barrier against transmissible genetic elements. These functions are usually carried out in collaboration with CRISPR-associated (Cas) proteins (i.e., CRISPR-Cas systems): the spacer sequences of CRISPR transcripts (crRNA) guide Cas proteins to complementary nucleic acids, resulting in target cleavage by a specific Cas endonuclease. The Cas proteins and other functional components of CRISPR-Cas systems vary depending on the particular system type. In consequence, these systems may differ in mechanistic aspects, requirements and target identity (RNA or DNA). Type II systems employs the Cas9 protein for DNA target recognition and double-strand cleavage (interference stage). In addition to the guide crRNA, a trans-activating CRISPR RNA (tracrRNA), partially complementary to the CRISPR sequence, is involved in this stage of type II CRISPR-Cas action. The three elements, Cas9, crRNA and tracrRNA, are the core of the CRISPR-Cas9 technology that enables a great variety of applications *in vivo* to achieve specific cell-immunization against infectious agents, trigger cell death, regulate gene expression, label multiple loci or edit genomic regions in both prokaryotic and eukaryotic hosts.

Adeno-associated vectors and friends – a brief overview on non-integrative viral vectors

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Non-integrating viral vectors are use as gene delivery tools in gene and cell therapy in case post-mitotic tissues such as liver, muscle, brain or the eye are the target. Furthermore, they are employed for transient modification of proliferating cells. Generally, non-integrating vectors lack an active integrase activity and thus episomes - formed following release of the vector genome from the protective capsid - serve as templates for transcription. Typically examples of non-integrative viral vectors in gene therapy are adenoviral (AdV) and adeno-associated viral (AAV) vectors. These vector systems will be introduced comparing their infection biology and reviewing their advantages and challenges.
As viruses have not evolved to serve us as tools in gene and cell therapy, viral vectors need to be optimized for improving efficiency and safety. Here, we will focus on the issue of tropism. AdV as well as AAV vectors use common receptors for cell binding and cellular uptake. As consequence, target as well as off-target cells are transduced when vectors are applied *in vivo*. Besides increasing the vector dose needed to achieve therapeutic efficacy, transgene expression in off-target cells may result in undesired adverse events.

Furthermore, locally as well as systemically applied AdV and AAV particles are transported via the blood to the liver, where they accumulate. Strategies to overcome this limitation and to re-direct viral vectors towards the cell type of choice will be presented and discussed. In addition, we will review the impact of cell responses on vector tropism.

Integrative viral vectors

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Gene therapy consists in the "use of genes as medicines". This involves the incorporation of a functional or therapeutic sequence of nucleic acids in certain cells of a patient to correct or alleviate a genetic defect. It can be used to replace a defective gene or to introduce a new gene whose expression cures a patient or improves its clinical condition or evolution. The range of conditions that could benefit from these new techniques is extensive, including the treatment of numerous hereditary diseases, some cancers and even certain viral infections such as HIV-1.

This therapy can be performed either in vivo or ex vivo, but in the case of hematopoietic gene therapy, the approaches that are more frequently used are ex vivo because cells obtained from the patient can be directly exposed to the vector and then re-infused after the correction. Gene therapy has shown clinical efficacy in a variety of monogenic diseases, such as X-linked severe combined immunodeficiency (SCID-X1), adenosine deaminase-severe combined immunodeficiency (ADA-SCID), Wiskott-Aldrich syndrome (WAS), chronic granulomatous disease (CGD), adrenoleukodystrophy, metachromatic leukodystrophy and hemoglobinopathies. Gene therapy can be performed using different strategies such as gene substitution, gene suppression or gene addition. When a healthy copy of the defective gene is introduced in the target cells we speak about gene addition, mainly performed by integrative viral vectors.

Viral vectors, by harnessing the machinery of natural virus infection, are the most effective and the most used in gene therapy protocols. There are different types of viral vectors and the choice of one or the other will depend on the goal that we want. In hereditary hematologic diseases, the γ-retroviral (γ-RV) and lentiviral vectors (LV) are the vectors most commonly used both in preclinical and clinical studies.

Hematopoietic stem cells are characterized by their self-renewal capacity, multipotency and long term reconstitution potential, for these reasons, the ideal target cell to perform gene therapy in hematopoietic diseases would be these primitive hematopoietic stem cells.

Insertional mutagenesis is an essential factor to be considered when performing gene therapy with integrative viral vectors. Both γ-RVs and LVs preferentially integrate in transcriptionally active areas, but γ-RVs have more preference to integrate into promoter and regulatory regions around the transcriptional start sites of genes. Therefore γ-RVs present more safety concerns than LVs. For this reason, the design of the viral vector is critical.

Consequently, in order to increase the safety of these vectors they have been modified with self-inactivating (SIN) Long Terminal Repeats (LTRs) and the use of weak or tissue specific promoters and insulators that protect surrounded genes from the vector influence.

Therefore, pre-clinical studies must include the description of LV integration sites of the designed viral vector. In the inherited bone marrow failure syndrome, Fanconi anemia, we have shown for the first time combined evidence of therapeutic efficacy together with hitherto uninvestigated genotoxicity risks of gene therapy in an inherited disease associated with DNA repair defects and genome instability.

Engineering the genome with the *Sleeping Beauty* transposon system

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A non-viral vector system that unites favorable characteristics of integrating viral vectors (i.e., stable chromosomal integration and long-lasting transgene expression) with those of non-viral delivery systems (i.e., lower immunogenicity, enhanced safety profile and reduced costs of GMP manufacture) is the *Sleeping Beauty* (SB) transposon. SB is a non-viral gene delivery system system that supports stable genomic integration of transgenes. However, in contrast to viral vectors, transposon vectors can be maintained and propagated as plasmid DNA, which makes them simple and inexpensive to manufacture, an important consideration for implementation and scale-up in clinical practice. Further advantages of SB as gene-transfer system compared to viral vectors include its lower immunogenicity, a greater capacity for genetic cargo and a superior biosafety profile. Because transposition proceeds through a cut-and-paste mechanism that only involves DNA, transposon vectors are not prone to incorporate mutations by reverse transcription (that are generated in retroviral stocks at reasonable frequencies), and can tolerate larger and more complex transgenes. Owing to permanent genomic insertion of transgene constructs, transposition-mediated gene delivery can lead to sustained and efficient transgene expression in preclinical animal models. SB yields efficient stable gene transfer following gene delivery into the germline of several mammalian model species as well as into pluripotent and multipotent stem cells that are relevant targets for regenerative medicine and gene- and cell-based therapies of complex genetic diseases.

We have recently established a robust preclinical protocol for transposition-mediated stable gene transfer into human T cells by SB vectors supplied as minicircles (MCs) and synthetic mRNA as a source of the transposase. In the context of CAR gene delivery, the SB protocol is i) as efficient as lentiviral gene transfer with respect to overall stable gene transfer, sustained transgene expression and biological activity of genetically engineered cells, ii) associated with a far safer genomic integration profile than any viral system currently in clinical use and iii) fully non-viral, thereby allowing GMP vector production at significantly reduced costs on a per patient basis. We adapted the MC/mRNA gene delivery protocol to $CD34^+$ hematopoietic cells, and we are in the process of setting up preclinical gene therapy in mouse models.

Gene editing

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Gene editing using nucleases has emerged as an incredible tool to potentially modify any sequence in the genome, allowing the generation of knock out models or the correction of genes mutated in different diseases.
Targeted nucleases have the ability to induce a site specific DNA cleavage in the genome that leads to a double strand break (DSB) in that specific locus. Once generated, this DSB can be repaired using the endogenous mechanism of DNA repair in the cell: Non homologous end joining (NHEJ) or Homologous Recombination (HR) when a donor template is present. In this context gene editing mediated by targeted nucleases increases HR efficiency at least two order of magnitude, opening the possibility to use this approach in the future for the treatment of different diseases. In contrast, the repair of the DSB by NHEJ generates integrations and deletions (indels) that can interrupt the targeted locus. This strategy has allowed the generation of many different knock out models.
During the last years several programmable nucleases have been described, being the most frequently used the Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR-associated 9 protein (Cas9). Although all of them finally lead to the generation of DSBs, unique characteristics regarding specificity, efficiency and the feasibility to deliver them allows the selection of the most appropriate tool for a range of genome editing applications.
In this talk we will review the most relevant characteristics of the different targeted nucleases, the criteria to select the ideal one for our gene editing approach, the tools designed to test the efficiency of our nuclease and the main applications described up to now: from the generation of knock out mouse models to gene editing in human cells already tested in patients.

Tissue engineering: a fast changing field

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The field of tissue engineering has evolved a great deal since the early days, when Langer and Vacanti published a landmark paper[1] that defined the essential concepts of this then novel area of research. In its initial phases, the *ex vivo* engineering of tissues was an artisanal craft that required relevant multidisciplinary expertise in both materials science and cell biology. As a result, the replicability and scalability of these techniques in order to attain the quality and logistical requirements for clinical application was a struggle, and few tissue engineered constructs made it to the late stage clinical phases. In the last few years however, new stem cell biology developments such as the use of induced pluripotent stem

cells or direct reprogramming for source cells, the accidental discovery of *in vitro* self-organizing 3D organoids and the steady progress in biofabrication technologies (such as 3D printing, hydrogels, and others) have created a number of novel opportunities to bring these technologies from the proof of concept study stage to become clinical realities. In this class, we will review the major historical developments in this field by using examples for a number of tissue engineered products, and will make informed predictions about the future research as showed by the endless possibilities arising from *in vivo* tissue reprogramming[2] and organ engineering[3].

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Cellular and animal models of neurodegenerative diseases: Focus on Parkinson´s disease

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There is an important number of neurodegenerative disorders, although a few major diseases constitute the most common causes of neurological disability and death. Many models of neurodegenerative diseases have been established in order to better understand the underlying mechanisms and to find more efficient treatments. It is not possible to analyze or even enumerate all these models in a short presentation. First, we will comment on common aspects of animal and cell models of neurodegenerative diseases. Then, we will focus on models of Parkinson´s disease (PD), one of the major neurodegenerative diseases. There is no ideal model of neurodegeneration as models cannot perfectly reflect all aspects of human disease. Furthermore, it is normally expected that models, unlike the slow progression of neurodegenerative disease, develops pathology quickly and reliably. However, experimental studies can use more than one model, each of them exploring different aspects of human disease, or may be tailored to address specific hypotheses. In the case of Parkinson´s disease, classic'' models are based on neurotoxins and are particularly adequate for studying the neurodegenerative processes and for testing symptomatic treatments. More recent models employ genetic manipulations that introduce mutations similar to those observed in familial cases of PD. However, genetic models also have shown a number of problems, and other models such as the delivery of alpha-synuclein with viral vectors targeted directly at dopaminergic neurons are an attractive alternative for modeling the disease process. Rodent and, particularly, non-human primate models are closer to human physiology. However, cellular models develop pathology more quickly, are less costly and do not require ethical approval. Between both types of models, invertebrate animals such as *Drosophila melanogaster* and *Caenorhabditis elegans*, as well as vertebrates such as zebrafish are also used.

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Human pluripotent stem cells: iPSCs and hESCs

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Human Pluripotent stem cells (hPSCs) include both human embryonic stem cells (hESCs) and induced Pluripotent stem cells (iPSCs). These cells can be grown undifferentiated for long periods of time (self-renewal) and they have the potential to be differentiated to all cell types present in human adult organism. hESCs were firstly isolated from the inner cell mass from human embryos in 1998. This year we celebrate the tenth anniversary of the human iPSC discovery. This breakthrough has been a huge revolution not only in the field of regenerative medicine but also it has broken well-established dogmas in cell and developmental biology.
We will review the initial experimental approaches to generate hPSCs and the most relevant new methodologies currently employed. These achievements have enabled the accessibility of the technique to many laboratories around the world and the establishment of international consortiums and stem cell banks specialized in the standardization, storage and distribution of hPSCs.
We will discuss some of the potential applications of hPSC: human developmental biology, drug screening, regenerative medicine and disease modeling. We will see some of the most relevant present and future research lines in regenerative medicine focusing our attention on iPSCs. Next, we will give a general overview of the use of hPSCs as disease models for the discovery of molecular mechanisms responsible for several common pathologies. Finally, we will show of our experience in the application of cellular reprogramming to the study of an extremely rare human disease in our lab: Bernard-Soulier Syndrome (BSS).

Gene and cell therapy strategies for immune and haematological disorders

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More than 50 years of scientific progress and of medical experience in bone marrow transplantation have enabled the development of hematopoietic gene therapy. The approach is based on the gene modification of the patient's own hematopoietic stem cells and has been first used to treat severe genetic disorders of the blood or immune system. Several clinical trials of hematopoietic gene therapy are currently ongoing and very recently, the first cell and gene therapy medicinal product has been registered as a drug to treat the immune deficit ADA-SCID. The last 15 years of hematopoietic gene therapy trials have clearly shown the importance of engrafting sufficient numbers of gene-corrected

hematopoietic stem cells in patients. We will ftherefore discuss the different types of gene transfer vector systems and transduction additives that can be used to gene modify hematopoeitic stem cells. We will review how gene-modified hematopoietic stem cell engraftment can be measured in patients. Finally, we will present the new trends in the field and challenges that lie ahead. Overall, this presentation will provide an overview of clinical applications and scientific progress in the field of gene and cell therapy for immune and haematological disease

Gene therapy in congenic bone marrow failure syndromes

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Inherited bone marrow failure syndromes (BMFS) are a group of disorders characterized by peripheral cytopenia and hypoplastic bone marrow. Some of these syndromes, such as Fanconi anemia (FA) are associated with DNA repair defects, while others are related with defects in the ribosomal apparatus (Diamond Blackfan Anemia) or in the elongation of telomeres (Dyskeratosis Congenita). In these congenital disorders, hematopoietic stem cell transplantation (HSCT) is the preferential therapeutic option. However, new tools for improving the collection of HSCs and advances in vector development to facilitate the safe and efficient transfer of therapeutic genes into these cells have opened new alternatives for the treatment of BMFS based on gene therapy. Based on these advances, in our laboratory we have developed a lentiviral-mediated gene therapy approach for FA patients.
In contrast to other disorders already treated by gene therapy, marked proliferation and differentiation defects have been observed in FA hematopoietic stem cells (HSC), complicating the harvesting of a high number of CD34+ cells to be used in gene therapy protocols. On the other hand, the proliferation advantage of gene-corrected FA HSCs may facilitate the hematopoietic reconstitution of the patient by a low number of transduced HSCs. In our clinical studies, the collection of FA HSCs was based on the mobilization of these cells using G-CSF and plerixafor. The short transduction of small aliquots of mPB $CD34^+$ samples from these patients with a GMP-produced lentiviral vector harboring the *FANCA* therapeutic gene corrected the phenotype of 20-40% of the progenitor cells. To assess the repopulating ability of transduced FA-A $CD34^+$ cells, samples were transplanted into immunodeficient NSG mice. Remarkably, most of the transplanted samples engrafted the NSG mice, and an *in vivo* proliferation advantage of gene-corrected $CD34^+$ FA-A cells was observed in transplanted mice. Based on these preclinical studies, a gene therapy trial of FA-A patients is currently open in Spain.

Gene therapy of CNS disorders

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[insert abstract]

Cell therapy of Parkinson's disease

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Intrastriatal carotid body (CB) grafts produce trophic protection and restoration of the dopaminergic nigrostriatal pathway in rodent and primate models of Parkinson's disease (PD), which is mediated by high levels of glial cell line-derived neurotrophic factor (GDNF) produced by CB implants. Phase I/II open trials showed that CB autotrasplantation improve motor symptoms in PD patients. However, the efficiency of CB cell therapy observed in clinical trials is lower than in experimental models, being patient age one of the factors influencing the clinical outcome. To explore limiting factors that affect the efficacy of human CB transplants, we have studied how aging and chronic hypoxia present in intracerebral grafts can modify CB GDNF expression. Chronic hypoxia induced an up-regulation of CB GDNF expression in young mice, while the same treatment in aged mice decreased CB GDNF expression. This age-related differential regulation of GDNF is also present in the intrastriatal graft and affects the efficacy of mice antiparkinsonian CB cell therapy. Moreover, human CB xenografts from young (≤40 years) donors induced an important protection of the nigrostriatal dopaminergic neurons of parkinsonian mice, while CB implants from aged (≥60 years) donors failed to produce a significant effect. Finally, we performed a study of the methylation status of human and murine GDNF promoter from young and aged CBs, identifying hypoxia-related regions that could explain the differential regulation of GDNF expression. These findings provide a molecular explanation of the outcome of previous clinical trials and offer insights for the design of new antiparkinsonian cell therapy treatments.

Gene therapy of retinal degeneration

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Over 120 genes that have been identified that lead to various forms of inherited retinal dystrophy. In order to decide which gene defects are the most suitable targets for the first gene therapy approaches, various parameters must be considered. These include disease severity and prevalence, the availability and suitability of disease models and the efficacy of the treatment in proof-of-concept studies. Severity of disease in particular is an important consideration, as on the one hand a rapid loss of photoreceptor cells severely limits the window of opportunity for treatment, but on the other hand allows an efficient read-out of treatment efficacy in a clinical trial. Over the past decade, we have developed gene therapy protocols in over a dozen different animal models of retinal dystrophy and we have conducted a clinical trial of *RPE65* gene therapy for Leber congenital amaurosis (LCA) type 2. Here I will discuss our pipeline of therapies. Our strategy is to begin our initial studies in rare, but amenable disorders, such as RPE65-deficiency and AIPL1-deficiency and move towards more common, but more complicated disorders for which to develop treatments, for example development of gene therapy for X-linked RP caused by defects in *RPGR*.

Cardiovascular cell therapy

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Acute Myocardial Infarction (AMI) stills an unsolved issue nowadays and the promising results obtained over the last years in the treatment of AMI with mesenchymal stem cells (MSC) make them a good alternative therapy. Previous reports demonstrated that hypoxia improves MSC self-renewal and therapeutic effect. Hypoxia-inducible factor-1α (HIF-1α) is a master regulator of the adaptive response to hypoxia and our group showed that overexpression of HIF-1α improves the therapeutic potential of the cell by reducing the scar tissue, infarct size and enhancing or stimulating the angiogenic process. MSC cells overexpressing HIF-1α (MSC-HIF) could be a good strategy to improve therapeutic potential of these cells. Since the paracrine hypothesis is the most extended mechanism to explain the therapeutic effect of the mesenchymal stem cells, we study the implication of exosomes from MSC and MSC-HIF in the angiogenic process focusing in the Notch pathway. We pointed out that exosomes from MSC and MSC-HIF are loaded with a broad repertoire of miRNA in a HIF-1α expression dependent manner. In addition, two recently published papers by our group have demonstrated the role of exosomes in cellular communication and in cell survival. This conference will be focused on the recent advances in MSC genetic modifications to improve their survival or immunomodulatory properties under various stress conditions, both at the level of cellular contact as transfer level exosomes.

New cell therapies in inflammatory and autoimmune disorders

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The immune system is faced with the daunting job of defending the organism against invading pathogens, while at the same time preserving body's integrity and maintaining tolerance to its own tissues. Loss of self-tolerance compromises immune homeostasis, resulting in the onset of autoimmune disorders. Identification of factors and cells that control immune tolerance and inflammation is a key goal for immunologists. Evidences from the last decade indicate that regulatory T cells, tolerogenic dendritic cells and mesenchymal stromal cells exerts potent anti-inflammatory actions and participates in the maintenance of immune tolerance at multiple levels, especially in immunological disorders. I will describe how these cells, especially mesenchymal stromal cells have emerged as attractive candidates to treat inflammatory and autoimmune disorders in humans and will discuss challenges and new therapeutic strategies that we should address in the future to improve their use in the clinic.

Skin rare diseases: Thinking outside the box

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The skin is a highly specialized organ that serves as a barrier between the organism and its environment, protecting it from harmful external factors and maintaining internal systems intact. The skin consists of three compartments: the epidermis, the dermis and the hypodermis. The epidermis is mainly composed of keratinocytes, cells of epithelial origin. The epidermis is a self-renewed tissue that occurs thanks to the proliferation of a subpopulation of keratinocytes: the epidermal stem cells. The keratinocytes in the epidermis are organized into four layers: the basal, granular, spinous and cornified layer. These layers represent different stages of differentiation. Other specialized cell types are, the melanocytes, Langerhans cells and Merkel cells. The dermis is a connective tissue, composed mainly of collagen and elastic fibers in which the predominant cell type is the fibroblast. Blood vessels, nerve endings, glands and hair follicles, are also involved in this connective tissue.
The genodermatosis represent a significant part of dermatological pathology and constitute about 10% of the set of all rare diseases. Currently the genetic basis of more thab 430 genodermatosis, mostly monogenic, have been elucidated allowing accurate classification, molecular diagnostics and understanding of some of the pathogenic mechanism. However, the vast majority of patients living with rare diseases are still without treatment even though their conditions are potentially lethal or chronically disabling. The genodermatosis that will be the subject of this training course belong to the families in which more knowledge has accumulated in recent years. These are: a) disorders of the epithelial adhesion, b) disorders of the keratinization, c) DNA repair diseases.

ADVANCED THERAPIES FOR GENODERMATOSES
At present there is no effective, only symptomatic, treatments for genodermatoses. However, some forms of epidermolysis bullosa are currently the paradigm model in pursuit of new effective treatments. In fact, gene therapy protocol for dystrophic epidermolysis bullosa was recently translated to clinical trials. Also, there has been significant progress in preclinical gene therapy strategies for other genodermatoses. Thus, ex vivo gene therapy has been used successfully in humanized models Syndrome Netherton and Xeroderma Pigmentosum. In the case of congenital Pachyonychia innovative strategies such as mRNA silencing have been successfully tested. Finally, other strategies such as enzyme replacement, in the case of lamellar ichthyosis, could be translated to the clinical practice in the near future. Advanced therapies (cell-, gene therapy and tissue engineering) will soon be translated to clinical trials and, according to their results, probably to clinical practice. In addition, the new tools of masive sequencing and other high throughput "*omics*" approaches are giving us with a comprehensive understanding of the pathogenic mechanisms and perhaps new therapeutic targets. Clearly this is a time in which basic and clinical researchers must interact.

Genome editing for the treatment of AIDS

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Over the last few years, new and powerful molecular tools, ZFNs, TALENs and CRISPR/Cas9, capable of editing the genome of livings cells have emerged. Their ability to engineer site-specific changes at chosen locations in the genome, in combination with recent advances in

stem cell biology, regenerative medicine and systems biology, has the potential to change the medical practice, as we know it. So far, the efforts to develop new clinical applications have centered mostly on genetic diseases, but other uses are starting to emerge, for example, in the treatment of AIDS.

The approach is inspired on a clinical case reported in Berlin, in which an AIDS patient suffering also from acute myeloid leukemia received a bone marrow transplant from a healthy matched donor, which was also homozygous for the CCR5-D32 mutation. CCR5 is the main co-receptor used by the HIV virus to enter into human cells. D32, a deletion of 32 bp. in the coding region of the gene, results in a defective receptor, not accessible to the HIV but without any noticeable deleterious effect on human health. As a result of the bone marrow transplant, the so-called ¨Berlin Patient¨ fully recovered from both leukemia and AIDS. 8 years later the patient has no detectable HIV in his organism.

The Berlin case oriented the efforts of the biomedical community in the direction of using genome editing tools to modify autologous peripheral blood T cells or HSCs in a way that would mimic the naturally occurring CCR5 $^{D32/D32}$ genotype. Recent developments in the field will be discussed.

Safety considerations of gene therapy strategies

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Gene and immune therapy represent a promising innovative treatment strategy for the treatment of inherited and acquired diseases. The clinical application spectra range from rare or infectious diseases to autoimmune disease and cancer. Notably, gene transfer changes the biological properties of its target cells, with the risk that also unwanted side effects occur in the affected cells, tissues and orgnaisms. As with any other clinical trials, robust pharmacokinetics and pharmacodynamics studies have to accompany each trial. In GT, these studies comprise measurement of transgene expression, vector copy number and clonality of the gene-corrected cell pool. To identify vector insertion sites, a variety of PCR-based, and, more recently, direct sequencing based methodologies are available. Gene editing technologies by CRISPR Cas9 or other systems pose additional challenges because their clinical use requires the detection of on and off target activities that has been followed by homology repair or non homologous end joining, and is usually devoid of any vector sequences. The technological aspects of tracking DNA modifications in target cells, tissues and organisms as well as its functional consequences such as immune reconstitution will be discussed

Gene therapy for liver inherited diseases

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The liver plays a critical role in the metabolism of lipids, carbohydrates, and proteins. Genetic deficiencies that lead to disease in each of these critical liver functions have been

identified; the majority of them belonging to the group of rare diseases. For this reason the liver is a major target for gene therapy. The work of our group is focus on the development on gene therapy strategies for liver inherited metabolic diseases using AAV vectors.
Wilson disease (WD) and Primary Hyperoxaluria type I (PH1) are two of the disease in which we are currently working employing different therapeutic approaches. WD is due to mutation in the copper transporter protein ATP7B that leads to an accumulation of copper in the liver resulting in a wide variety of hepatic and neurological problems. The only curative therapy is liver transplantation. The gene therapy approach for WD is based on the expression of ATP7B gene in the liver, which results in elimination of copper from the liver and the improvement of a number of pathological aspects of the disease. PH1 is due to mutations in the AGXT gene that results in the accumulation of oxalate stones in the kidney and kidney failure. In this case we use a substrate reduction therapy (SRT) based on the elimination of the expression of protein acting upstream AGXT, glycolate oxidase (GO). For that purpose AAV delivery of CrispR-Cas9 targeting GO were used with promising therapeutic results in PH1 mice.
In conclusion, two completely different gene therapy strategies using AAV-vector for liver inherited disease will be presented and discussed.

Lysosome storage disorders

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Lysosomal Storage Disorders (LSDs) are severe childhood conditions caused by inherited defects of lysosomal function and often characterized by a neurodegenerative course. There is no cure for the central nervous system (CNS) pathology in these diseases. The main goal of our laboratory is to develop AAV-mediated gene transfer approaches to treat the CNS in LSDs with minimal invasiveness and high CNS targeting efficiency. Intrathecal (IT) injection into the cerebrospinal fluid is an attractive administration route to deliver therapeutic genes to the CNS with potentially minimally invasiveness. We recently characterized the CNS transduction pattern of several AAV serotypes upon IT administration in a large animal model (*wt* pigs). Based on this work, we are developing and testing IT AAV-mediated gene transfer therapies for different LSDs, including the Mucopolysaccharidosis type IIIA (MPS-IIIA), one of the most severe forms of neurodegenerative LSDs. These strategies are based on specific AAV serotypes selected for their tropism to specific CNS regions/cells and on the use of modified versions of lysosomal enzymes with enhanced therapeutic potential. Intravascular administration is an alternative and non-invasive route for delivering therapeutic genes. However, it is generally inefficacious for the treatment of the CNS. We are also testing the therapeutic potential of a gene transfer strategy based on the systemic delivery of AAV serotype 8 to target the liver and convert it into a factory organ for lysosomal enzymes engineered to cross the blood-brain barrier and target the brain. This approach has been successfully tested in MPS-IIIA mice and is now being tested in other animal models of MPSs.

Towards a gene therapy for neurological and somatic mucopolysaccharidosis

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Mucopolysaccharidosis Type II (MPSII), Hunter Syndrome, and Type III (MPSIII), Sanfilippo Syndrome, comprises 5 autosomic recessive disorders caused by mutations in genes that encode for enzymes involved in the stepwise degradation of glycosaminoglycans (GAGs). Accumulation of GAGs in lysosomes leads to lysosomal pathology, and affected patients undergo severe neurodegeneration with mild somatic disease, and usually die during adolescence. There is no cure and MPS diseases constitute an unmet medical need. This presentation will discuss the potentiality of intracerebrospinal fluid AAV vector-mediated gene therapy to counteract neurologic and somatic MPS. Using this approach to treat for MPSII and MPSIII, expression of the different therapeutic genes was detected in widespread brain regions and in the liver, leading to increased enzyme activity in CNS and serum and simultaneous correction of both central and somatic disease. The results of this study provide strong evidence supporting the clinical translation of the approach.

Engineered T cells for cancer treatment

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Cancer immunotherapy, and in particular T cell therapy, have recently produced impressive clinical results. Expanded tumor specific T cells, genetically engineered T cells, such as CAR T cells, represent a new class of anti-cancer drugs that are rapidly entering the clinical arena. Gene transfer tools have been critical for reaching this phase of development. Technological advancements today allow not only to add a gene and function to a target cell, but - thanks to the genome editing technology - to completely and permanently substitute one or more biological functions in a cell of choice. T lymphocytes have the unique ability to recognize specific targets, on microbes, infected cells, or tumors, through their T cell receptors (TCR). Recognition induces T cell activation and efficient killing of targets, and results in long-term protection against diseases. To be effective against cancer, T cells needs to be 1. Specific for cancer antigens, 2. Able to expand and persist long enough to mediate a long lasting clinical response, 3. Able to counteract the immunosuppressive tumor microenvironment. TCR genetic engineering represents a suitable approach to generate large numbers of tumor specific T cells. The core of this approach is the transfer in patients' T cells of genes encoding for rare tumor-specific TCR. However, the simple transfer of tumor specific TCR genes into T cells is affected by some limitations: genetically modified T cells shall express four different TCR chains, that might mispair, leading to unpredictable toxicity and to an overall dilution of the tumor specific TCR on lymphocyte surface, thus limiting the efficacy of therapeutic cellular product. To overcome these issues, we developed a TCR gene editing procedure, based on the knockout of the endogenous TCR genes by transient exposure to alfa and/or beta chain specific Zinc Finger Nucleases (ZFNs), followed by the introduction of tumor-specific TCR genes by lentiviral vectors. The TCR gene editing technology, proved safer and more effective than conventional TCR gene transfer in vitro and in animal studies, in models of acute myeloid leukemia and multiple myeloma. We developed protocols to generate high numbers of TCR edited memory stem T cells and central memory T cells, lymphocyte subsets endowed with long term persistence capacity. The immunosuppressive

environment that such innovative cellular products will encounter once infused to cancer patients represents an additional challenge that will be discussed during the presentation.

Virotherapy, basic concepts

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Virotherapy is the use of viruses to treat cancer. Different types of natural, that is "wild type", or genetically modified viruses with tumor-selective or "oncolytic" tropism are used intratumorally or intravenously in cancer patients. Among them, an herpes virus has recently been approved for early stage melanoma. Tumor debulking or lysis combined with immune activation are the main mechanisms of action. However, much work needs to be done to improve their efficacy. Main hurdles have been identified in systemic tumor-targeting, intratumoral spread across stromal barriers, and the activation of antitumor immunity besides antiviral immunity. Adenoviruses have a unique life cycle and epithelial cell infectivity particularly suitable for oncolysis of solid tumors. This lecture will present an overview of the field and strategies to overcome these limitations, with a special focus on oncolytic adenovires.

Virotherapy, clinical applications

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In recent years we have seen an increased interest in the application of oncolytic virotherapy for the treatment of human tumors These treatments have already reached the clinical arena, and the first results of clinical trials have been published. Oncolytic viruses may become a new option in the antitumor armamentarium, although there is still a long way ahead for clarifying indications, dosage, treatment guidelines and other aspects that will determine the optimization of their use in humans. The therapeutic potential of oncolytic viruses has been demonstrated over and over in different in vitro models, however, the administration in experimental animals first, but especially in patients, has put in a more realistic light the therapeutic capacity of oncolytic virotherapy. There are some limitations in the clinical use of oncolytic viruses that decrease their effectiveness very significantly. On the one hand the antiviral immune response that the body develops causes the elimination of the therapeutic effect following a first administration, or even with the first dose in pre-immunized patients. Moreover, the poor natural ability of oncolytic viruses to infect micrometastatic lesions significantly minimizes the effective dose of virus; increasing the dosage does not ensure a greater effect due to increased toxicity, and to the role of the immune system commented before. This problem does not occur in localized tumors, but it is significant in metastatic tumor disease, which is the leading cause of cancer death.

Translating gene therapy tools to clinical applications in inherited immunodeficiencies

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At the start of the 1990s, the first clinical trials of gene therapy were attempted for an inherited severe combined immunodeficiency (SCID) caused by deficiency of the intracellular enzyme adenosine deaminase. In the absence of definitive treatment, SCID of any molecular type is usually fatal within the first year of life, although patients with ADA deficiency can be supported by administration of exogenous enzyme replacement. Even so, this is often only partially effective, and is extremely expensive. The rationale for the development of gene therapy for SCID therefore derives from the severity of the illness, the inadequacy of conventional therapy, and the considerable morbidity and mortality associated with stem-cell transplantation, particularly from a mismatched donor. Efficacy in these early studies was limited, but a decade further on, gene transfer technology and cell handling protocols had been refined sufficiently to produce real clinical benefit. Four recent studies have demonstrated highly effective gene therapy for the X-linked form of SCID (SCID-X1) and ADA deficiency, using retroviruses to deliver the therapeutic genes into haematopoietic stem cells *ex vivo* (two examples from our own centre referenced below). Similar 'proof of principle' studies have been conducted in patients with Chronic Granulomatopus Disease and Wiskott-Aldrich Syndrome. Bearing in mind the outcome and adverse effects of conventional therapy, these are remarkable results and the first clear indication that gene therapy can offer a cure for some human diseases. In a few patients the treatment has failed, indicating that there is more to learn about the effective dose of corrected cells and the potential for host factors to influence immune cell development.

Many different types of vector have been tested in laboratory experiments to deliver therapeutic genes, and their effectiveness is largely determined by the host and tissue type. For stable gene transfer to dividing cells, such as haematopoietic cells, the new genetic material has to be retained through cell division and passed on to daughter cells. Although retroviruses are highly effective for this, their dependence on chromosomal integration brings with it the risk of inadvertent gene activation or inactivation. Having initially achieved successful immunological reconstitution, several patients with SCID-X1 (out of a total of 19 treated worldwide) developed T cell lymphoproliferative disease up to 6 years after the gene therapy procedure. In four of these patients, the enhancer sequences in the retroviral vector, which are responsible for effective transgene expression, had activated the *LMO-2* proto-oncogene. Reports of similar adverse events in other applications, once again in the context of early generation gammaretroviral vectors, paved the way to the development of refined vector technologies including use of self-inactivating vectors in which the powerful viral enhancer sequences are deleted.

Clinical trials using self-inactivating gammaretroviral and lentiviral vectors have now been reported, and one again demonstrate the huge potential of gene therapy for haematopoietic disorders including SCID-X1, Wiskott-Aldrich Syndrome, and ADA-SCID. At the same time, the safety profile appears to have been significantly enhanced, and efforts are being made to develop protocols for medicinal licensing. New technologies including homologous recombination or gene repair to accurately correct genetic mutations may eventually supersede gene addition once limitations of efficiency and toxicity have been addressed.

Gene modified HSCs as therapeutic tool

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Current results from ongoing trials of Hematopoietic Stem Cell (HSC) gene therapy performed with lentiviral vectors for the treatment of some primary immunodeficiencies (like Wiskott Aldrich syndrome, WAS) and storage diseases (like metachromatic leukodystrophy, MLD) show stable and extensive genetic engineering of hematopoiesis with polyclonal reconstitution by gene modified HSC with substantial therapeutic benefit. These findings provide a new therapeutic perspective for patients affected by these diseases and, conceivably, several other ones. Autologous HSC gene therapy may eventually become a first treatment option for those patients candidate to allogenic HSC transplant (HSCT) who lack a fully matched normal donor. Autologous HSC gene therapy would not only be available to virtually any patient but may also substantially reduce the morbidity of the treatment as compared to allogenic HSCT, because there is no risk of graft-vs-host disease and therapeutic benefit may often be achieved by partial chimerism with transduced HSC, thus relieving the need for fully myeloablative and immunosuppressive preparatory conditioning. In some diseases, such as MLD, genetic engineering of HSC may even surpass the benefit of conventional HSCT, because it may engage novel therapeutic mechanisms, such as increased dosage and biodistribution of the replaced gene product over what can be achieved by transplanting normal HSC. These clinical results also prove the feasibility to manipulate HSC ex vivo without hampering their long-term repopulation potential and open the way to design improved gene therapy strategies. To further enhance the safety and efficacy of gene transfer, we have devised novel strategies to target gene expression to selected lineages by transcriptional and post-transcriptional, micro-RNA mediated regulation and to precisely edit the genome by artificial endonucleases. These strategies are now being translated into new therapeutic strategies for treating more common diseases, such as cancer. More precise genetic engineering can be achieved by correcting disease-causing mutations in situ, thus restoring both the function of the gene and its physiological expression control. Targeted gene editing, however, is constrained in HSC by quiescence and low expression of the DNA repair machinery. We could overcome these barriers and provide evidence of correction of SCID-X1 causing mutations in the IL2RG gene. We have validated this approach in an ad hoc humanized SCID-X1 mouse model to support the scientific rationale and safety of the proposed treatment, and identify the conditioning regimen and degree of chimerism with edited cells required to correct the disease. As the first ex vivo gene therapies have now progressed to the market, constant engagement of the drug companies and regulatory agencies becomes essential to define appropriate quality standards for manufacturing and release and to build suitable pipelines for supplying these personalized and expensive therapies. Concomitantly, the scientific community has engaged in a debate open to all society stakeholders to address the ethical implications raised by the prospective application of genome editing to a growing range of uses, including the possibility to modify the human germline.