

Stem cell therapy: hype or hope?



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III Forum

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Presentation

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The Forum “Stem cell therapy: hype or hope?” was a successful, highly inspiring meeting. The meeting brought together stem cell leaders from different countries. The purpose was foster collaboration between stem cell researchers, emphasize the light and shadow aspects of stem cell based therapy, update the knowledge on regenerative medicine and identify the yet unsolved open questions in the field, in order to stimulate creative solutions.

Over 100 participants, belonging to different research centers, attended the meeting to listen and discuss about the latest scientific results in the field of research and clinical use of stem cells.

The meeting was opened by Paolo Bianco from Rome, one of the most recognized leader in the field of mesenchymal stem cells, who emphasized the dark side of stem cells research and the risks of uncontrolled translation of basic science to clinic, with enormous troubles for the patients. Austin Smith gave a brilliant lecture on embryonic stem cells and the naïve pluripotency of stem cells cell renewal. He described how, under the right conditions, stem cells can give rise to all the different cell types that make up the organism, and provided evidence of the transcriptional circuits controlling the stemness as well as the differentiative potential of embryonic stem cells. Neuronal stem-cell based therapeutic approaches for the cure of Parkinson’s diseases were discussed by Vania Broccoli and Andres Björklund, who respectively focalized on the potential of reprogrammed dopaminergic neurons, and the most advanced clinical trials for cell replacement therapy of Parkinson’s disease. A fascinating link between stem cells and the pluripotency was described by Pierre Gönczy, who talked about asymmetric cell division of stem cells and the mechanisms of spindle positioning during mitosis. Michele De Luca revisited the world of epidermal stem cells. He described the fascinating and challenging actions of clinical applications of transplan-

tation of sheets of cultured keratinocytes into patients with extensive burns as well as on patients with genetic skin diseases.

The talks of Shahrghim Tajbakhsh, Thomas A. Rando and Giulio Cossu gave a wide overview on the role of muscle stem cells in myogenesis and muscle regeneration. Professor Tajbakhsh and Rando illustrated the process of satellite cells lineage determination, highlighting the role of stem cells microenvironment or “niche” as the dynamic environment supporting the stem fate maintenance. Professor Cossu, one of the leader in the regenerative medicine approaches in the treatment of patients with Duchenne muscular dystrophy (DMD), the most severe and dramatic type of muscular dystrophy, described the exciting albeit still partially satisfying, results of clinical trials, based on mesoangioblast stem cell transplantation, on young patients with DMD. Detailed emphasis on the role of inflammation and immune response in the regeneration processes was provided by Prof. Nadia Rosenthal, who described the impact of inflammatory cells in the regenerative processes in various animal models, ranging from amphibians to the human, revealing that stem cells activity is deeply influenced by supporting cells of the immune system. Any perturbation of these interactions is predicted to alter stem cells function. At the end, Elisabetta Montesoro from the Italian Medicines Agency (AIFA) illustrated the Italian and European regulatory view on stem cells based medicine. Overall, the meeting provided a platform for exchanging and discussing over many practical aspects of this fascinating world of stem cells. The lessons learned from extensive studies in the field of gene therapy will hopefully be applied by the leaders of this new field so as to hasten and facilitate the clinical translation of safe stem cell technology. The very formidable challenge is to develop safe and effective transplantation protocols for stem cells, a challenge which may take decades to overcome.

The “take-home message” we received is that there is potential for stem cells to be incorporated in clinical interventions, either as a cellular therapy to modify the natural history of disease or as a component of engineered tissue constructs that can replace diseased or damaged tissues. However, the public and private excitement surrounding the development of stem cells for regenerative medicine can lead to a potentially troublesome bias.

Introduction

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One of the most exciting aspirations of modern medical science is the possibility of regenerating every single damaged part of the human body. The idea of tissue regeneration first appears in the 5th-century B.C. Prometheus narrative written by Aeschylus. Prometheus stole fire from Olympus and gave it to human kind, thus infringing a hard and fast rule of the ancient gods. In revenge, Zeus chained Prometheus to the side of Mount Caucasus, where a vulture preyed daily on his liver, which was renewed during the night. The tribulation would be resumed the next morning. Fortunately, Prometheus' skin and liver were ready for the daily regeneration, since both these organs possess the highest regenerative potential in the entire human body.

As a dramatist, Aeschylus was likely to ignore molecular and cellular mechanisms behind wound healing or liver regeneration; that choice, however, was not accidental, because if the vulture had chosen a different organ on which to dine, e.g. brain or heart, poor Prometheus might not have survived his ordeal.

Nowadays we all know tissues have the ability to regenerate themselves and possess reserves of special cells, which are prepared to renew or repair them in case of damage. These are the *stem cells*.

The “Stem cell therapy: hype or hope?” Forum was aimed to define the current state of the art in the biology of stem cells, while highlighting certainties, hopes and illusions hidden behind potential stem cell therapies.

Several questions remain unanswered: What can be cured with stem cells? If different tissues possess a stem cell compartment why several tissues fail to regenerate under pathological conditions?

The answer to the first question is, not much! So far, there have been just a few examples of clinically proven stem cell therapies, including those treating skin and corneal burns, cartilage and bone graft and those treating some types of blood cancers.

Moreover, the hostile microenvironment characterizing various affected tissues might have an influence on survival of stem cells and limit their efficacy.

Today, we know one thing for certain: stem cells “cure” tissues in which they reside. Skin stem cells cure skin burns; corneal limbus stem cells cure corneal damage, e.g. cauterization; hematopoietic stem cells cure blood cell diseases.

The idea to cure neurodegenerative diseases, such as ALS, multiple sclerosis and spinal cord injuries, as well as to form neurons by means of mesenchymal stem cells (deputed to form bone, cartilage and adipose tissue) is sheer fantasy and a dangerous illusion.

An effective treatment is focused on the full understanding of benefits, risks and limits of stem cells. Therefore, it is puzzling to witness this muddled situation, in which medical practice, legal disputes and political powerlessness are mixed together.

Providing patients with safe, effective, non-commercial therapies should be researchers and clinicians’ main concern.

SESSION 1

Out of the hype or hope dilemma: stem cells and the commercialization of science

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The “hype or hope” question has become a commonplace in the kind of literature that surrounds true science in the stem cell field. The phrase alludes to circumstances that are most visible in the stem cell field, but also apply to some extent of other areas of medicine. At this time, for every individual on the planet who receives a genuine stem cell based, life-saving treatment, at least three others in the US only traverse the southern border to receive a treatment that is said to be stem cell based, but is in fact neither stem cell based nor a treatment. Stem cells have emerged as a commercial entity, not just an object of the natural world that can be possibly be used in medicine in the future. This commercial entity is actually impinging on the natural object, changing its definition, nature, properties at least as conveyed to the public opinion. A number of different kinds of stem cells are subjected to this commercial hijacking, including cord blood derived hematopoietic stem cells, so-called mesenchymal stem cells, and many others. In the western world, strict regulation forbids the commerce of medicinal products that have not been shown to be safe and efficacious through proper, established parameters of evidence based medicine. This has resulted in the flourishing, in most other countries, of a constellation of commercial enterprises that offer putative stem cell treatments to patients worldwide, with extensive advertisement and direct recruitment via the web. This phenomenon, commonly referred to as “stem cell tourism”, is often linked to the boosting of so called emerging economies, and, most importantly, is not entirely foreign to the operation of biotech companies that operate in the West. For many of these, stem cell clinics disseminated in other regions of the globe solely represent an off-shore station for a kind of marketing that is not allowed in the US or EU.

The marketing of unproven stem cell treatments outside of regulated regions of the globe is linked on the other hand to potent pressure towards abrogation of re-

gulation [1]. Arguments used in a profusion of communication directed to the lay public as well as to politicians read that patients are to be made free to choose, that regulators (FDA, EMA) are bureaucratic monsters that stand in the way to life-saving treatments, and that, of course, the offered stem cell treatments represent the cutting edge of medical innovation. A constellation of ultra-conservative (libertarian) think tanks in the US provide the main source of arguments, which are then potently disseminated through old and novel media. Pressure is exerted directly on Governments in order to promote deregulation of stem cell commerce through legislative action. This has happened for example in Mexico, and many other countries, including Europe and North America. A common scheme is followed: political lobbying, creation of infrastructure, advertisement, and public opinion campaigning.

Some kinds of stem cells have been most potently hijacked for commercial purposes. As we speak, over 380 clinical trials are recorded in www.clinicaltrials.gov that purportedly test the safety and efficacy of so-called mesenchymal stem cells for a variety of illnesses such as autism, heart attacks, strokes, renal failure, liver fibrosis, arthritis, neurodegenerative diseases, sepsis, even urinary incontinence. None of these trials have ever provided evidence for anything, and none of them lists results in the www.clinicaltrials.gov website. A recent meta-analysis of randomized controlled trials employing MSCs for ischemic heart diseases have revealed major flaws in all studies that had reported benefits, and zero benefits in all studies that were apparently flawless. The very existence of trials is used as an argument testifying to the safety at least of the treatments proposed. In fact, there is no evidence that intravenous infusions of MSCs (what is done in virtually all trials and offered in virtually all MSC marketing activities) is either safe or effective in any disease. Evidence in fact suggests that intravenously infused MSCs die within hours, and do not engraft. Similar considerations apply to the use of alternative kinds of stem cells as applied to disease that cannot be treated by stem cells as of today. Many of the so-called trials in which stem cells are used do not truly qualify as such. Patients must pay to access the trial in the first place, and the trial is as a rule uncontrolled and interventional. In other words, a mere cover-up for marketing unproven therapies.

Worldwide, a campaign is being conducted that aims at deregulating the market of stem cell therapies [2, 3]. In brief, the proposal reads that such therapies should access the market prior to gaining evidence of efficacy. It would then be legal to market unproven, ineffective therapies, after a phase I trial, presented as sufficient to establish safety. In fact, a phase I trial per se does not prove safety, and efficacy can never be proven in a post-marketing setting, in which proper controls, proper recruitment of homogeneous patient cohorts, and proper reporting of data is not possible. What the Western world faces is the most powerful attack to the regulation of the drug markets even known in history. This attack would have the effect of abrogating not just regulation, but the very notion of Western medicine, which is based on science on the one hand, and on a non-market type relationship between patient and doctor on

the other. In the “free-to-choose” scheme (such is the name given to the deregulated layout of drug markets) the patient is a mere consumer of therapies, freely offered on the market. The patient is told to be free to choose, whereas he/she typically has no choice (suffers from a disease with no alternative). The offer of a “therapy” justifies the fee; the “innovative” (experimental) nature of the therapy justifies the failure; and the abominable exploitation of hopeless patients is then called “compassionate” treatment, in a sinister distortion of science, medicine and ethics together.

The scientific community is realizing the need to mark sharply the watershed between real expectation and commercial illusion. This however implies a better awareness of more subtle but equally misleading views that have pervaded the scientific community itself. Such as, the notion that science is only as good as it provides products to be put on the market, or the idea that fundamental knowledge is costly and useless and only translational activities are justified. A profound reversal of this view, emanating from a precise as much as disruptive commercial and political agenda, is urgent.

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The nature of embryonic stem cells

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Embryonic stem (ES) cells are capable of unlimited proliferation in vitro and retain the unique capability to give rise to all somatic lineages and to germ cells, a property called pluripotency. ES cells were firstly isolated by Martin [1] and by Evans and Kaufman [2], who captured and placed in culture the transient inner cell mass or epiblast compartment of the pre-implantation mouse blastocyst, with the hope of arresting development at this very early stage. Thanks to this experiment, they obtained cellular identities now known as ES cells, which can be expanded indefinitely as embryonic stem (ES) cells ex vivo, but exposed to an appropriate environment, can also differentiate into all the lineages, and even re-enter the embryo development [1-4]. Research with mouse embryonic stem cells over the past 10 years has identified the master genes that control pluripotency. However, still many questions about the intriguing and fascinating properties of these cells remain unsolved. First of all, the unlimited existence of these cells in vitro, since in vivo such a phase is transient and exists only for at most 24 hours. Second, it is unknown how the pluripotent cells remain naïve and how do they choose to canalize along different lineages and make different types of tissue. The aim of this work is to address how the ES cells retain the naïve state, avoiding entering into differentiation and which cues are critical for their differentiation. The goal is to unravel the determinants of the decision to retain or exit pluripotency and the mechanism of lineage choice. We are also interested in the relationship between stem cell lines propagated in culture and progenitor cells in vivo. The hope is to apply the knowledge gained to control the derivation, expansion and differentiation of stem cells to the bio industry to facilitate use of stem cells in the study of disease mechanisms and for drug discovery.

In the three decades since their first description, ES cells have been derived and maintained in culture by using various empirical combinations of feeder cells, con-

ditioned media, cytokines, growth factors, hormones, fetal calf serum, and serum extracts. One unique feature of ES cells, unlike any other non-cancer cell culture, is that they proliferate autonomously in a minimal environment and do not need serum or growth factors for their proliferation *in vitro*, thus revealing that ES cells have an innate program for self-replication that does not require extrinsic instruction. This property may account for their latent tumorigenicity.

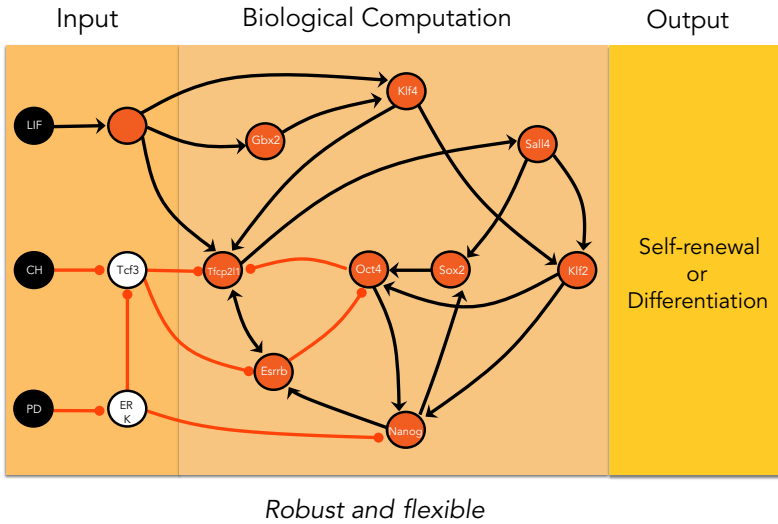
Several years of intensive studies led to the concept that the key for self-renewal is suppression of differentiation. A defined platform of dedicated transcriptional circuitries, which suppress differentiation, is strictly required to maintain the ground naïve state of these cells. The first extrinsic stimuli which is an absolute requirement for the propagation and pluripotency of ES cells is the cytokine leukaemia inhibitory factor (LIF), which is routinely used in the derivation and culture of mouse ES cells [5]. LIF binds to the gp130 protein and leads to activation of JAK kinases [6] that, in turn, phosphorylate the transcription factor Stat3 [7]. When phosphorylated, Stat3 dimerizes and enters the nucleus, thereby activating the expression of target genes [7-9]. Indeed, forced activation of Stat3 is sufficient to sustain ES cell self-renewal in the absence of LIF [10, 11], thus demonstrating that Stat3 is the key mediator of LIF action in ES cells. LIF also activates via JAK other signalling pathways that have been proposed to play a role in ES cell maintenance [12, 13] but are not essential. A second external signal involved in the suppression of ES cells differentiation is the bone morphogenetic factor 4 (BMP4). Ying *et al.* have uncovered a role for BMP4 in ES cell growth and demonstrated that in the presence of LIF, it can replace the requirement for serum. ES cell self-renewal is also enabled by the elimination of differentiation-inducing signalling from mitogen-activated protein kinase (MAPK). Additional inhibition of glycogen synthase kinase 3 (Gsk3) consolidates biosynthetic capacity and suppresses residual differentiation. As a consequence, ES cells can be derived and maintained using small molecule inhibitors of Gsk3 and the Fgf-MAPK signaling cascade (CHIRON99021 and PD0325901, respectively) [14], and genetic or pharmacological inhibition of the Fgf-MAPK pathway blocks efficient ES cell differentiation. With these developments, it is now possible to grow ES cells with defined factors in the absence of serum or feeder cells [15].

The picture that emerges has been revealed by combinatorial studies of external signal inhibition through small inhibiting molecules, which inhibit FGFR, MEK, and GSK3 (3i) or MEK and GSK3 (2i) [14]. The 2i/3i (two or three types of differentiation inhibiting molecules) medium is used successfully to maintain mouse stem cells *in vitro* in combination with or without LIF. The ES cell maintained in 2i/3i medium is in a *ground (naïve) state*, unbiased to any developmental specification.

While in *in vitro* cultures ES cells retain the powerful potential of unlimited propagation, such a property is restricted to embryonic day 4.5 (E4.5) during mouse development [16]. A crucial question is what confers the pluripotency at this precise stage?

We sought to dissect the molecular dynamics governing the transition from and to-

• **Figure 1**



wards naïve stem cell state through elucidation of molecular interactions that regulate lineage commitment. Efforts to delineate the transcriptional program governing ES cells behavior have identified a cluster of transcription factors, as forming a core regulatory network that plays a fundamental role both in mouse and human embryonic stem cell pluripotency, commitment to differentiate and reprogramming. Oct4 and Sox2 confer pluripotency and are expressed throughout the embryonic development and not only in the E4.5 precise moment. Other transcription factors, as Nanog, Esrrb, Tfc211, Klf2 and Klf4, show potent gain of function effects, but individually none of them are essential. These factors interconnect one to each other forming a system extensively redundant, since none of these factors are indispensable and deletion of individual member of the circle does not prevent ES cell self-renewal. To provide mechanistic insights into the ground state of naïve ES cells and to unravel the topology of the transcriptional circuitry governing ES cells pluripotency, we adopted a computational modelling approach to test correlations between key pluripotency factors [17]. We found some obligate interactions and other optional links. We applied computational software to reduce the enormous number of possible models. This allowed a definition of a minimal regulatory circuit comprising just 3 inputs and 12 transcription factors which can both reproduce known ES cell self-renewal behavior and predict behavior in response to previously untested perturbations (• **Figure 1**). This circuitry is presumed to activate expression of genes necessary to maintain ES state, while simultaneously repressing genes encoding lineage-specific transcription factors whose absence helps pre-

vent exit from the pluripotent state. We aim to extend our modelling approach to the molecular interaction dynamics governing the landscape of gene expression in mouse and human ES cells, to unravel the transition between naïve and primed ES cells, and to explore the pathway of molecular reprogramming that creates induced pluripotent stem (iPS) cells.

In conclusion, recent studies have delineated the transcription factor circuitry that defines ES cells and sustains them in a stable ground state. This recursive, robust and flexible control circuit is specific to the naïve pluripotent state. Pivotal elements of this circuit are down regulated in later phases of pluripotency in rodents and are absent in current human pluripotent stem cells. Functional implementation of the ground state transcription factor circuit in human cells is therefore a key challenge.

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In vivo phenotypic and functional stability of fibroblasts-derived dopaminergic neurons ensures their therapeutic potential

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The human body is a large collection of more than two hundred different fully differentiated somatic cell types that are the final heritage of a long process occurring from the pluripotent stem cell during the embryonic development. Till few years ago it was generally assumed that cells, once terminally differentiated, are locked in their cell fate. However, the ancient assumption that lineage fate decision is permanent has been challenged with the recent exploration of a cellular plasticity, demonstrating the incredible versatility of adult somatic cells. A broad number of studies in the recent years have shed light on the possibility of lineage reprogramming, as the ability of converting a mature, terminally differentiated cell type into another mature without undergoing dedifferentiation. The concept of cell reprogramming holds the potential to provide researches and physicians to transform the way we treat a number of diseases requiring cell replacement resulting from liver failure, heart disease, diabetes, Parkinson's disease, Alzheimer's disease, and blood diseases.

The first example of cell reprogramming came from the pioneering studies of Weintraub's group in the middle of the '80s, which demonstrated that expression of the single myogenic transcription factor, MyoD, is sufficient to convert a skin fibroblasts directly into a fully differentiated myotube, functional and with the contractile ability, a fate change process that was termed transdifferentiation, a direct route to lineage specification [1]. Since then, other remarkable examples of programming cell fate by the overexpression of specific transcription factors have been reported, including those of the neural, cardiac, blood, and pancreatic lineages [2-4]. Nuclear reprogramming can be achieved by nuclear transfer into oocytes [5], by cell fusion between ES cells and somatic cells [6, 7], and by the ectopic expression of transcription factors in somatic cells [8-10]. These transcription factors are also called "masters" or "selectors" of cell identity. They are generally expressed during development or during cell lineage

commitment. Many of these developmental transcription factors are specific of the different cell lineages and most of them are well conserved in animal evolution. Surprisingly enough, adult cells remains responsive to these factors even in adulthood. Cell reprogramming experiments acquired much more emphasis in the 2006, with the study of the Nobel Prize's awarded Prof. S. Yamanaka, who showed that mouse embryonic and adult fibroblasts can be reprogrammed into an embryonic-like state using a cocktail of transcription factors – often referred to as the “Yamanaka factors” – Oct3/4, Sox2, c-Myc, and Klf4 [9]. The reprogrammed cells, called induced pluripotent stem (iPS) cells, exhibit functional similarities to ES cells and present an exciting area of research. The ability to reprogram somatic cells into iPS cells that are pluripotent and can self-renew has transformed the fields of developmental biology and regenerative medicine. In the following years, regenerative medicine has seen a recent upward surge of investigation with regard to lineage reprogramming and several groups found it possible to convert adult human fibroblasts into induced pluripotent stem (iPS) cells using the same Yamanaka factors [8, 11-14] as well as different set of factors, [10, 15, 16].

Reprogramming of adult somatic cells has been efficiently applied also to generation of functional neurons. Genetic based approaches demonstrated that functional neurons could be generated independently of stem cells by direct cell conversion through genetics-based approaches [17]. More recently, fibroblasts have been directly converted into induced neuronal cells (iNS) through the forced expression of the three neuro-developmental factors Mash1, Brn2 and Myt1l [18]. iNS cells retain cell morphology, gene expression, differentiation potential, self-renewing capacity and *in vitro* and *in vivo* functionality similar to those of wild-type NSCs. However, iNS cells represent a heterogeneous population of glutamatergic and GABAergic neurons and their degree of global reprogramming remains to be properly characterized.

In the last years, my lab has focused on the possibility to reprogram differentiated somatic cells into a subtype of neuronal cell: the dopaminergic neuron. The dopaminergic neurons arise from the ventral side of the developing midbrain, and during their differentiation, a number of transcriptional factors, intrinsically expressed in the progenitors, is activated at either, early or late stages, to impose a progenitor cells to become a fully differentiated dopaminergic neuron. We took advantage of the transgenic mouse line TH-GFP, in which the thyrosine hydroxylase (TH) promoter drives the expression of the green fluorescent protein GFP. Thus, early-generated dopaminergic neurons, which produce TH as the first step in the dopamine biosynthesis pathway, will activate the GFP reporter [19]. By using this approach, we identified a minimal set of three transcription factors – Mash1 (also known as Ascl1), Nurr1 (also known as Nr4a2) and Lmx1a – as the minimal combination of transcription factors driving the direct generation of functional dopaminergic neurons from mouse and human fibroblasts without reverting to a progenitor cell stage [19]. After 2 weeks of expression of these three factors, fibroblasts changed completely their morpholo-

gy acquiring a neuronal like morphology, expressed TH and all the specific markers of dopaminergic neurons. Most importantly, the generated induced dopaminergic (iDA) cells were capable of dopamine production and releasing and showed spontaneous electrical activity organized in regular spikes consistent with the pacemaker activity featured by brain dopaminergic neurons. The possibility to produce functional iDA from fibroblasts holds the great hope to integrate successfully the generated cells into the fetal brain with a beneficial effect on symptoms of the neurotoxic rat Parkinson disease (PD) model [16]. Furthermore, it opens the possibility to rescue the autosomal recessive PD variants in the skin fibroblast model and use the derived dopaminergic neurons as material for the identification of biomarkers and mechanistic insights. Thus, this discovery might have significant implications for understanding critical processes for neuronal development, *in vitro* disease modeling and cell replacement therapies. To explore these possibilities, we verified the capacity of the three novel identified transcription factors to convert fibroblasts of donor individuals. Strikingly, we found that the three factors were able to elicit dopaminergic neuronal conversion in prenatal and adult fibroblasts from healthy donors and Parkinson's disease patients [19].

Sixteen days after reprogramming, a large number of iDA expressed many of the typical components of the dopaminergic machinery, such as TH, vesicular monoamine transporter 2 (VMAT2), dopamine transporter (DAT), as well as aldehyde dehydrogenase 1a1 (ALDH1A1) and calbindin. As a further confirm of the specificity of the conversion, these cells did not express markers associated with either adrenergic (dopamine- β -hydroxylase (DBH)) or serotonergic (tryptophan hydroxylase 1 or 2 (TPH1/2)) serotonin transporter (SERT; also known as SLC6A4). Finally, we tested the functionality of the produced iDA neurons. Strikingly, these cells showed a correct localization of the dopaminergic synaptic terminals as well as active synaptic processes execution. Patch-clamp recordings demonstrated that iDA neurons showed normal resting membrane potential, normal overshooting action potentials and prominent K1 currents after spike hyperpolarizations.

An important feature of the produced iDA neurons was the expression of high levels of the dopamine receptor D2. Strikingly, these autoreceptors were found to be functional and able of silencing the activity of the induced neurons. Thus, we demonstrated that a minimal combination of three transcription factors is able to reprogram somatic cells into new identity with several major properties of dopaminergic neurons in terms of spontaneous spiking activity, temporal parameters of action potentials. Importantly, this cell conversion diverges with respect to developmental neuronal lineage commitment, as it does not progress through detectable intermediate neuronal progenitor stages.

Until recently, cellular differentiation and development was thought to be a one-directional process. The breakthrough discoveries of nuclear transfer and reprogramming of adult somatic cells have shown that indeed it is possible to generate induced

pluripotent stem (iPS) cells by reprogramming differentiated cells to a pluripotent state, which can then be re-differentiated into any cell type in the body. The notion of cell plasticity holds the potential to provide researchers and physicians the opportunity to offer patient-specific treatments and transform the way we treat a number of diseases requiring cell replacement resulting from liver failure, heart disease, diabetes, Parkinson's disease, Alzheimer's disease, and blood diseases. Direct generation of iDA neurons from somatic cells might have significant implications for understanding critical processes for neuronal development, *in vitro* disease modelling and cell replacement therapies.

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Mechanisms of spindle positioning: from *C. elegans* to human cells

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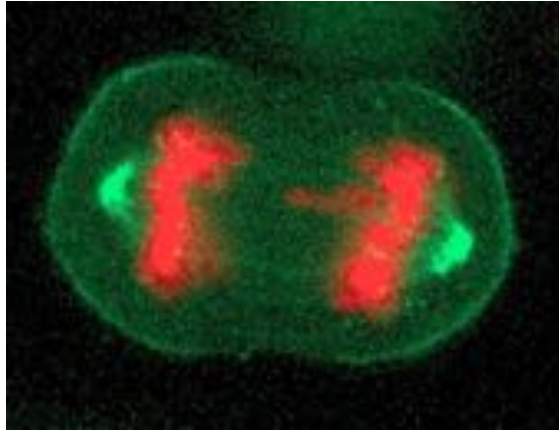
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During development and in adulthood, asymmetric cell divisions contribute to ensuring the balance between proliferation and differentiation. Regulated asymmetric cell division is thus necessary to generate cellular diversity during development and to maintain adult tissue homeostasis [1]. Disruption of this balance may result in premature depletion of the stem/progenitor cell pool, or instead in abnormal proliferation. During mitosis, the microtubule cytoskeleton is reorganized and assembled into a bipolar mitotic spindle [2]. Accurate positioning of the mitotic spindle is critical for proper asymmetric division during development and in stem cell lineages [1].

To allow proper spindle positioning, molecular motors anchored on the cell cortex exert pulling forces on astral microtubules that emanate from the two spindle poles [3, 4]. One such motor is the protein cytoplasmic dynein [5-7]. Dynein is a multisubunit ATPase motor protein complex of >1.5 MD also important for proper organelle distribution, spindle assembly, and kinetochore function [8]. Work in *C. elegans* and other systems revealed that spindle positioning relies on an evolutionary conserved ternary complex. In human cells, the members of this ternary complex are NuMA/LGN/G α . Their interaction with the cell membrane anchors the cytoplasmic dynein complex in that location during metaphase. Cortically anchored dynein regulates spindle positioning by exerting a pull on the plus-end of astral microtubules and/or by its capability to remain associated with force-generating depolymerizing microtubules [1].

Our laboratory has analyzed the mechanisms controlling spindle positioning during mitotic progression in human cells. In contrast to *C. elegans* embryos, most tissue culture human cells are not polarized, but polarization can be imposed upon them for instance by plating on a fibronectin substrate. In this manner, spindle positioning occurs in a stereotyped manner in control conditions. We found that depletion of members of the ternary complex LGN, NuMA and G α or of the dynein heavy chain

- **Figure 1.** Anaphase human HeLa Kyoto cell transfected with GFP-NuMA (green) and mCherry-H2B (red). Note presence of GFP-NuMA at the cell cortex in addition to the spindle poles



Source: courtesy of Sachin Kotak.

DYNC1H1 through siRNAs results in the random positioning of the spindle, confirming that the ternary complex is needed for proper spindle positioning during metaphase in HeLa cells [9, 10]. Furthermore, we demonstrated that cortical dynein levels markedly increase during anaphase, and that such increase is mediated by NuMA [11]. NuMA exhibits a dynamic distribution across the cell cycle: during interphase, NuMA is present in the nucleus, whereas following nuclear envelope breakdown (NEBD), the protein is released into the cytoplasm, and concentrates primarily in the vicinity of the two spindle poles. Moreover, NuMA is enriched slightly at the cell cortex during metaphase, whereas its distribution in that location is markedly enriched during anaphase (● **Figure 1**). This distribution correlates with that of dynein and of the dynein-associated protein p150^{Glued}, suggesting that NuMA may be critical for directing dynein anchorage to the cell membrane. Indeed, NuMA depletion severely impairs dynein localization not only in metaphase, as was known previously, but also in anaphase, where this results in impaired spindle elongation and increased chromosome-chromosome distance [11]. NuMA harbors four consensus sites in its tail domain for the CDK1/CyclinB kinase complex. We demonstrated that, during metaphase, CDK1 phosphorylates NuMA at T2055 and thus negatively regulates NuMA cortical localization; this phosphorylation event is counteracted by PPP2CA phosphatase activity. Moreover, we found that upon CDK1 inactivation in anaphase, the rise in dephosphorylated NuMA at the cell cortex leads to cortical dynein enrichment, and thus to robust spindle elongation. A NuMA mutant protein bearing a

non-phosphorylatable residue at T2055 (T2055A) exhibits increased localization at the plasma membrane, and defective spindle positioning during metaphase [12].

Overall, these findings reveal a mechanism whereby the status of NuMA phosphorylation coordinates mitotic progression with proper spindle positioning, ensuring that low levels of cortical dynein are present during metaphase. These and follow-up studies are expected to provide mechanistic insights into asymmetric cell division and thus prove fundamental for our understanding of stem cell biology.

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SESSION 2

Regenerative medicine by somatic stem cells: the paradigm of epithelial stem cells

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Squamous epithelia are constantly renewed. Being the first protective barrier against the external environment, these epithelia receive daily assaults, such as wounds, which need timely repair. Epithelial stem cells are responsible for such regeneration and repair processes [1, 2]. Stem cells have the unique capacity to self-renew and to generate committed progenitors – often referred to as transient amplifying (TA) cells – that differentiate into the cell lineages of the tissue of origin after a limited number of cell divisions. TA cells increase the number of differentiated progeny produced by each stem cell division, enabling stem cells to divide infrequently, at least under normal homeostasis.

Epithelial stem cells can be studied at a single cell level. Clonal analysis of human epidermal cultures has identified three types of clonogenic cells giving rise to distinct clones, referred to as holoclones, meroclones and paraclones [3]. The holoclone-forming cell is the smallest colony founding keratinocyte, has the highest proliferative capacity and is the stem cell of the epidermis and the hair follicle. It soon became clear that the holoclone-forming keratinocyte is the stem cell of virtually all human squamous epithelia [4]. Holoclones have all the hallmarks of stem cells, including self-renewal capacity, telomerase activity and long telomeres, an impressive proliferative potential and the capacity of generating all the epithelial lineages of the tissue of origin. During its clonal evolution, the holoclone produces paraclone-forming cells, which have a very limited proliferative capability, generate aborted colonies containing only terminal cells and have the properties expected of TA cells. Meroclones have an intermediate proliferative and clonogenic potential and are a reservoir of paraclones [4, 5].

The culture of keratinocyte stem cells holds the potential for a wide spectrum of clinical applications and has also enabled a gene therapy for a genetic disorder of the skin. Thanks to a discovery made in the 1980's by Howard Green, epidermal stem cells

can be taken from a patient, multiplied [6] and used to grow sheets of epidermis in the laboratory [7]. The new epidermis can then be transplanted back onto the patient as a skin graft [8, 9]. This life-saving cell therapy initiated the age of regenerative medicine by means of cultured stem cells. Similarly, identification, molecular characterization and cultivation of stem cells of the corneal epithelium allowed the restoration of severely damaged cornea, allowing restoration of sight in patients with severe ocular burns and poor alternative therapies [10-13].

The anterior part of the eye is covered by corneal, limbal and conjunctival squamous epithelia. The corneal epithelium is flattened, transparent and contains a basal layer of cuboidal cells lying on the Bowman's membrane of the avascular corneal stroma. The limbus, which is the narrow zone between the cornea and the bulbar conjunctiva, harbors the stem cells of the corneal epithelium [14]. Clonal analyses of the human ocular surface have shown that limbal cells are clonogenic and endowed with high proliferative potential. By contrast, central and paracentral corneal keratinocytes generate only aborted colonies with a negligible proliferative capacity. The peripheral cornea (the area of the cornea adjacent to the limbus) contains some clonogenic cells, which, however, have only a limited capacity for multiplication. Indeed, only limbal cells are able to generate holoclones, with a frequency of 3-10% [4]. Human corneal renewal and repair are executed by stem cells located in the limbus.

Transparency of the cornea is essential to visual acuity and depends on stromal avascularity, highly organized stromal collagen fibrils and epithelial integrity. Allogeneic corneal transplantation (keratoplasty) can restore scarred corneal stroma and inner endothelium. However, its clinical success relies on patients' own limbal stem cells, which generate the host-derived corneal epithelium needed to resurface the donor corneal stroma. In the case of chemical or thermal destruction of the limbus leading to limbal stem cell deficiency (LSCD) a functional corneal epithelium can no longer be formed, the keratoplasty is unsuccessful and the cornea (re)acquires an epithelium by invasion of bulbar conjunctival cells originating beyond the destroyed limbus. This process leads to corneal vascularization and opacification, and loss of vision. The only way to prevent corneal "conjunctivalization" is to restore the limbus, which can be attained in unilateral LSCD through the grafting of limbal fragments from the uninjured eye [15].

The notion that limbal cultures contain stem cells detectable as holoclones inspired the first therapeutic use of such cultures for the regeneration of a corneal epithelium. This technology significantly reduced the size of limbal withdrawal from the donor eye and, more importantly, allowed permanent regeneration of a fully functional corneal epithelium not only in patients with unilateral LSCD, but also in patients with severe bilateral corneal damage. Indeed, 1-2 mm² of spared limbus in one eye is sufficient to generate limbal cultures able to restore the corneal epithelium of both eyes [11].

We performed long-term clinical studies with homogeneous groups of patients presenting with corneal opacification and visual loss due to chemical and thermal

burn-dependent LSCD [11, 12]. In two third of these patients, the visual acuity was below the light perception. We obtained 66-78% of full success, in that grafts of autologous cultured limbal keratinocytes permanently restored the corneal epithelium and the visual acuity of these patients. We tried to get further insight the molecular reasons for the failures, and observed that success was closely associated to the percentage stem cells in culture. Long-term (over 14 years) follow-up on hundreds of patients provided conclusive evidence that the most important biological criterion to assess graft quality and the likelihood of a successful treatment is a rather precise evaluation of the number of stem cells detected as holoclones: a minimum of 3% of clonogenic cells should be holoclones [11, 12]. This number is consistent with the notion that the human limbus contains approximately 5% stem cells and that limbal holoclones progressively decrease during aging, dropping below the threshold of 3.5% in the elderly. Neither total number of clonogenic cells (CFU) nor colony size could predict clinical outcomes, confirming that the vast majority (~95%) of clonogenic keratinocytes (meroclones and paraclones) behave as transient progenitors. This does not mean that the number of clonogenic cells is an irrelevant parameter. First, clonogenic progenitors represent an integral part of any squamous epithelium and are likely to contribute to the initial coverage of the corneal lesion, hence fostering stem cell engraftment. Second, it is unlikely that an epithelial culture devoid of a proper number of clonogenic cells contains a sufficient number of holoclones, which are a subset of such cells. Thus, an adequate CFU is a necessary, although not sufficient, parameter to define the potency of the culture.

Clinical data provided conclusive evidence that holoclones can be identified by quantitative immunodetection of p63 [16], confirming the notion that p63 is a determinant of the regenerative potential of epithelial stem cells [17]. The protein p63 is a transcription factor with remarkable properties. Ablation of the p63 gene results in the absence of stratified epithelia [18, 19]. Mutations of p63 cause disorders of the epithelia and of non-epithelial structures whose development depends on the epithelial functions, particularly in the cranio-facial region [20].

Human limbal stem cells can be identified, both *in vivo* and *in vitro*, through their expression of p63 [21-23]. The TP63 gene generates six isoforms. Transcription from different promoters generates two different pre-messenger RNAs: TAp63 and Δ Np63. Alternative splicing of each transcript produces α , β , and γ isoforms [16]. Ocular keratinocytes may contain all the Δ N isoforms, but Δ Np63 α (hereafter referred to as p63) is by far the most abundant; it is present in the limbus but not in the uninjured central cornea and is expressed in holoclones but not in paraclones [22, 23]. The protein p63 sustains the proliferative potential of limbal stem cells, and the C/EBP δ transcription factor maintains the self-renewal of these cells and regulates their mitotic rate [21]. During corneal repair, limbal stem cells that stain intensely (hereafter referred to as p63^{bright} cells) are released from C/EBP δ -dependent mitotic constraints, multiply, and migrate to regenerate corneal epithelium [21].

We observed that a minimum of approximately 3000 stem cells, detected as p63^{bright} holoclone-forming cells, is required to achieve full clinical success of autologous limbal cultures [11, 12].

The knowledge attained during years of clinical application of epidermal and limbal cultures allowed the development of *ex vivo* epidermal-stem cell mediated gene therapy of inherited epidermis bullosa (EB), a devastating and disabling skin adhesion disorder [24]. EB is characterized by structural and mechanical fragility of the integuments, leading to recurrent skin and mucosal blistering and erosions that severely impair the quality of life of EB patients. Junctional EB (JEB) is marked by blister formation at the level of the lamina lucida of the basement membrane and absence (or severe alteration) of hemidesmosomes. JEB has been divided into three categories: Herlitz (JEB-H), non-Herlitz (JEB-nH), and JEB with pyloric atresia (JEB-PA). JEB-H is an early lethal form and is usually due to deleterious mutations in *LAMA3*, *LAMB3*, or *LAMC2* genes causing a total absence of laminin 332 (previously known as laminin 5), a heterotrimeric protein that consists of α , β , and γ chains, and links 6 integrins to collagen VII dermal fibrils. Mutations of the same genes cause JEB-nH, which is characterized by reduced expression of laminin 332. JEB-nH can also arise from mutations in *COL17A1*, the gene encoding collagen XVII, whereas JEB-PA is due to mutations in genes encoding the $\alpha 6\beta 4$ integrin [24]. There is no cure for EB; treatments are palliative and focused on relieving the devastating clinical manifestations [25].

A phase I/II clinical trial showed that autologous epidermal cultures containing genetically modified holoclones restored a normal epidermis on both upper legs of a patient (Claudio) suffering from a severe form of laminin 332- $\beta 3$ -dependent JEB-nH. Epidermal keratinocytes were taken from his palm skin, which, at variance with other affected body sites, contained an appropriate number of holoclones. Cells were transduced *ex vivo* with a murine leukemia virus (MLV)-based retroviral (RV) vector expressing long terminal repeat (LTR)-driven *LAMB3* cDNA and used to prepare transgenic epidermal grafts, which were transplanted onto surgically prepared regions of Claudio's upper legs [26].

The critical goals of the trial were to evaluate the safety and long-term persistence of genetically modified epidermis. During the 6.5-year follow-up, the epidermis of both of Claudio's upper legs was normal looking, normally pigmented, robust and did not itch or form blisters, either spontaneously or after induced mechanical stress (such as biopsy withdrawal). Synthesis of normal levels of functional laminin 332 was observed together with absence of infections, inflammation, or immune response. In situ hybridization using vector-specific laminin 332- $\beta 3$ probes showed that the regenerated epidermis consisted only of transgenic keratinocytes [26, 27]. A normal epidermal-dermal junction was restored and the regenerated transgenic epidermis was found to be fully functional and virtually indistinguishable from a normal control [26, 27]. The density and organization of collagen bundles in the papillary dermis

were consistent with restoration of mechanical strength. Transmission electron microscopy showed that the thickness and continuity of the basement membrane and the number and morphology of hemidesmosomes were virtually indistinguishable between control and transgenic skin. We estimate the presence of approximately 10 independently transduced stem cells in 10 mm² of epidermis [27]. Since virtually all keratinocytes contain LAM332-β3 transcripts, it is clear that the vast majority of transduced keratinocytes grafted onto the patient's legs were transient-amplifying progenitors that were lost within a few months after grafting, and the regenerated epidermis is sustained by a discrete number of engrafted, long-lasting, self-renewing transgenic stem cells. These data pave the way for the safe use of epidermal stem cells in combined cell and gene therapy for ex vivo gene therapy of all types of EB.

The difficulties encountered in keratinocyte therapy have been great, but much progress has been made, even if much of this progress has not yet been assimilated by the large number of scientists and physicians who have an interest in the subject [28]. Therapy with cultured keratinocytes is ready for more widespread use because the necessary criteria for graftable cultures and for their surgical use are now well understood. These criteria are relevant to future use of any cultured cell type for therapeutic purposes.

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Molecular regulation of skeletal muscle stem cell states

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Skeletal muscle satellite cells are mononucleated myogenic cells, located between the sarcolemma and basement membrane of terminally differentiated muscle fibres and are named for their intimate positional or “satellite” relationship with fibres. These cells are the principal myogenic stem cells and are normally quiescent in adult muscle, but act as a reserve population of cells, able to proliferate in response to injury and give rise to regenerated muscle and to more satellite cells. Following their discovery in 1961 by Mauro, these cells were considered to be a quiescent post-mitotic population of myogenic cells. The demonstration that satellite cells are able to self-renew and support several rounds of muscle regeneration conferred them the status of *bona fide* muscle “stem cells”. In the adult, quiescent satellite cells exhibit limited gene expression and protein synthesis but they can be activated in response to an injury. What triggers the transition between quiescence and activation, remains largely unknown. Quiescent satellite cells express *Pax7*, a paired-box transcription factor marking specifically quiescent and newly activated muscle precursor cells, as demonstrated by the absence of myogenic cells in young *Pax7* null skeletal muscle [1]. Upon exposure to an injured environment, individual satellite cells leave their niche and enter the cell cycle, co-expressing *Pax7*, *Myf5* and *MyoD*. Activated satellite cells then undergo multiple rounds of cell division, downregulate *Pax7*, and express Myogenin and later *Mrf4* [1-4]. Powerful inhibitors of muscle differentiation are serum and growth factors, and withdrawal of these mitogens leads to cell fusion to form multinucleated myotubes, irreversible commitment of muscle cells to the postmitotic state and transcriptional activation of downstream differentiation genes [5]. Differentiation is intimately coupled to the cell cycle, and transcription of differentiation genes begins when myoblasts are growth arrested in the G1-G0 phase.

Although all skeletal muscles in the body arise from muscle founder stem cells located in somites, the muscle body composition varies remarkably in the different musculature (trunk, limb and head muscle groups) [6], indicating that the regulatory genes are not used in the same combinatorial manner in all regions where muscles are established. For example, Pax3 plays important roles in the embryo in regulating muscles in the trunk and limbs, but head myogenesis has evolved with a distinct transcriptional code independent of Pax3. Indeed, recent studies underscored the notion that the site of origin of the founder stem cells is important in determining the regulatory genes that govern their behavior [7, 8]. These findings are relevant in the context of myopathies, some of which affect only a subset of muscles, whilst other muscle groups escape the disease [9, 10]. Therefore, the developmental ontology of skeletal muscle might provide insights into the susceptibility of certain muscles to succumb to the disease.

The regulatory cell state of muscle stem cells fluctuates during perinatal growth, after entering quiescence, and following muscle injury when amplification of the stem cell pool results in the production of myoblasts and future self-renewing cells. We observed heterogeneous properties in the muscle stem cell pool during quiescence and proliferation and performed serial transplantations of subpopulations of these cells isolated directly from the quiescent state. We demonstrated that a major regulator of cellular quiescence in muscle is Notch signaling, which is highest in quiescent muscle satellite cells, and is lost rapidly following cell cycle reentry, then restored during self-renewal [11,12]. Notch signaling regulates cell proliferation, differentiation, and cell fate determination [13, 14]. Upon the binding of Delta and Jagged family of ligands to Notch transmembrane receptors, sequential enzymatic cleavages of the Notch receptor release the active truncated form, the Notch intracellular domain (NICD). Then, the NICD translocates from the cytoplasm to the nucleus and activates the CSL (CBF1, Suppressor of Hairless and Lag-1) family of transcription factors, leading to target gene expression. During skeletal muscle regeneration, the Notch ligand Delta is quickly up-regulated following injury in committed myogenic cells. Activation of Notch signaling stimulates the proliferation of satellite cells and their progeny and thus leads to the expansion of proliferating myoblasts. Indeed, inhibition of Notch signaling abolishes satellite cell activation and impairs muscle regeneration. At the onset of myogenic differentiation, however, the inactivation of Notch signaling in myoblasts is required for their fusion. Conversely, forced expression of NICD results in a block of the commitment of mouse embryonic muscle stem/progenitor cells to differentiation [11]. The arrested cells continue to proliferate, thus indicating that sustained Notch signaling is sufficient for their cell-autonomous maintenance. Furthermore, we uncovered a paradoxical effect of Notch, whereby sustained Notch activity is associated with stem/progenitor cell expansion in the mouse embryo, yet at later stages during foetal development it promotes reversible cell cycle exit [11]. In the adult genetic ablation of Rbpj, the main effector for canonical Notch signaling,

results in a break in cellular quiescence of the muscle stem cells and promotes their differentiation. Interestingly, this is associated with a bypass of S-phase DNA replication and a direct G0 quiescence to G1 differentiation transition during homeostasis [12]. These findings support the notion that active Notch signalling is sufficient to sustain an upstream population of muscle founder stem cells while suppressing differentiation.

To gain further insights into the behavior of satellite cells in their microenvironment, we generated a transgenic *Tg:Pax7-nGFP* mouse that marks all satellite cells, from the embryo to the adult [8]. We reasoned that muscle stem cell heterogeneity might be correlated with the expression levels of the upstream marker Pax7. Indeed, by FACS at opposite ends of the spectrum of green fluorescent protein (GFP) expression, we isolated two subpopulations of Pax7 positive cells: Pax7 high and low [15]. In doing so, we identified a novel dormant stem cell state during cellular quiescence, characterized by high expression of Pax7 (Pax7-nGFP^{Hi}), that comprises lower metabolic activity, higher expression of stem cell genes and a longer lag before the first mitosis [12]. Transplantations of these satellite cells demonstrated their long term capacity to self-renew after 6 rounds of serial transplantations in immunocompromised mice [15]. Furthermore, we discovered that during muscle regeneration, the majority of Pax7-nGFP^{Hi} cells asymmetrically segregating template DNA strands to the stem cell, and they generate distinct daughter cell fates [15]. Notably, old DNA template strands are inherited by the daughter cell fated to be stem (Pax7+), whereas new DNA strands are inherited by the daughter cell that will differentiate (Myogenin+). Although muscle cell fate in asymmetrically dividing cells correlates with asymmetric DNA segregation, this correlation is not absolute as asymmetric DNA segregation can also occur in Pax7-Pax7 daughter cell pairs. How the balance between asymmetric and symmetric cell divisions relates to the stem cell niche is being investigated with micropattern technologies where individual cell fates can be altered by modifying the geometry of the micropattern design – symmetric or asymmetric [16]. In a different study, we analyzed the remarkable resistance of satellite cells to survive post-mortem, raising the possibility that stem cells can be isolated from tissues and organs of deceased individuals long after death. Strikingly, seventeen days after death, some satellite cells remained viable and adopted a reversible dormant state characterized by reduced metabolic activity, a prolonged lag phase before the first cell division, elevated levels of reactive oxygen species and a transcriptional status less primed for commitment. In these studies we showed that in post mortem tissue, severe environmental conditions (anoxia, necrosis) lead muscle stem cells to adopt the dormant stem cell state thereby retaining their regenerative potential after transplantation into regenerating muscle [17]. We came to the conclusion that a key regulator of stem cell survival is the oxygen status and that severe hypoxia, or anoxia is critical for maintaining stem cell viability and regenerative capacity. Thus, these cells provide a useful resource for studying stem cell biology.

In conclusion, skeletal muscle provides an excellent paradigm to investigate the biomedical and regenerative potential of stem cells in the context of pathologies that involve degeneration and regeneration. The remarkable ability of satellite cells to regenerate even after multiple rounds of injury make these cells amenable for investigating the role of stem cells in these processes, and over extended periods. An understanding of how stem and associated cells construct a tissue can provide insights into the development of clinical strategies to combat the normal decline in skeletal muscle performance during ageing, or its reconstitution after trauma and during disease.

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Muscle stem cell therapy in injury and disease

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Adult skeletal muscle is a postmitotic tissue that exhibits an extremely low turnover in the absence of disease or injury [1]. Following an injury, skeletal muscle undergoes a highly orchestrated degeneration and regenerative process that takes place at tissue, cellular, and molecular levels [2]. This regeneration process relies on the dynamic interplay between muscle stem cells (MuSCs), or “satellite cells”, and their environment (stem cell niche), and results in the reformation of innervated, vascularized contractile muscle apparatuses. MuSCs play an indispensable role in the repair process. The self-renewing proliferation of MuSCs not only maintains the stem cell population but also provides numerous myogenic progenitors capable of proliferating, differentiating, and fusing, finally allowing new myofiber formation and reconstitution of a functional contractile apparatus [2]. The complex behavior of MuSCs during skeletal muscle regeneration is tightly regulated through intrinsic and extrinsic factors constituting the muscle stem cell niche/microenvironment. After anatomical identification in 1961 by Mauro, satellite cells were intensively investigated in terms of their behavior in response to growth and regeneration. For the last half century, advances of molecular biology, cell biology, and genetics have greatly improved our understanding of skeletal muscle biology. Extensive research on MuSCs and their niche have elucidated many cellular and molecular mechanisms, and these studies have also promoted research on novel therapeutic strategies to alleviate the physiological and pathological conditions associated with myopathies, sarcopenia and muscular dystrophies. In the new era of regeneration medicine, MuSCs represents a great hope for the development of therapeutic strategies against muscular pathologies, especially since cell transplantation is increasingly studied in a wide variety of tissues and diseases.

My lab focuses on the use of MuSCs for the treatment of degenerative disorders of muscle, such as the muscular dystrophies, and a specific kind of muscle injury referred

to as volumetric muscle loss (VML) [3]. Our purpose is to dissect the characteristics of mouse and human MuSCs that render them most potent in transplantation for restoring normal structure and function to diseased or injured muscle. We have explored both the transcriptional and epigenetic states of MuSCs to understand how treatment *in vitro* can maintain, restore, or induce a highly potent state. One of the most important criteria to increase the grafting potential of MuSCs is to maintain them in intimate physical contact with the naïve niche. The stem cell niche refers to the microenvironment that maintains “stemness” [4]. The concept of a stem cell niche was originally described by Schofield in 1978, with reference to mammalian hematopoiesis [4]. It was proposed that the niche comprises cells whose secretory products interact with and govern stem cell behavior. Accordingly to this model, the niche composition would be essential in maintaining stem cell quiescence in the absence of any external activating cues but would promote proliferation and maturation of the progenitors should the need arise, as well as ensuring self-renewal of the stem cell pool. Schofield’s model states that the stem cell niche can be identified by a set of well-defined features: a) a specific anatomical location, b) a site supporting cells reproduction, c) a place where differentiation is inhibited, and d) a space that also limits the numbers of stem cells. In recent years, several lines of evidence have supported the notion that the muscle fiber is a key component of the MuSC niche. Indeed, removal from association with the myofiber plasmalemma leads to the activation of quiescent satellite cells and their entry into the cell cycle, suggesting a role in inhibition of mitogen-induced cell cycle entry by the myofiber [5]. Studies on freshly isolated single muscle fibers have shown that a subset of quiescent satellite cells retains its ability to proliferate and return to quiescence, a process enhanced by contact with the muscle fiber, while the differentiation commitment is impaired [5, 6], thus suggesting that the muscle fiber fulfills three of Schofield’s criteria of a stem cell niche.

We have concentrated our studies on the functions of satellite cells and the regulation of their niche during the process of skeletal muscle regeneration. Toward this end, we have focused on important signaling pathways such as the Notch and the p38 MAPK pathways and how modulation of those pathways impacts MuSC state and function [7]. Our group has shown that Notch signaling plays a prominent role in regulating proliferation and differentiation of activated MuSCs [7]. Interestingly, Notch3 is expressed by quiescent MuSCs [8], and its disruption leads to misregulation of SC proliferation [9]. Furthermore, Notch signaling downstream targets Hes1, Hey1 and HeyL are highly expressed in quiescent SCs [8], suggesting that behind its function in regulating proliferative expansion of activated SCs, Notch may also be important in the regulation of SC quiescence. To gain further insight into the role of Notch signaling in adult MuSCs, we genetically depleted RBP-J specifically in MuSCs using an inducible system. We found that RBP-J is required for the maintenance of the MuSC population in a state of quiescence by preventing their activation [10]. These results demonstrate that Notch signaling plays an additional and unexpected

role in the regulation of MuSC quiescence in adults. Furthermore, RBP-J-deficient MuSCs, once activated, fail to self-renew suggesting that Notch signaling regulates not only the maintenance of the quiescent state but also the return to the quiescent state. A further signal regulating myogenesis is the Wnt pathway [11]. By directly acting on myogenic progenitors, Wnt promotes the transition of progenitors from the proliferative phase to the differentiation phase during postnatal myogenesis. Furthermore, the increase in Wnt signaling dictates a critical transition from high Notch activation to high Wnt activation, a molecular switch that is essential for effective muscle regeneration. Importantly, GSK3 β is a pivotal determinant of fate of MuSC progeny, integrating inputs from the Notch and Wnt signaling pathways. Indeed, the balance between these pathways is reflected by the state of activation of GSK3 β , which is maintained in the active state by Notch signaling and inactivated by Wnt signaling [11].

A second interest of my research group has focused on efforts in improving the potential of MuSCs for regenerative therapies. Although intensive studies have shed light on the complex muscle regeneration process, there are still many interesting questions that remain to be answered. We asked what are the intrinsic and extrinsic mechanisms that determine satellite cell proliferation, with the purpose to generate sufficient numbers of myogenic cells for muscle regeneration. Similarly, we have been interested in the mechanisms regulating the ability of muscle to regenerate in response to variable levels of damage. Our studies have focused on the generation of artificial scaffolds on which to seed purified mouse or human MuSCs prior to transplantation in order to enhance transplantation efficacy. Using both anatomical and physiological analyses, we have optimized the *ex vivo* approaches in a xenograft model of regenerative therapy of VML. Furthermore, we are currently combining this approach with the analysis of the effects of physical activity on the efficacy of muscle regeneration. Our long-term goals are to establish scalable processes for the isolation, expansion and transplantation of MuSCs as a therapeutic approach to disease and conditions for which no effective therapies currently exist. As such, our research on MuSCs is turning toward therapeutic applications. A major emphasis of our recent effort is pre-clinical studies using MuSCs in models of cell transplantation to treat muscular dystrophies with an eye toward future human trials.

Conclusions. The ability to maintain quiescence is critical for the long-term maintenance of a functional stem cell pool. The seminal observations of the satellite cell in its niche made by Alexander Mauro almost fifty years ago have spawned many advances in our understanding of this tissue-specific stem cell. The aforementioned observations, together with those from other studies, have formed the basis of our current view of satellite cells as MuSCs whose functions are dictated by their surrounding niche. We are just beginning to unravel the microenvironmental influences that mediate stem cell properties and how epigenetic regulation governs stable properties of MuSCs during homeostasis and repair.

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Immune cell regulation of regeneration

Nadia Rosenthal

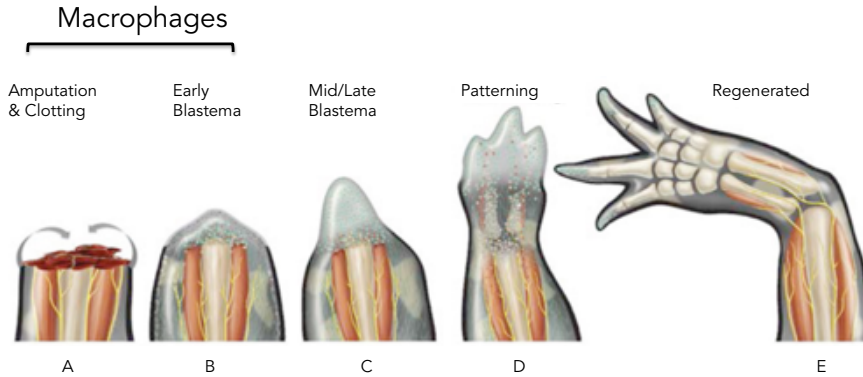
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Regenerative potential, robust in lower vertebrates, is gradually lost in higher vertebrates such as mammals. In contrast to the effective regeneration of other vertebrates, the limited restorative capacity of many adult mammalian tissues has been attributed to the loss of adequate cell replacement. However, although gradually lost in adult mammals, repair capacity is retained in the embryo.

In adult mammals, fibrotic scarring and cellular infiltration and immune signaling are major impediments to tissue regeneration [1, 2]. Macrophages are an important source of both inflammatory and anti-inflammatory signals; they reach the injury site within 48-96 hours, and clear dead cells, release proinflammatory cytokines, and produce factors responsible for dampening inflammation and stimulating angiogenesis, fibroblast migration, and replication [3]. Indeed, macrophage depletion or disruption of macrophage transcriptional regulation after skeletal muscle injury results in incomplete muscle repair and induction of fibrotic scarring [4, 5].

We have investigated the role of growth factors and the immune system in the restoration of injured tissues. In particular, we aimed to address the role of macrophages during scar-free tissue repair in a regeneration model of limbs of the axolotl aquatic salamander (*Ambystoma mexicanum*). Salamanders are ideal models for regeneration studies, since they have the remarkable ability to extensively replace complex structures such as limbs, tails, retina, and spinal cord, along with some sections of the heart and brain, during any stage of their life cycle [6]. Salamanders are also able to perform scar-free repair of deep tissue wounds after injury [7] (• **Figure 1**). Recently, it has been reported that as in mammals, the salamander immunological response and cytokine microenvironment play a key role in the proper execution of tissue repair [8-10]. We observed that, during the early stages of axolotl limb regeneration, a dynamic signaling microenvironment was accompanied by early myeloid cell recruit-

● **Figure 1.** Stages of limb regeneration in the salamander and the requirement for macrophages

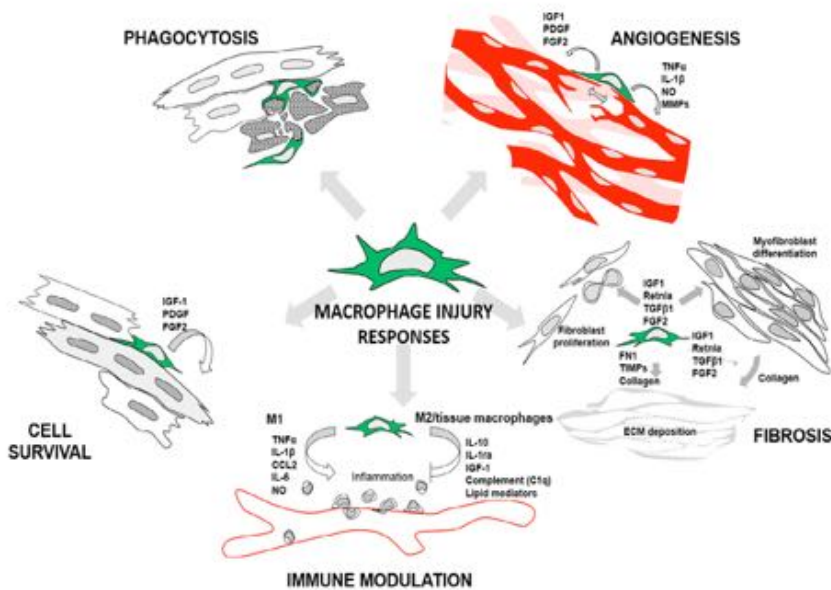


A-B: Wound healing phase requiring macrophage action. C: Proliferative blastema. D: Out-growth and patterning phase to generate functional limb.

Source: courtesy of J. Godwin.

ment. To gain further insight into the role of macrophages during tissue regeneration, we depleted the macrophage population by using the phagocytic uptake of liposome-encapsulated clodronate (Clo-Lipo), an established method for specific ablation of macrophages [11]. Following Clo-Lipo administration, we observed effective depletion of circulating axolotl monocytes and tissue resident macrophages, while the neutrophil numbers were unaffected (● **Figure 2**). The first week after amputation, before macrophages return, was characterized by changes in macrophage chemoattractants, increased levels of inflammatory cytokines, and reduced levels of anti-inflammatory cytokines. Molecular profiles analysis demonstrated a concomitant decrease in the expression of the ECM-degrading enzymes matrix metalloproteinase 9 and 3 (MMP9 and MMP3, respectively) and in the expression of *Msx2* and the blastemal marker genes *Prrx1* and *Sp9*. Macrophage depletion also caused a failure in the activation of *Tgf-β1* and TGF target genes *Runx2* and fibronectin. Since TGF-β represents a key component of mammalian wound healing process and constitutes an important signaling pathway in salamander limb regeneration [12], we speculated that the lack of the appropriate levels of TGF-β1 activation in macrophage-depleted limbs following amputation might account for the failure to regenerate in these animals. Furthermore, failure to up-regulate the activated wound epithelial marker WE3 in Clo-Lipo animals suggested that in this condition, the wound epithelium is not functional. Thus, we concluded that macrophage depletion before amputation

● **Figure 2.** Diverse role of macrophages in tissue regeneration



(Clockwise from top left) Macrophage-mediated removal of cellular and tissue debris by phagocytosis. Angiogenesis mediated by macrophage paracrine factors and direct interaction of macrophage with endothelial tip cells. Induction of tissue fibrosis by various paracrine factors. Macrophage-derived factors can induce fibroblasts proliferation and myofibroblast proliferation, and can directly and indirectly contribute to extracellular matrix (ECM) deposition. Macrophage can promote and suppress inflammation, respectively by enhancing or inhibiting leukocyte infiltration and secretion of inflammatory mediators by local cells. Macrophage-derived factors can promote cell survival.

Source: courtesy of A. Pinto.

disrupts specific gene pathways important for the progression from wound healing to regeneration within the first six days. These findings support the possibility of improving human regeneration by modulating the immune system, providing new targets for clinical intervention and improving prospects for molecular and cellular combination therapies.

A crucial focus of my laboratory over the past years has been the dissection of the molecular determinants governing the regeneration processes in mammals. Tissue repair occurs in four subsequent phases: degeneration, inflammation, regeneration and fibrosis [13, 14]. Among other factors, recent experimental models have implicated insulin-like growth factor-1 (IGF-1) as a modulator of growth and regeneration in skeletal muscle.

During the muscle repair process, neutrophils invading the muscle potentially contribute to the muscle damage through the release of free radicals. The activity of superoxide dismutase (SOD) is important for decreasing the levels of free radicals, counteracts the damage and eventually prevents tissue degeneration [13]. Interestingly, IGF-1 protects the injured muscles in mutant SOD1 (G93A) mice, through a reduction in the damage caused by free radicals and attenuation of muscle degeneration [15]. These data suggest that IGF-1 acts as a survival factor, maintaining muscle integrity and delaying the degeneration and paralysis of the skeletal muscle, as well as the motor neurons. As a confirmation, treatment by viral administration of recombinant IGF-1 in mouse models has been demonstrated to decelerate the progression of amyotrophic lateral sclerosis, a neurodegenerative disorder that causes progressive muscle weakness and loss of motor neurons [16]. IGF-1 also has an ameliorating effect on muscular dystrophy, as demonstrated by studies in mdx mice in which transgenic expression of IGF-1 clearly reduces the breakdown of myofibres and therefore plays a protective role against muscle degeneration [17, 18]. Finally, IGF-1 also has a protective role on senescent muscle [19]. In the heart, we have characterized a novel, abundant population of cardiac tissue macrophages with a unique gene expression profile [20] that changes rapidly with age, including IGF-1 as well as canonical macrophage markers [21], suggesting a role for resident macrophages in cardiac homeostasis and tissue maintenance.

We demonstrated that local damage leads to the recruitment of circulating cells into regenerating myofibres [22, 23] and, interestingly, the expression of IGF-1 enhances this recruitment of bone marrow stem cells at the sites of muscle injury [22]. Our data showed that IGF-1-enhanced muscle regeneration involving multiple precursor cell types, both endogenous to the muscle and attracted from the circulating stem cell pool. Two alternately acting forms of IGF-1 have been characterized: the locally acting propeptide with an autocrine/paracrine action and the circulating fully processed peptide with endocrine effects [24]. We demonstrated that the selective production of the two forms triggers different effects on muscle regeneration and myogenesis [25].

In conclusion, an emerging concept in the tissue regeneration field is that a rapid and fine tuned inflammatory response is a mandatory requirement for a full recovery upon an injury. Both excessive or restricted macrophage involvement can hamper the outcome of the repair. These works uncover a complex interaction between local repair mechanisms and circulating immune cells that remove necrotic cells, secrete growth factors that limit inflammation, and promote tissue replacement. Concerted strategies are needed to optimize the immune response and its humoral components in the context of tissue repair. Our work supports the feasibility of improving mammalian regenerative capacity by modulating key signaling pathways controlling specific components of the immune system, providing new targets for clinical intervention and improving prospects for molecular and cellular combination therapies.

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Cell therapy for muscular dystrophy: a phase I/IIa study

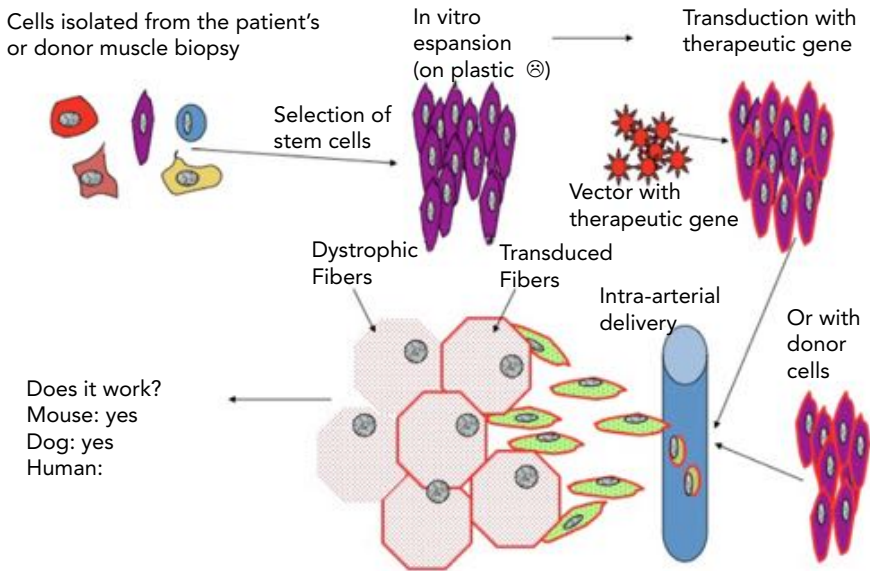
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Muscular dystrophies are inherited diseases characterized by fragility of muscle fibers which are unusually susceptible to damage [1]. Duchenne muscular dystrophy (DMD) is one of the most severe forms of muscular dystrophy and among the most common fatal genetic disorders diagnosed in childhood, affecting approximately 1 in every 3,500 live male births (about 20,000 new cases each year). Since the mutated gene (named dystrophin) is on the X-chromosome, it affects boys and knows no cultural, economic, or social boundaries. DMD appears in early childhood and worsens progressively resulting in progressive loss of muscle strength which confines the patient to wheelchair in his teen ages (and sometimes earlier) and ends with an almost complete paralysis and need for assisted ventilation. The loss in muscle mass affects primarily the “proximal” muscles around the hips and the shoulders. This means that fine movements, such as those using the hands and fingers, are less affected than movements like walking [2]. Muscular weakness and skeletal deformities frequently contribute to breathing disorders. Cardiomyopathy occurs in almost all cases, beginning in the early teens in some, and in all after the age of 18 years [3]. Although there are medical treatments (mainly glucocorticoids) that slow its progression, there is currently no cure for Duchenne.

As stated previously, DMD is caused by a mutation in the largest known gene, dystrophin, which encodes for an important muscle protein that confers resistance to stress associated with contraction of the muscle fiber [3]. Dystrophin is also believed to carry signals between the inside and outside of muscle fibers. Without dystrophin, muscles are unable to operate properly, and eventually suffer progressive damage. Gene therapy for DMD aims at successfully introducing a normal copy or correcting the dystrophin gene into muscle cells, thereby restoring dystrophin production, and, ultimately, curing the disorder. This is however a formidable task given the size of the

• **Figure 1.** Mesangioblasts for the cell therapy of primary myopathies



gene and the fact that the correction must be carried out in most of the skeletal muscle, the most abundant tissue of our body. Cell therapy, on the other hand aims to deliver cells to the dystrophic muscle. Two alternative cell therapy approaches can be followed: (i) using cells obtained from an healthy donor (ideally HLA-identical, as for bone marrow transplantation), which express the normal copy of the mutated gene but induce an immune response that must be controlled through immunosuppression; (ii) using cells obtained from the patient, which do not require immune suppression but must be genetically corrected in vitro to restore the expression of the mutated protein. In order to avoid the side effects derived from a permanent immune suppression, the second approach seems to be preferable. A scheme of the protocol is shown in • **Figure 1.**

However, the exceptional length of the protein and the complexity of its gene make the attempts to correct it all but simple. Moreover, both cell and gene therapy will only work if efficient methods are developed to deliver the cells or the genes into each (or at least most of) muscle cell in the body. Many scientists working with gene therapy are using viruses, modified in the lab and termed viral vectors, to transport this genetic information, since viruses have evolved to deposit their own genetic code into cells.

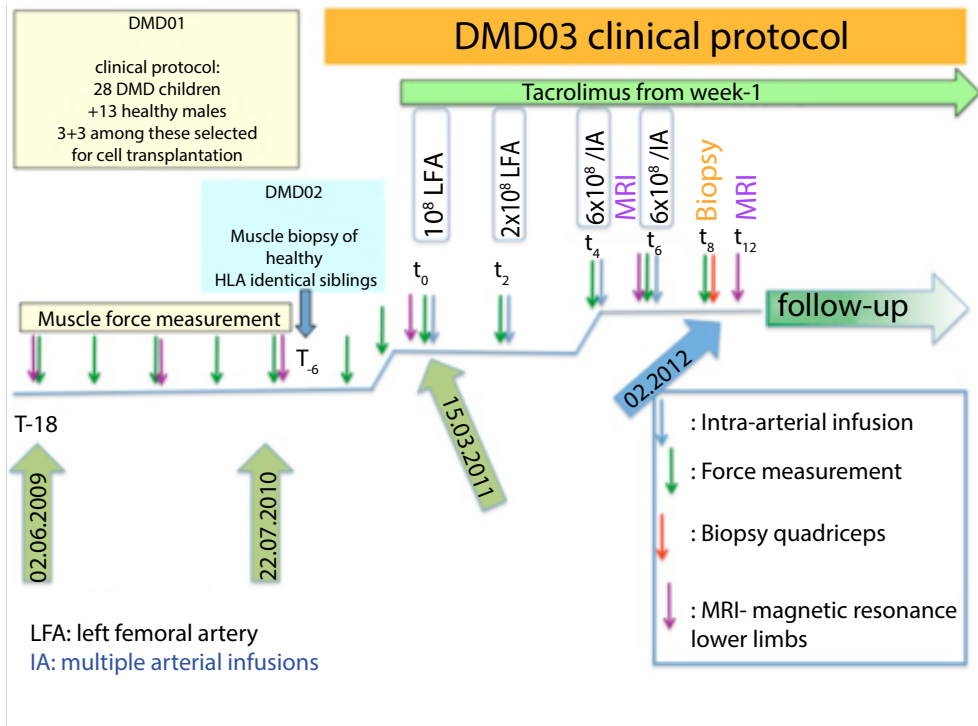
My research activity has intensively focused on development of cell therapy techniques based on muscle stem cells with the aim to coax muscle cells into producing dystrophin protein. Ideally, an eligible stem cell suitable for gene therapy should be: i) easily obtainable from accessible anatomical sites, ii) expandable in vitro to

the large number of cells required for systemic treatment (10^9 or more), iii) easily transducible with viral vectors, iv) able to reach skeletal muscle through a systemic route and, finally v) able to differentiate into skeletal muscle cells *in vivo* while maintaining a self-renewal ability. Since their discovery in the sixties, satellite cells have represented the elected cell type retaining the status of “muscle stem cells” and a major hope for cell-based therapy of muscular dystrophy [4, 5]. However, soon it became clear that some of their biological features limit their potential use for the treatment of primary myopathies. In fact satellite cells lack the ability to cross the muscle endothelium when delivered systemically and must be injected intramuscularly every 2 mm³ of all, or at least of life essential, muscles of the patients, since this is the maximal distance they apparently can migrate from the site of injection [6]. This feature alone makes their use in cell therapy protocols extremely difficult, at least with current technology. A second problem is represented by the reduced proliferation potency of satellite cells from dystrophic patients and also by the recent observation that *in vitro* expansion reduces their *in vivo* differentiation potency [7].

A large body of evidence demonstrated the existence of other myogenic cell types beside the satellite cells [8-12]. Among them, we identified “mesoangioblasts”, as the *in vitro* expandable counterpart of a subtype of pericytes, the cells that wrap capillaries and small vessels. Mesoangioblasts appear as a common progenitor for extravascular and vascular mesodermal derivatives [13]. Mesoangioblasts (MABS) are able to differentiate into different types of mesoderm, including skeletal muscle [14]. Freshly isolated mesangioblasts express some of the proteins that leukocytes use to adhere to and cross the endothelium, and this makes them capable of diffusing into the interstitium of skeletal muscle when delivered intra-arterially. This is a striking distinct advantage over resident satellite cells that cannot do the same. When MABS were delivered intra-arterially to muscles of dystrophic mice and dogs they resulted in a significant functional amelioration [15-16]. Human adult MABS, isolated and expanded *in vitro* from muscle biopsies, were shown to correspond to alkaline phosphatase expressing pericytes [17]. When transplanted into dystrophic immune-incompetent mice, they give rise to new fibers expressing human dystrophin. Moreover, these cells differ from other mesoderm stem/progenitor cells because their method of isolation (explant rather than proteolytic digestion) and their myogenic differentiation potency which is higher than any other cell in the body, beside resident satellite cells. We performed transplantation of normal canine MABS, obtaining promising results in the Golden Retriever dystrophic dog, the most reliable animal model that shows a form of dystrophy very similar to and even more severe than human DMD [16]. Collectively, these data formally suggest that these cells fulfill many of the criteria for a successful cell therapy protocol in muscular dystrophy.

Based on these results, and after careful studies of toxicology and biodistribution, a one-year preliminary study (involving 28 DMD patients) was conducted to validate outcome measures [18]. Starting on March 2011, three out of these patients

• **Figure 2.** A simplified scheme of the clinical protocol



(with an HLA-identical donor) underwent successive intra-arterial transplantations at escalating doses of cells, under a continuous regime of immune suppression. Two more patients have been treated the following year. The study was a mono-centre, prospective, non-randomized, clinical phase I/IIa study of cell therapy with HLA-matched donor human mesoangioblasts in DMD patients under a regime of immune suppression. A scheme of the treatment is shown in • **Figure 2.**

The inclusions criteria required having obtained informed consensus, having completed the observational study and availability of an HLA identical donor. Cells were expanded under clinical grade conditions to about two billion cells and frozen as intermediate medicinal product. One week before the injection, cells were thawed and expanded till the injection. Each patient was subjected to four consecutive intra-arterial infusions (at two months interval) of donor mesoangioblasts at escalating doses in the femoral and (in the first three patients), also in the subclavian arteries. The first endpoint of the trial was safety. However we also monitored the effect of the therapy by a number of standardized tests, such as the six-minute walk test (the child has to walk for six minutes and the number of meters completed is a quite precise prediction of his motor function: when the patient becomes unable

to cover more than 300 meters, he will most likely lose the ability to walk within about six months). Furthermore, every six months patients were subjected to MRI of the lower limbs, whose results correlate well with the progression of the disease.

At date, the five patients are alive and well indicating safety of the treatment. Results are under analysis and will be published at a later time.

Overall clinical efficacy appears still to be reached and new strategies are being devised to this aim. Future trials could very much benefit from the ongoing experimental studies such as genetic correction of autologous cells corrected with new generations of viral vectors and new generations of embryonic or reprogrammed stem cells.

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Stem cell therapy for Parkinson's disease

IBSA Foundation scientific editorial staff (summary of Prof. Anders Björklund's lecture, Wallenberg Neuroscience Center, Lund University, Lund, Sweden)

Parkinson's Disease (PD) is a chronic neurodegenerative disease that develops over many years, leading to impairments of movement and deficits in mental functioning. Parkinson's is characterized by a gradual loss in the ability of the patient to control muscle movement. Initially, PD manifests in the form of tremors in one or both hands. As the disease progresses, patients lose total postural control and are often confined to a wheelchair. It affects around 7-10 million of people worldwide. There is no cure for Parkinson's, but a combination of drugs and physical therapy can effectively relieve the symptoms in many patients. In particular, the main clinical treatment for PD is dopamine replacement therapy using l-dihydroxyphenylalanine (l-DOPA) and/or dopamine receptor agonists [1, 2]. Although pharmacotherapy can improve the symptoms during the initial stage of PD, its efficacy is gradually lost during long-term treatment, and the on-off phenomenon, wearing-off phenomenon and drug-induced dyskinesia occur in later stages. In addition, this therapy cannot delay the progression of the loss of DA neurons, and also cannot recover the lost DA neurons [3, 4].

The leading cause of PD is a progressive loss of neuronal cells producing dopamine – a chemical that allows messages to be sent to the parts of the brain that control movement – which are located in a part of the brain called *substantia nigra*. Parkinson's is also linked to formation of clumps of a protein called alpha-synuclein in the brain. The abnormal protein clumps are called Lewy bodies. As dopamine nerve cells die, Parkinson's patients develop tremors and rigidity, and their movements slow down. They might also lose their sense of smell or suffer from sleep disorders, depression, constipation and sometimes dementia in the later stages of the disease. The underlying reason for the dopamine-producing neurons cell death is still largely unknown. In about 1 in 10 cases, it is caused by an inherited genetic problem that affects production of the alpha-synuclein protein. What causes the remaining 90 per cent of

cases is not clear. It mainly affects people over 40 but can appear in younger people. Men are more at risk than women. Some research has made a link with pesticides, while smoking and coffee appear to reduce the risk of getting the disease, though it is not known why.

Although the causes of Parkinson's disease are unknown, it is clearly known which cells and areas of the brain are involved. Thereby, one of the most promising therapeutic strategies is the use of stem cells to grow dopamine-producing nerve cells in the laboratory to replace the lost nerve cells with healthy new ones. Stem cells based strategies to replace neurons could be persuaded by using several different approaches. One is to dissect the precursors of dopamine-producing neurons from aborted human fetuses, and then graft them into the brains of Parkinson's patients. Upon transplantation, these cells begin to integrate themselves into the existing circuitry, forming connections with other cells. Cell replacement studies started in the eighties, when Prof. Björklund's group transplanted dopamine-producing neurons from aborted fetuses into the patients' brains [5]. This was the first time dopamine-producing tissue had been transplanted into the human brain and it provided proof-of-principle for the cell replacement strategy in this disorder. The grafted dopaminergic neurons can reinnervate the denervated striatum, restore regulated dopamine (DA) release and movement-related frontal cortical activation, and give rise to significant symptomatic relief [6]. So far, more than 400 advanced PD patients have been treated as part of open-label clinical trials [7, 8]. Some grafted PD patients have shown striking improvements in their symptoms [9]. In the most successful cases, patients have been able to withdraw L-dopa treatment after transplantation and resume an independent life [10, 11]. Overall, the clinical trials with fetal mesencephalic grafts in PD patients have provided proof-of-principle that cell replacement can restore function in the parkinsonian brain. However, there are several problems linked to the use of fetal tissue: 1) lack of sufficient amounts of tissue for transplantation in a large number of patients, 2) variability of functional outcome with some patients showing major improvement and others modest if any clinical benefit, and 3) occurrence of troublesome dyskinesias in a significant proportion of patients after transplantation [7, 8, 12].

Barker reported a summary of the data obtained from four different trials involving sixty-five patients, highlighting that, albeit with an high variability, 27 out of 65 patients showed an improvement higher than 40%. Several trials are still on going, providing convincing indications that dopaminergic cell transplantation can offer very long-term symptomatic relief in patients with Parkinson disease and supporting for future clinical trials using fetal or stem cell therapies [13].

Prof. Björklund's laboratory is working on the possibility to make the process of embryonic stem cell differentiation as efficient as possible, and is further exploring how to reprogram fibroblasts – a type of connective tissue cell found in skin – directly into dopamine-producing neurons. The aim is to develop clinical trials involving patients at very early stages of the disease, in order to get a big impact on the PD

patients. We seek to develop techniques for isolation and identification of neural progenitor cells with the capacity to generate fully functional dopamine neurons after transplantation to the Parkinsonian brain. We use cell sorting and cell engineering techniques to generate dopamine neuron precursor cells specifically for transplantation purposes, and explore techniques for insertion of genes into stem cells to direct the development of stem cells into transplantable midbrain dopamine neurons.

In a second line of research, we plan to explore rodent models of Parkinson's disease to study the mechanisms underlying development of L-DOPA-induced dyskinesias, and use recombinant viral vectors as tools to model Parkinson-like neurodegeneration in rodents, and for delivery of putative therapeutic factors as the GDNF and its close relative neurturin (Nurr-1) to the brain. Indeed, glial cell line-derived neurotrophic factor (GDNF) is a potent survival factor for injured nigrostriatal dopamine (DA) neurons and is currently being evaluated as a potential treatment for Parkinson's disease (PD) [14]. Interestingly, by using recombinant lentiviral (rLV) vector delivery of GDNF to the striatum or *substantia nigra* (SN) in the rat, we found that GDNF initially induced an increase in DA turnover (1-6 weeks), accompanied by significant contralateral turning in response to amphetamine, suggesting an enhancement of the DA system on the injected side. Furthermore, we observed a selective down-regulation of tyrosine hydroxylase (TH) protein throughout the end of the experiment (24 weeks). The magnitude of TH down-regulation was related to the level of GDNF expression [15].

A clinically competitive cell therapy has to provide advantages over current, rather effective treatments for alleviation of motor symptoms in PD patients. Cell-based approaches should give rise to long-lasting, major improvements of mobility and suppression of dyskinesias without the need for further therapeutic interventions. Therefore, it is necessary to develop alternative efficient methods to delay the onset or progress of Parkinson's disease.

A major hope is the generation of midbrain DA neurons from pluripotent stem cells, such as ESCs and/or iPSCs, which could offer an alternative source of new cells for Parkinson's patients. iPS can be generated in large numbers and then reprogrammed into dopamine-producing neurons for transplantation. Furthermore, unlike embryonic stem cells, iPS cells are avoid from tumorigenic side effects and might therefore represent a safer tool for transplantation studies. Typically, it takes about 50 days to generate dopamine-producing neurons in this way. We now know, however, that the cells can be transplanted at an earlier stage and that they continue to mature to form mature dopamine-producing neurons after being grafted into the brain. An alternative approach is based on generation of dopamine-producing neurons from human embryonic stem cells, which have been shown to significantly improve movement deficits when grafted into the brains of rats with symptoms resembling Parkinson's. One problem with this approach is that the grafts can become contaminated with dividing cells, and consequently, some of the animals that receive grafts go on to develop tu-

mours. One of the immediate challenges is, therefore, to make sure the cells are safe and totally free of tumour-forming cells.

In conclusion, while during the past two decades, there has been rapid and remarkable advancements in PD cell therapy research, stem cell therapies for Parkinson's disease are not yet ready for use in patients. Much work still needs to be done before clinical trials can go ahead. The main challenges for scientists are: a. to identify the type of cells that has the most potential for research and new treatments; b. to find out how to grow neurons in sufficient quantities and at high enough safety standards to treat patients; c. to establish exactly how and where to transplant the cells so that they work properly in the brain without causing side effects. The scientists hope early clinical trials may be able to start in 2014 or 2015. A European Consortium called "TRANSEURO" has been generated, directed by Prof. Roger Barker in Cambridge. It has the principal objective to develop an efficacious and safe treatment methodology for Parkinson's disease suffering patients using fetal cell based treatments. The consortium has gathered international experts including leading clinicians, scientists, industrial partners, ethicists and patients' representatives who have joined forces in a new round of experimental work and cell therapy trials in Parkinson's disease.

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Stem cell therapy: the italian regulatory view

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The term “stem cell based products (SCBP)” refers to products containing or deriving from stem cells, intended to be administered to a patient [1]. According to the European legislation, advanced therapy products – namely gene therapy, somatic cell therapy and tissue engineered products - are classified as medicines: Advanced Therapy Medicinal Products (ATMPs). Consequently, their production and quality control must comply with the rules established for medicines.

Translating basic stem cell research into routine therapies is a complex multi-step process and it entails the challenge to manage the expected therapeutic benefits with the potential risks while complying with the existing regulations and guidelines. Importantly, preparation of a cell- and tissue-based therapy for clinical and commercial use strictly requires consideration of several regulatory issues related to the safety, efficacy, and quality of SCBPs. In a first phase it is essential the safety testing, including assays for potential microbial, fungal, endotoxin, mycoplasma, and viral contamination; karyotype testing; and enrichment for the required cell population. Once safety has been established, the product must pass *in vitro* functional assays designed to act as surrogate measures for clinical effectiveness [2]. Then, the potency assays must be fully validated to meet regulatory requirements, including appropriate standards and controls. The final product has to be made to a certain set of specifications, ensuring high quality. All animal models have inherent limitations, like, for example, the application of human cells in a xenogenic milieu [3]. Thereby, it is required the use of severely immuno-compromised small animals. While in the United States (US) and European Union (EU) regulations are in place, in many countries there are not well-defined regulatory frameworks for “stem cell based products (SCBP)”.

Since cells and tissues may also be used as transplants, sharp definitions have been set by the European Parliament and the Council in the Regulation (EC) No 1394/2007

in order to clearly distinguish between the two different types of products. This classification is particularly important since, differently from the transplants, both manufacturing and quality control of medicines must be performed in compliance with the European Good Manufacturing Practices (EU GMP). EU GMP defines requirements which have to be accomplished by all the medicinal products for human use, and are particularly strict for ATMPs. In fact, besides quality, safety and efficacy that have to be guaranteed for all the medicinal products, ATMPs show specific issues related to their biological origin (such as contamination and transmission of bacterial and/or viral infections, transmission of genetic diseases, transmission of transmissible diseases, induction of immunological responses or of tumours) which have to be carefully addressed. Consequently, in addition to the manufacturing process, also the premises where the ATMPs are manufactured and controlled must comply with the EU GMP requirements.

The Italian Medicines Agency (AIFA) is the national *authority* responsible for drugs regulation in Italy. It is a public body operating autonomously, transparently and according to cost-effectiveness criteria, under the direction of the Ministry of Health and under the vigilance of the Ministry of Health and the Ministry of Economy. It cooperates with the Regional Authorities, the National Institute of Health, Research Institutes, Patients' Associations, Health Professionals, Scientific Associations the Pharmaceutical Industry and the Distributors. The AIFA is the competent authority that, after the assessment of the dossier, releases the manufacturing authorization for the production of ATMPs and, by regular inspections, verifies that GMP and applicable rules are regularly and duly respected. The milestone in the Regulatory framework is the Directive 2001/83/EC, which was implemented in Italy with the Decreto legislativo 219/2006, the community code for regulating the medicines for human use at European level. This directive has been amended many times and in particular, the Directive 2009/120/EC concerns the advanced therapies (implemented in Italy by the Decreto Ministeriale 18/05/2010).

Commercial clinics worldwide are currently advertising so-called stem cell “therapies” for a host of diseases. Unfortunately, most of the clinics providing stem cell based interventions do not operate within the context of a formal clinical trial (CT), with extremely high risks of physical harm and financial exploitation to patients. Appropriate regulation of SCBP is essential to ensure public safety and trust while minimizing unnecessary barriers to product development, but presents numerous regulatory challenges.

Finally, the EU and Italian directives recognize that conventional nonclinical pharmacology and toxicological studies may be different for cell-based drugs, but should be strictly necessary for predicting response in humans. The EU regulation (1394/2007) on Advanced Therapy Medicinal Products (ATMPs) became effective from December 2008 and is binding in its entirety and directly applicable in all Member States of the European Parliament and of the council. ATMPs include gene therapy medicinal

products, somatic cell therapy products (as defined in Directive 2001/83/EC), and tissue engineered products [4]. Cells fall under this regulation, in case they have been subjected to substantial manipulation, resulting in a change of their biological characteristics, physiological functions or structural properties relevant for the intended therapeutic application. The Committee for Advanced Therapies (CAT) within European Medicines Agency (EMA) is responsible, among other tasks, for preparing a draft opinion on the quality, safety, and efficacy of ATMPs that follow the centralized marketing authorization (MA) procedure. Yet, no MA has been granted for any stem cell based medical product (SCBPM) in the EU (Committee for Advanced Therapies and CAT Lancet, 2010).

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Conclusions


Stem cells bring new hope for the treatment of many diseases, thanks to the exceptional potential to regenerate and to impact the development of disease-modifying treatments. Ever since their first discovery, researchers have dreamed of using stem cells to cure a huge range of disorders. As novel techniques make it now possible to correct genetic defects within stem cells, gene therapy strategies in stem cells are rapidly providing new types of disease-specific genetic interventions. A huge boost in the field came from the exciting discovery by Takahashi and Yamanaka that differentiated somatic cells can be reprogrammed into pluripotent cells (induced pluripotent stem (iPS) cells) by the forced but transient expression of a set of defined transcription factors, a discovery that proved worthy of a Nobel Prize and which holds the potential to revolutionize the regenerative medicine approaches.

Time day, researchers have different sources of stem cells, ranging from embryonic (ES) to iPS, to adult stem cells. While ES and iPS retain the pluripotency potential, their clinical use is hampered by their tendency to form teratomas. In contrast, adult stem cells are avoid of teratoma-formation issues. So, although ES and now iPS cells hold great potential, we do not yet know which stem cell type ultimately will provide the greatest promise for the future.

At the same time stem cells based approaches also raise one of the most difficult dilemmas in medical research: as with most technological advances, the key question is rather whether will society use the new power responsibly.

The “hype or hope” dilemma has become a paramount concern. Some good basic science studies have been done over recent years but equally importantly, clinicians need to continue communicate openly on the success and failures with this emerging modality under evidence-based conditions. The field of stem cell research is yet evolving science and much work still needs to be overcome before stem cell-based

cell replacement therapies become a reality. Researchers require scientific freedom to pursue research on all types – including ES, adult and iPS cells – in order to yield results for patients, and developing effective and safe mode of intervention on human being.



Stem cells bring new hope for the treatment of many diseases, thanks to the exceptional potential to regenerate and to impact the development of disease-modifying treatments. At the same time they raise one of the most difficult dilemmas in medical research: will society use the new power responsibly?

The purpose of the successful, highly inspiring IBSA Forum, that brought together prominent international experts, was foster collaboration between stem cell researchers, emphasize the light and shadow aspects of stem cell based therapy, and identify the yet unsolved open questions in the field, in order to stimulate creative solutions.