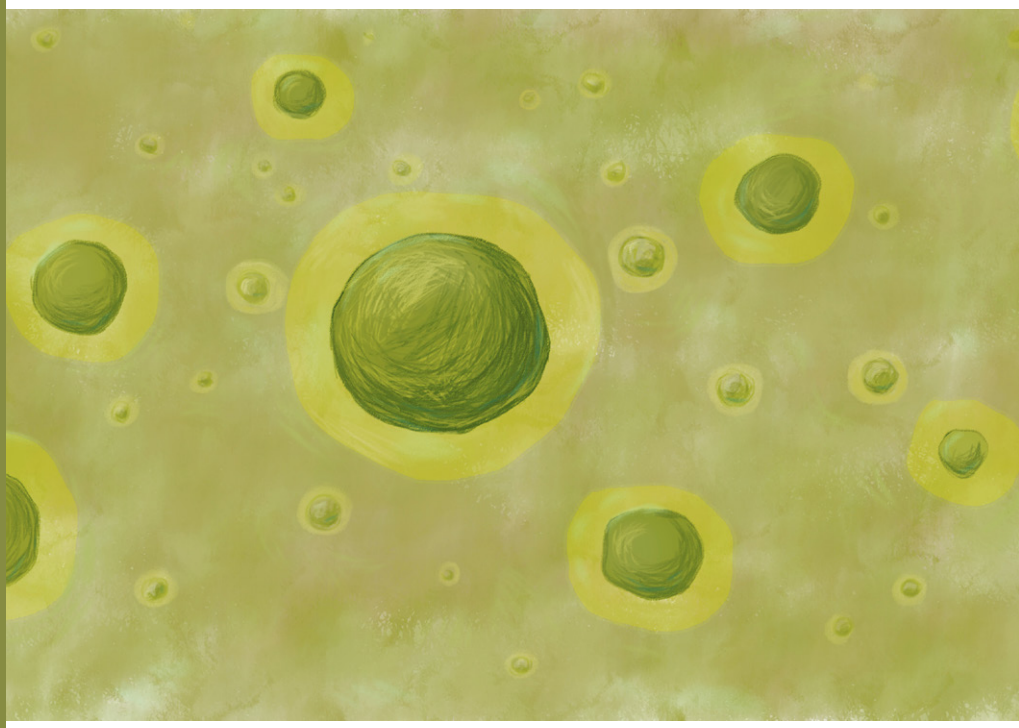
SCRIPTA VARIA 153

Edited by
Helen M. Blau
Elaine Fuchs

Stem Cells and Their Promise for Regenerative Medicine



Proceedings of a Workshop held at
Casina Pio IV, Vatican City, 5-6 May 2022

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Pontificiae Academiae Scientiarvm Scripta Varia 153

The Proceedings of the Workshop on

Stem Cells and Their Promise for Regenerative Medicine

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This work is dedicated to Nicole Le Douarin who, among the world's foremost development biologists, established the foundations of tissue stem cell biology with her fundamental discovery that precursor cells within the neural crest are multipotent. Through her active and integral roles in the Pontifical Academy of Sciences, Dr. Le Douarin has contributed greatly to the concept of this workshop and to educating the public on the importance of stem cells and their promise for regenerative medicine.

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ISBN: 978-88-266-0898-3

Let us thank the Lord for the progress that medical science has made, especially in recent times; new technologies have made it possible to prepare therapies that are of great benefit to the sick; research continues to make a valuable contribution to eliminating old and new pathologies; rehabilitation medicine has greatly expanded its expertise and skills. None of this, however, must make us forget the uniqueness of each patient, his or her dignity and frailties.

Message of His Holiness Pope Francis for the Thirtieth World Day of the Sick, February 11, 2022.

Contents

Concept Note

The discovery of stem cells was a remarkable breakthrough in biological research. Two major types of stem cells exist during the lifecycle of multicellular organisms: *embryonic stem cells*, resulting from the early divisions of the fertilized egg, characterized by their "pluripotency", i.e. the capacity to produce all the cell types found in the adult organism, and the *tissue-specific stem cells* present in the tissues and organs of the adult. Pluripotent stem cells can now be genetically engineered from any cell type in the body and used to model human diseases in culture. The tissue-specific stem cells play an important role in renewing the cells of the various organs throughout life. They are particularly active in tissues and organs in which the lifespan of the differentiated cells is short, like blood, skin and intestine, as well as skeletal muscle. Moreover, they are required to regenerate the tissues in response to damage. These adult stem cells are highly specialized and are dedicated to producing the tissue in which they reside. They are "unipotent" or "multipotent". These adult stem cells are the subject of this workshop. Scientists have learned to grow many of them in a dish into mini-versions of the mouse and human organs from which they derive. First established for skin and mammary gland, this so-called "organoid technology" has now been applied broadly to most tissues and organs of the body, opening new avenues for the study of development, physiology and disease and for personalized medicine. In the future, cultured mini-organs may replace organ transplants from donors and open the way to regenerative medicine.

ADVANCES IN iPSC TECHNOLOGY

Recent Progress in iPS Cell Research and Application

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Public Interest Incorporated Foundation, CiRA Foundation

Abstract

Induced pluripotent stem cells (iPSCs) can proliferate almost indefinitely and differentiate into multiple lineages, giving them wide medical applications. As a result, they are being used for new cell-based therapies, disease models, and drug development around the world. We are proceeding with an iPSC stock project in which clinical-grade iPSC clones are being established from healthy donors with homologous HLA haplotypes to lower the risk of transplant rejection. We started distributing the iPSC stock to organizations in Japan, and related clinical studies have begun for age-related macular degeneration (AMD), Parkinson's disease, corneal epithelial stem cell deficiency, cancer immunotherapy, and other diseases, giving expectation that iPSC-based regenerative medicine will be widely used in the future. However, donors with HLA homozygous are rare. Genome editing technology could be used as an alternative to reduce the transplant-rejection risk. Indeed, we reported HLA gene-edited iPSCs that could expand the range of patients who benefit from iPSC therapies faster than the homologous HLA haplotype strategy. This technology also has the potential to prevent or treat genetic diseases and gives great hope to patients. Finally, we are automating iPS cell production to reduce costs for autologous transplantation to deliver better regenerative medicine.

⋆⋆⋆

The genome is the code for our entire body. It decides the color of our hair, the size of our nose, and whether we look more like our mother or our father. It is now recognized that a single cell in one's body has all the genes required to produce an entire body, but this was not always the case. Scientific experiments had suggested that some genes were lost or permanently inactivated with development so that cells could only access the genes necessary for their function. However, in 1962, a landmark study by

John Gurdon would show otherwise. By inserting the nucleus of a frog's intestine cell into an enucleate egg, Gurdon successfully grew a normally developing tadpole, demonstrating that all the genetic code in a single intestinal cell was enough. [1]

Decades later, scientists would report embryonic stem (ES) cells. [2] These cells are extraordinary but also controversial. Extraordinary because they show incredible developmental potential, with the ability to differentiate into just about any cell type in the adult body. [2] They therefore have excited the scientific and medical community for the opportunities they provide to study human development and disease. The existence of ES cells has greatly advanced scientific understanding of how the brain, heart and other organs develop from an embryo. Controversial because they are procured from human embryos, a process that requires the destruction of the embryo. In response, national laws and international guidelines have restricted the use of ES cells compared with other human cell types.

Considering Gurdon's work, scientists hypothesized that any cell could be transformed into an ES cell-like state if the appropriate portions of the genome were activated and inactivated. The question had long remained, however, which parts? While it was certain that not all several billion base pairs in the human genome would need manipulation, narrowing down the precise combination was viewed a daunting task. And it was, but the answer turned out to be far simpler than anticipated.

We showed that by transiently activating just four genes (Yamanaka factors) in adult cells is sufficient to reprogram them into induced pluripotent stem (iPS) cells. [3] Adding to the amazement is that the same four genes successfully produced iPS cells from the adult cells of a number of species.4 The degree to which iPS cells behave as ES cells is remarkable, and years of study from laboratories around the world have confirmed that iPS cells and ES cells are functionally equivalent. From an ethical perspective, the impact of this research is profound, because we can acquire iPS cells from the very same blood samples one may donate to the Red Cross rather than an embryo. [5]

From a medical perspective, iPS cells have two attractive features. First, as mentioned above about ES cells, is their ability to differentiate into many cell types. Second, again like ES cells, is their ability to proliferate. The embryo is a rapidly growing structure, in which cells are replicating and developing constantly. iPS cells share this feature. Thus, from a relatively small sample of human blood, we can generate a massive number of iPS cells from which we can prepare an assortment of different cell types.

Regenerative medicine using iPS cells

Human iPS cells were first reported in 2007. Yet within seven years, cell products made from them were already being used as regenerative medicine in human trials, and many more clinical trials have since begun.

The first in-human transplantation was selected both because of remarkable scientific accomplishments and the relative safety of the treatment. Masayo Takahashi and her research team prepared retinal epithelial sheets prepared from iPS cells that they transplanted into one eye of a patient suffering from age-related macular degeneration (Fig. 1). [6]

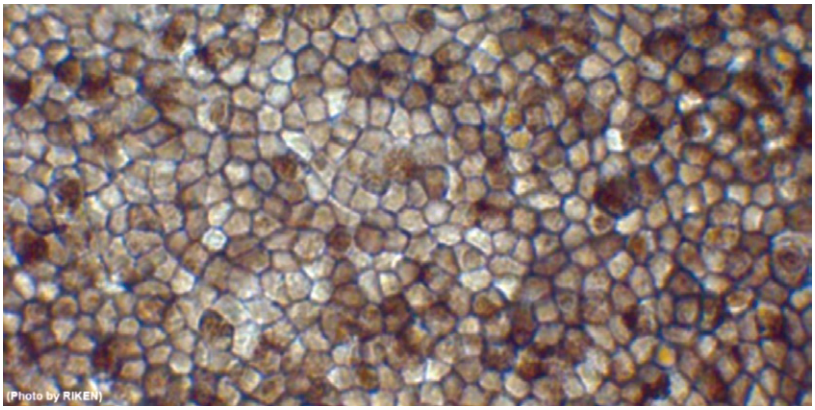

Figure 1. Images of the retinal epithelial sheets used in the first in-human iPS cell trial.

This condition can be treated symptomatically with drugs, but the transplantation was expected to retard the degeneration if not stop it completely. Indeed, several years later, the patient's vision has stabilized, and no serious complications have occurred.

This study is an example of autologous cell therapy, because the iPS cells were reprogrammed patient cells. However, it was obvious from this work that current reprogramming technology is inadequate for widescale iPS cell-based autologous therapies. The time and cost to reprogram the iPS cells, validate their safety and finally prepare the cell product for the therapy (in this case, retinal epithelial sheets) are too high. Particularly for degenerative diseases such as age-related macular degeneration, time is precious to the patient.

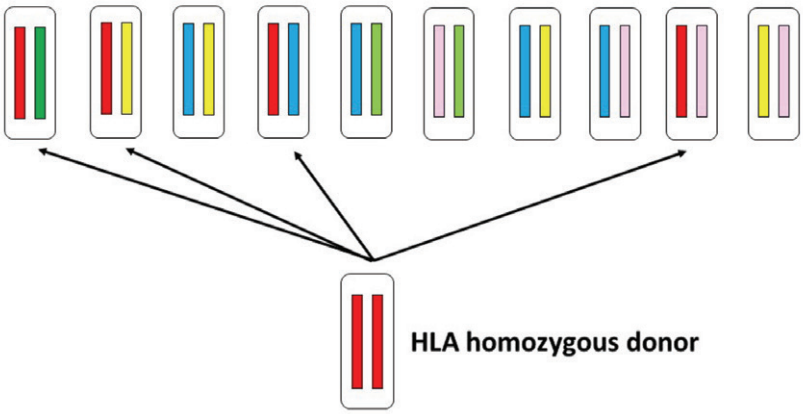

Figure 2. Whereas an HLA heterozygous donor must match both HLA haplotypes of the patient, an HLA homozygous donor need to match only one haplotype to minimize the risk of immune rejection.

In response, the manufacturing of clinical-grade iPS cell stocks has been investigated. However, like any transplantation, immune rejection is always a concern. Therefore, to prepare stocks that serves a wide population, in Japan, the CiRA Foundation has been cooperating with the Japanese Red Cross Society (JRCS) to recruit HLA homozygous donors. Compared to HLA heterozygous donors, cells from these donors have a higher probability of immune matching (Fig. 2).

This strategy does not completely eliminate the risk, which is why almost all iPS cell-based transplantation therapies will be accompanied by immune suppressants, but for now it is perceived to be the best choice when adding the considerations of time and cost. Along with the JRCS, the CiRA Foundation is working with cord blood banks to find more HLA homozygous donors.

Once finding these donors, the CiRA Foundation reprograms the blood cells into iPS cells while assuring homogeneously high quality. To date, the CiRA Foundation has manufactured and distributed the cell stock from HLA homozygous donors for 9 ongoing clinical trials, with many more planned (Fig. 3). Furthermore, the stock is being distributed to institutes across the world who aim to develop iPS cell-based therapies. Even the aforementioned age-related macular degeneration project has shifted to this allogeneic iPS cell strategy by using iPS cells provided by the CiRA Foundation.

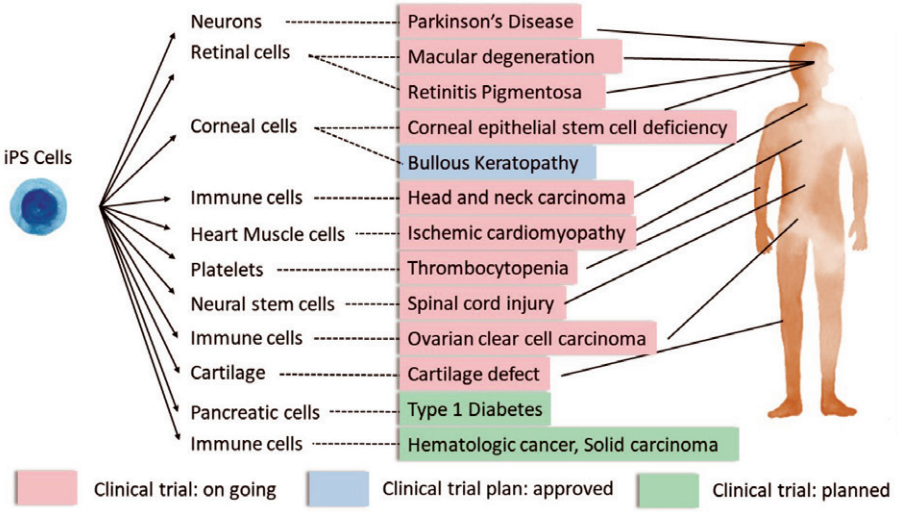

Figure 3. A list of all iPS cell-based regenerative medicine in Japan as of May 2022. All projects are using iPS cells prepared from the CiRA Foundation except head and neck carcinoma.

Degenerative diseases are not the only targets for iPS cell-based regenerative medicine. Koji Eto and his team have developed technology to produce a cell product as an alternative to current platelet transfusions. [7] Depending on the country, several million platelet transfusions are performed in a year. [8] All of these transfusions depend on donors, but with aging populations across the world, many nations are anticipating a serious donor shortage. Adding to the challenge is that platelets are particularly difficult to store, because they only have a shelf life of a few days. Therefore, a steady supply of donors is required in the current scheme. As a practical solution, Dr. Eto and colleagues are mimicking natural thrombopoiesis to produce platelets from iPS cells. [7] Following their iPS cell scheme, progenitor cells can be manufactured from the iPS cell stocks from HLA homozygous donors and stored for many months, and only when they are needed the platelets are produced from the stored progenitors. An in-human trial using these platelets is ongoing.

Another example is cancer. The latest generation of cancer therapy, following chemotherapy and radiotherapy, is immunotherapy, where a patient's immune cells are processed and transplanted. There have been many encouraging results from immunotherapy studies, but ultimately the

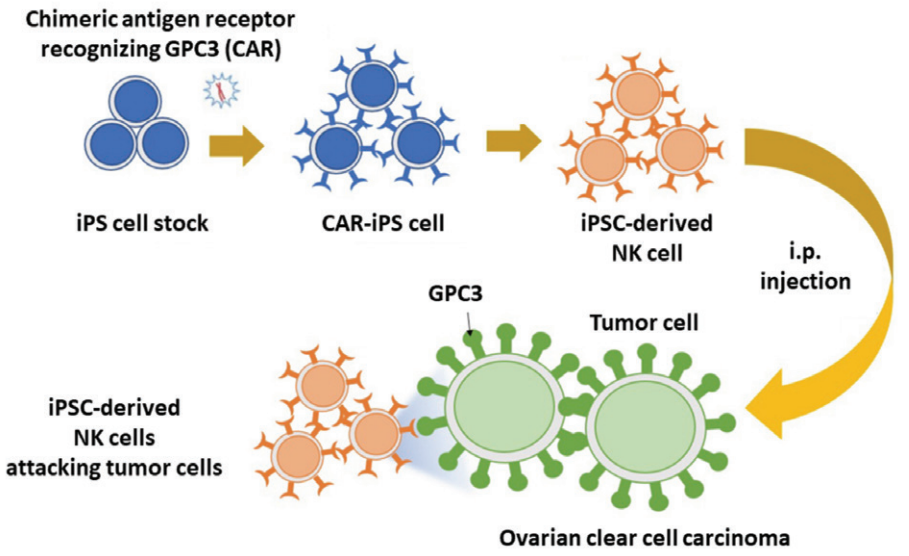

Figure 4. New immunotherapy that combines iPS cell and CAR technologies. Because iPS cells are easier to proliferate than other cell types, the CAR is genetically incorporated into them. In this example, iPS cells with CAR targeting GPC3 are then differentiated into anti-cancer cells (in this case, NK cells) for immunotherapy against ovarian clear cell carcinoma.

condition of the patient is a major factor in the outcome. As a solution, Dr. Shin Kaneko and his team are combining iPS cell and CAR (chimeric antigen receptor) technologies to manufacture anticancer immune cells for the next generation of immunotherapy.9 Here, CAR technology determines the effectiveness of the treatment by specifically attacking cancer cells but remaining unresponsive to healthy cells, while iPS cell technology assures the quality and abundance of the cells. Having adopting CAR that targets glypican-3 (GPC3), the research team is now scheduled to begin a therapy for ovarian clear cell carcinoma (Fig. 4).

Other iPS cells for clinical use

Currently, the iPS cell stock at the CiRA Foundation can match approximately 40% of the Japanese population, praiseworthy progress in just several years. However, to provide stock that matches the entire population and then the world's entire population, the task becomes exponentially more difficult, and finding donors for extremely rare HLA haplotypes is almost impossible.

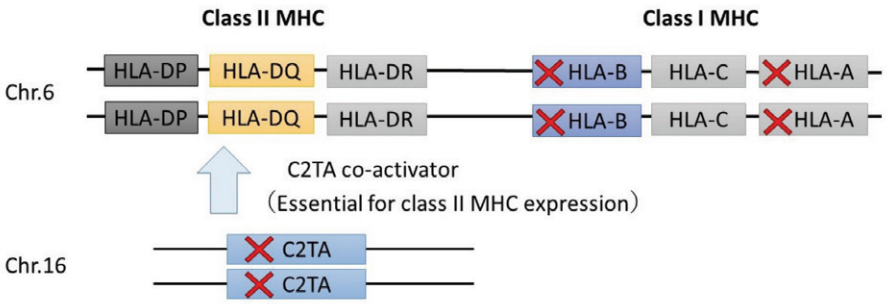

Figure 5. CRISPR-Cas gene editing of HLA to enhance the probability of donor-patient matching. HLA class 1 genes are deleted in chromosome 6, and C2TA is deleted in chromosome 16.

One solution may come in the form of gene editing technology, namely, CRISPR-Cas. Rather than trying to recruit HLA homozygous haplotype donors, some scientists are editing the HLA genes to increase the percentage of the population for which iPS cell products are available. Here, HLA-A and HLA-B are knocked out, but HLA-C is preserved so as to avoid an innate immune response. In addition to manipulating these HLA class I genes, we are knocking out C2TA trans co-activator to suppress all HLA class II genes10 (Fig. 5). If successful, it has been estimated that only 10 iPS cell lines made through this genome editing approach will sufficiently cover most of the world's population.

Finally, while autologous iPS cells are currently unfeasible, they are ideal, since one's own cells have the lowest risk of triggering an immune response. Cost (around US$ 400,000 per iPS cell line), however, prohibits this option. Automation is one way to reduce cost by at least a magnitude. While automation is a worthwhile consideration in any manufacturing, the infancy of iPS cell technology leaves many unknowns that require further study. Therefore, an equal or greater effort must be devoted to basic research before a full commitment to manufacturing. Nevertheless, the CiRA Foundation is pushing forward with the "myiPS" project, which aims to reduce the cost of one iPS cell line to just US$ 10,000. Optimistically, this service will open beginning 2025.

In summary then, to expand the number of patients who can benefit from iPS cells, three parallel tracts for generating clinical-grade iPS cells are being pursued. The first and most progressed is the reprogramming of cells from HLA homozygous donors. The second involves the gene editing

of HLA and related genes. The final is a system in which individuals can reprogram and store their own cells for future autologous treatments.

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Retinal Cell Therapy Using iPS Cells

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Vision Care Inc. & Kobe City Eye Hospital

Background

Fourteen years have passed since the publication of the human iPS cell paper in 2007 by Prof. Yamanaka's group. During that time, we conducted the first clinical study of iPS cells, that was autologous iPS cell-derived retinal pigment epithelial cell (iPSC-RPE) sheet transplantation, from 2013 to 2015 and the second clinical study of HLA-matched allogeneic iPSC-RPE suspension transplantation from 2017 to 2019. More than 10 different clinical studies using iPS cells in Japan, including a clinical trial for Parkinson's disease (dopamine cells), corneal epithelial cells, cardiac muscle sheets, spinal cord transplantation, and platelets has been approved. The fact that clinical applications are progressing at such a rapid pace is largely due to the environment and legal framework that is suitable for regenerative therapies, that is completely different from small molecule drugs.

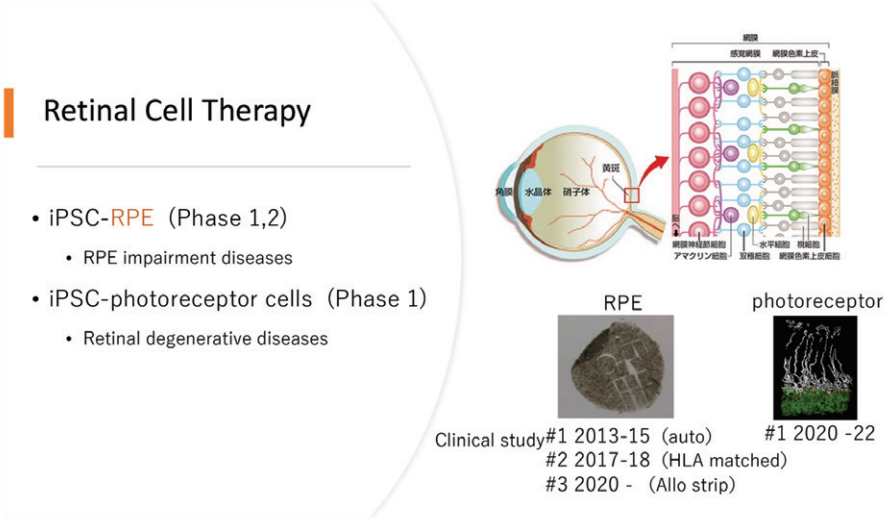

Figure 1.

The 1st Clinical Study – autologous iPSC-RPE sheet transplantation (1 case)

In 2012 we were the first in the world to submit a clinical research protocol using human iPS cells to the "Review Committee on Human Stem Cell Clinical Research". For autologous transplantation, it was necessary to be certain that (1) iPSCs of clinically usable quality could be generated from any case and (2) safe, mature iPSC-RPE could always be produced with a purity of almost 100% (effectively 99% or more = no more than 500 non-purpose cells mixed in the final product). Based on many years of experience in stem cell research, we thought that the special characteristics of RPE would be advantageous to make the cells absolutely safe nevertheless, given that stem cells are always a heterogeneous population even if they look the same and that cell culture is always subject to genetic mutation at each pass. Conversely, if the above two conditions can be achieved, RPEs can be used safely even if the slightest genetic mutation occurs, as far as RPEs with no reports of primary metastatic tumors are concerned. During the five years since the announcement of human iPS cells, we repeatedly verified the safety of the final product by modifying the differentiation method that had been established for human ES cells. Once we were confident of the safety of the final product, we applied for a protocol. In parallel with the scientific verification, we decided on the direction of the clinical study while keeping abreast of the direction and trends of the new regenerative medicine law that was being drafted. Finally, the cell risk was reduced to almost zero, and the cells went into clinical trials with only a few percent surgical risk, just as in ordinary retinal surgery.

Accumulation of information for risk

The reason why we can draw such a clear line of safety is because we performed a long period of stem cell research and also knew well about RPE cells' behavior in the eye through ophthalmic surgery. In other words, we knew the risks from basic research to clinical surgery, so that we could consider the risk matrix (significance and frequency of the risks) and apply the clinical study. The risks associated with RPE transplantation and the degree of efficacy in each case can be predicted to some extent based on our experience in treating age-related macular degeneration (AMD).

The RPE, which has been working almost irreplaceably in the eye for its entire life, has, as expected, survived in the same sheet shape even 5 years after transplantation, maintaining the photoreceptor cells only in the area where the transplanted sheet exists. Retinal photoreceptor cells will always degenerate without pigment epithelium, so it is clear that the

iPSC-RPE is still functioning in this case. Before the surgery, her visual acuity deteriorated although she got 13 times anti-VEGF injection. After the surgery her visual acuity is still stable.

We planned two cases for the first clinical study, but different from the first case, the patient's visual acuity in the second case had stabilized at 0.3 during the 10 months of cell preparation, so that the risk of surgery (visual acuity loss) was greater than in the first case, so we decided to stop RPE transplantation, although the safety was confirmed by intensive tumorigenicity tests.

Thus, only one case, but we showed the safe way to use the iPS cells, that many people thought dangerous to use for humans at that time.

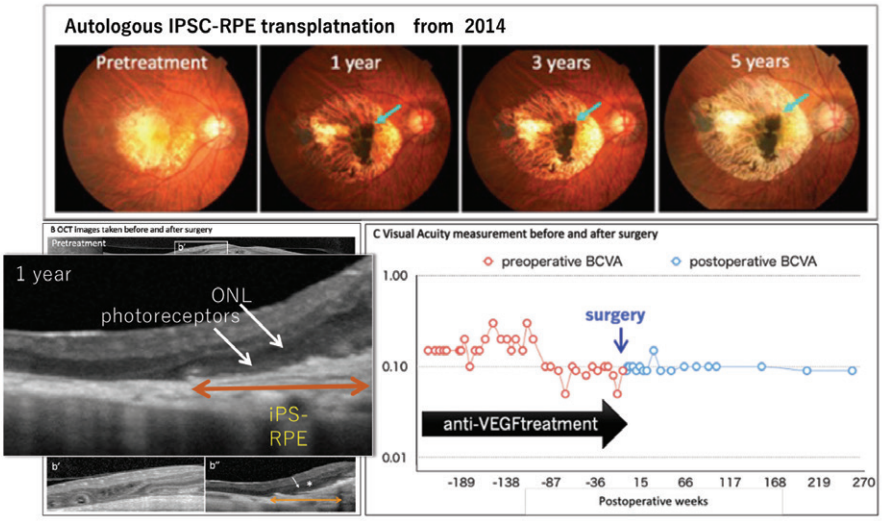

Figure 2. Postoperative course of the first case (rf. Mandai et al. N Engl J Med. 2017).

The 2nd Clinical Study – HLA-matched allogeneic iPSC-RPE suspension transplantation (5 cases)

The reason why we first started with autologous sheet transplants, which are technically difficult, was to do the scientifically best treatment for the first iPS application. Even though it is costly and time-consuming, autologous transplantation is the best treatment from the viewpoint of immunity. We think the autologous transplantation will eventually become an option, but for the time being, allogeneic transplantation, which can be

applied to many people by preparing several types of cells, is desired as the actual standard treatments.

CiRA in Kyoto University produced HLA6 homozygous iPS cells, which covered 17% of the Japanese population, so that we conducted clinical research on HLA-matched allogeneic transplants using this iPS cell line. The reason for using iPS cells in the development of a treatment for age-related macular degeneration, which had originally been conducted using ES cells, was because for the elder patients to use immunosuppressive drugs in the treatment of age-related macular degeneration, in which more than 70% of patients are over 70 years old, was not desirable. In fact, many of the side effects of retinal cell transplantation that have been reported in papers are caused by immunosuppressive drugs.[3]

The manufacturing method of RPE is the same, but in the second clinical study, the target was milder cases than the first clinical study and a suspension transplant was used. In those cases, RPE atrophy is often sparse and a large sheet is not necessary. It is less invasive and safer to inject the suspension through a small hole in the retina into the back of the retina (subretinal) to form a sheet within the eye to control the neovascularization.

Immune response – LGIR test

The primary endpoint of the second clinical study, as in the first study, was safety, but the key point was whether the rejection of HLA-matched allogeneic transplants could be suppressed by local treatment alone without systemic immune suppression. For this purpose, we created a test that can examine the patient's immune response in real time by examining the proliferation of mononuclear cells in mixed culture of patient peripheral blood mononuclear cells and transplanted RPE cells, which we call the LGIR (Lymph Graft Immune Reaction) test [4] (Figure 4). This test was very useful in clinical studies, and in the first case, we were able to confirm that the appearance of a small amount of subretinal fluid (a few μl) at 5 weeks after transplantation indicated the beginning of rejection, not the recurrence of the disease, which was suppressed by three injections of steroid outside the eye. In the other cases, there was no sign of rejection in this test or in the clinical findings during the one-year follow-up, and all five cases showed successful implantation of the transplanted RPE. In the case with the best control of the implantation site, polarized OCT showed that the transplanted cells were covering the neovascularization in a sheet-like pattern.[5]

In other cases, unlike in the animal model, it proved difficult to control the location of grafted suspension, and in all cases, epiretinal membrane

formation was observed due to backflow of cells from the injection site. In one of the cases, retinal edema appeared, and surgery was performed to remove the epiretinal membrane, a procedure that is frequently performed in ophthalmology, with no serious effects on visual function. These clinical experiences were immediately returned to the laboratory to improve the RPE formulation and surgical technique. We now reached to the RPE strip, that can be transplanted from a small hole in the retina and expand to form small sheets beneath the retina several weeks after the surgery. We think this is the safest and most effective formulation of RPE.

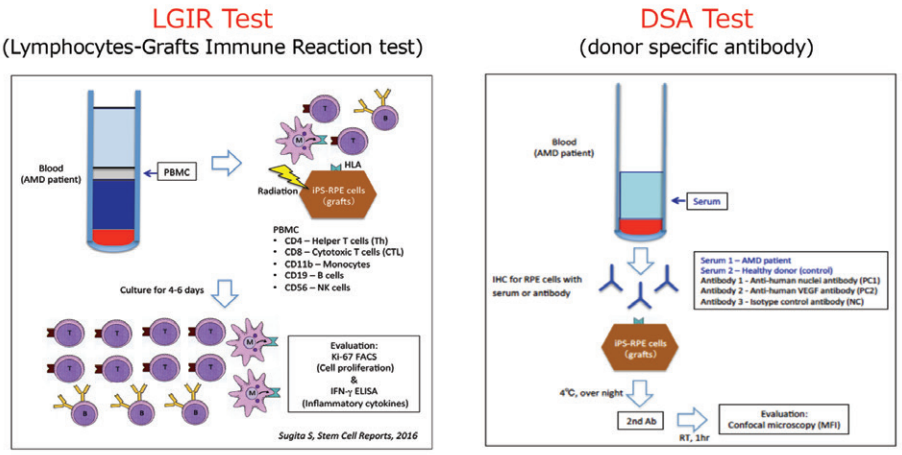

Figure 3. Real time immune reaction test.

Learning from Clinical Research – Effectiveness

We have learned a great deal from our experience with these six cases in total. There are good reasons for the RPE to be the first case of iPS application to humans. In most of the cell types, it is difficult to induce differentiation of pluripotent stem cells to functionally mature cells in a culture dish, but RPE is one of the few cells that can be differentiated to mature cells and transplanted with almost the same properties as in vivo. Furthermore, the inner layer of the eye cup during the development in the fetus becomes the neural retina and the outer layer becomes the RPE, and the neural retina and RPE have no cell adhesive apparatus but just contact with each other. Thus, monolayer of RPE cells with a basement mem-

brane is already a tissue, and the RPE sheet is a complete tissue graft. In other words, it is perfect tissue equivalent to the native RPE in the body.

Furthermore, the safety was obtained relatively easily because of the RPE that does not form tumors even with any gene mutations. These are the unique features of RPE that distinguish it from other tissues.

Thus, we proved the safety of autologous and allogeneic iPS-RPE. Now we move to the next step to evaluate the effectiveness of the RPE transplantation. For this, it is important to select the appropriate cases even in the same diseases while various conditions are referred to by a single disease name. The effect of the treatment is not determined by RPE cells that already have perfect function, but by patients' retinal environment, where the morphology and functions of cells change drastically depending on the microenvironment. That is the most different point of cell therapy compared to the current treatment. In regenerative medicine, the end products do not immediately make treatments like small molecule drugs.

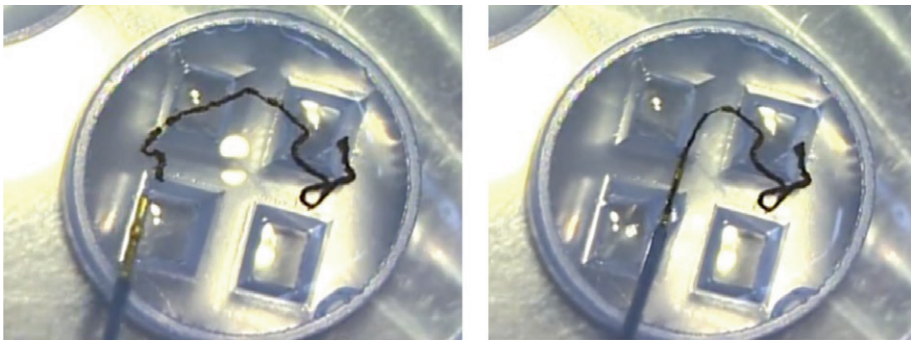

Figure 4. RPE strip (rf. Nishida et al. Sci Rep. 2021).

Retinal Organoids Transplantation – photoreceptor replacement

In 2011, Dr. Yoshiki Sasai's group invented a method called "organoids" to create a three-dimensional retina from ES or iPS cells. Since then, organoid research is flourishing for various parts of the body in the world. Again, in the case of photoreceptor cells, the world's first organoid transplantation was done in the field of retina.

Dr. Michiko Mandai in our group worked diligently to create the POC. It took about 7 years to prove 1) whether the grafted immature retinal organoid sheets would mature and become a photoreceptor cell, 2) whether

it would re-create synapses with the retina in the body, 3) whether it would function electrophysiologically, and 4) whether the photoreceptor degenerated model animals would be able to see the light after transplantation. After obtaining these POC in the animal, the Kobe City Eye Hospital performed the retinal sheet (photoreceptor) transplantation clinical study with two patients. The hospital reported the results at the Japanese Society of Clinical Ophthalmology meeting last month. In both cases, photoreceptor cells were successfully transplanted into the patient without any abnormal proliferation and survived for more than one year with some hint of functional recovery.

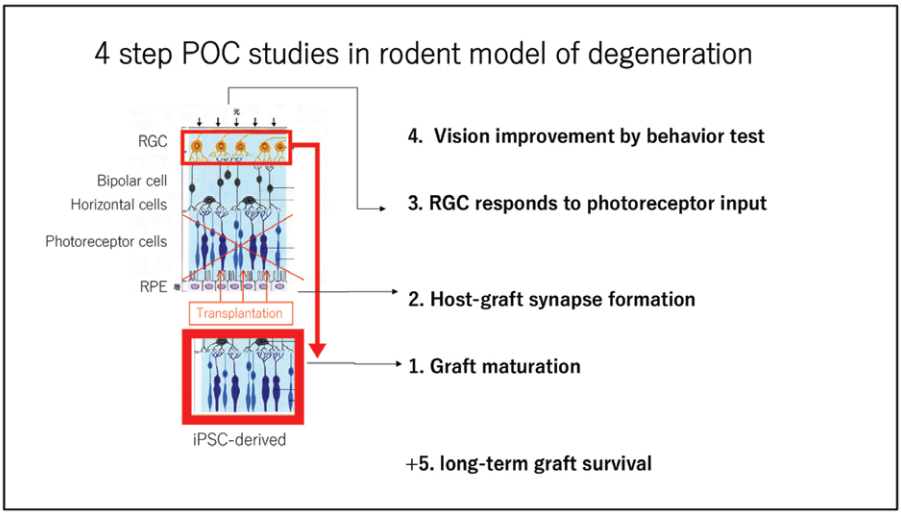

Figure 5. Photoreceptor transplantation POC in animal model.

In ophthalmology, microstructures such as OCT are observed to make decisions on pathological conditions and treatment. In replacement therapy in ophthalmology, anatomical effects (tissue reconstruction) are used as an indicator of effectiveness. We hope that we will be able to show the effect in the near future.

Conclusion

Surgical treatment has gone through a process of trial and error in its history, and at one point, through the ingenuity of many doctors, it was perfected. Having witnessed the development of cataract surgery these past

30 years, I think that regenerative medicine will trace the same course. Since safe and functional cells have been created, many doctors should be involved to accomplish the effective new treatment. A suitable path for regenerative medicine is necessary to deliver effective treatments to patients quickly and inexpensively.

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Pluripotent Stem Cell for Treating Parkinson's Disease

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Parkinson's disease was first described by James Parkinson, an apothecary and surgeon in London, more than 200 years ago. In his essay, reprinted recently,[1] he defined the disease as "shaking palsy" to stress the fact that patients show aspects of both aberrant movement (such as shaking/tremor) as well as lack or slowness of it. In honor of James Parkinson, the famous French neurologist Jean Martin Charcot was the first to refer to the disorder as Parkinson's disease,[2] and to describe in great scientific detail the specific motor as well as autonomic symptoms of the disease (Figure 1).

PD is the second most common neurodegenerative disorder affecting approximately 1 million patients in the US, and causes enormous health and financial burden, estimated at $52 billion of direct and indirect costs to society within the US alone.[3] Today, we understand that the key mo-

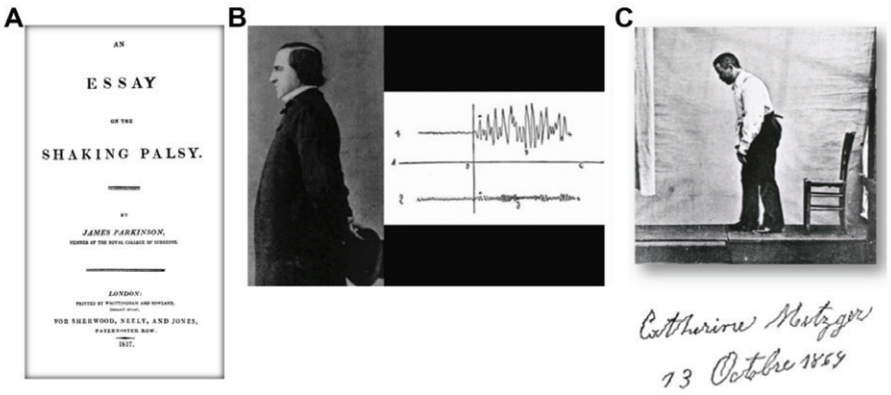

Figure 1. First description of Parkinson's disease in the scientific literature. A) Title page of James Parkinson's essay on Shaking Palsy, published in 1817. B) Depiction of Jean Martin Charcot at the Salpêtrière Hospital in Paris. Among the clinical features, he described "myographic curves" to distinguish tremor present in patients with multiple sclerosis (upper trace) versus spontaneous tremor in patients with Parkinson's disease (lower trace). C) Depiction of the characteristic posture and gate disturbance in a PD patient and illustration of micrography, one of the lesser-known symptoms of PD, which is the tendency of patients to write smaller and smaller.

tor symptoms of PD are caused by the loss of dopamine neurons in the midbrain. A healthy individual has about 300,000-400,000 midbrain dopamine neurons[4] and the loss of greater than 50% of those neurons is thought to trigger symptoms of Parkinson's disease and bring the patient to seek help from a neurologist. As the brain is estimated to comprise about 50-100 billion neurons, dopamine neurons represent a tiny fraction of total neurons. The rather discrete loss of dopamine neurons in a defined brain region makes PD an attractive target for regenerative medicine, as only a limited number of new neurons might suffice to significantly impact motor function in an individual patient. On the other hand, while motor symptoms are the classic feature of the disease, it is important to stress that PD affects many systems of the human body beyond those relevant to the movement disorder. Early symptoms that often precede movement-related symptoms include a loss of smell, sleep disturbances such as restless leg syndrome and chronic constipation. Some of the feared long-term complications include a progressive cognitive loss which can occur many years after onset of motor symptoms and can lead to PD-associated dementia.[5] It is important to note that both cognitive loss and peripheral disease symptoms are thought to be mostly independent of the dopamine dysfunction. Therefore, even a permanent "cure" of the dopamine-related movement disorder would likely not result in a true cure of the broader disease.

There is an increasing understanding of specific genetic factors that predispose an individual to PD. Those findings are based on studies of familial forms of the disease implicating more than 20 PD genes and on human genetic studies that define about 90 PD genetic risk loci in sporadic patients.[6] PD-related genes point to vulnerabilities in mitochondrial and lysosomal function that may underlie disease pathogenesis. Vulnerabilities related to energy demand and protein homeostasis may be particularly acute in midbrain dopamine neurons, given the remarkable size and complexity of their neurite arbors. It is estimated that a single human midbrain dopamine neuron in the substantia nigra has an axonal arbor with a combined length of about 4-5 meters and comprises 1-2.4 million synapses each.[4] The enormous size of dopamine neurons may also contribute to challenges in protein homeostasis, particularly for synaptic proteins of high abundance such as a-synuclein. The aggregation of a-syn into Lewy bodies is one of the key pathological hallmarks of the disease.[7]

Despite the considerable progress in unraveling genetic and biochemical pathways involved in PD pathogenesis, no disease-modifying therapy is available today that can halt or significantly slow down disease progression.

The currently approved therapies are symptomatic and include strategies to replace the neurotransmitter dopamine by supplying L-Dopa pharmacologically. L-Dopa is taken as an oral drug that enters the brain, where it gets converted into dopamine in the remaining dopamine neurons present in the brain. While highly effective at early stages of the disease, as the disease progresses, L-Dopa treatment becomes less and less effective and can trigger significant side effects such L-Dopa-induced abnormal movements (dyskinesia). The use of deep brain stimulation (DBS) represents another currently approved clinical treatment for PD patients that can improve several symptoms of the disease such as tremor or dyskinesia. However, DBS is not suitable for all patients and requires the implantation of hardware in the brain that needs to be serviced throughout life. Furthermore, the procedure can worsen speech-related or psychiatric symptoms in some of the patients. An experimental treatment approach that is not approved yet is gene therapy to either protect the remaining dopamine neurons or to boost their function via delivery of neurotrophic factors or genes that enhance the biochemical function respectively. Finally, there is the long-standing goal of replacing the dopamine neurons degenerated in PD via cell therapy, the main topic of this current paper.

The possibility of dopamine neuron replacement

While a plethora of cell types have been proposed in the past as a potential source of dopamine-producing neurons (ranging from adrenal medulla cells to peripheral neurons coaxed to produce dopamine), none of those non-midbrain cells has shown convincing evidence of long-term survival and dopamine function. In contrast, the use of human fetal midbrain dopamine neurons has offered valuable insights into the potential of achieving dopamine neuron replacement in PD patients.

This approach started in the late 1980s and has been performed since on more than 300 patients worldwide. The results showed convincingly that fetal dopamine neurons can survive when injected into the striatum of PD patients as illustrated by both histological data as well as by functional PET imaging using a radioactive F-Dopa tracer (Figure 2). However, two placebo controlled clinical trials,[10,11] assessing the efficacy of the approach at 12 months post-surgery in a larger set of patients, failed in their primary end point and showed some evidence of efficacy only in younger patients (< 60 years of age).[10] Furthermore, a subset of the patients showed unexpected side effects referred to as graft-induced dyskinesia that – combined with the modest evidence of efficacy – halted most fetal dopamine neuron

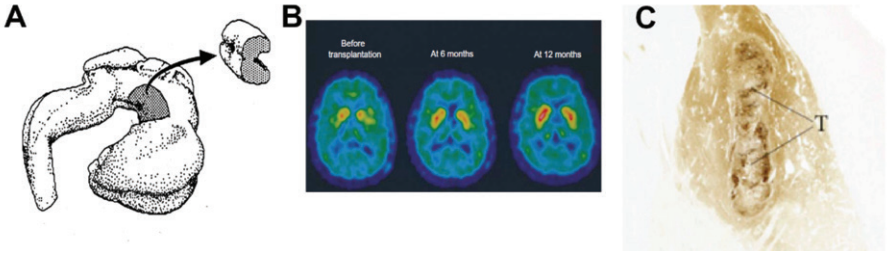

Figure 2. Demonstration of long-term survival of midbrain dopamine neurons following fetal tissue transplantation. A) Midbrain dopamine neurons were isolated from fetal tissue of 6.5-10 weeks post conception. B) PET imaging of PD patient brain after receiving fetal dopamine neurons at 6 months and 12 months after transplantation. Increase in the red signal suggests increased update of F-Dopa due to the surviving dopamine neurons. Image is modified from.8 C) Autopsy of a brain 24 years after receiving fetal dopamine neurons showing in brown expression of tyrosine-hydroxylase (TH), the rate-limiting enzyme in the production of dopamine (from the publication by Li et al.).9 TH staining suggests graft survival and the presence of dopaminergic fibers more than two decades old in a PD patient brain.

grafting trials. Nevertheless, some of the treated patients were followed for more extended time periods in studies that suggest that the optimal effects of the treatment may develop only at 2-3 years after transplantation.[12] In fact, a subset of patients was reported to do particularly well after being followed for 10 years and beyond, with several patients off any L-Dopa medication,[13] which is highly unusual for patients suffering from a chronic progressive disease such as PD. This suggested that the cell replacement approach can work at least in a subset of PD patients. However, it remained unclear which factors predict success regarding patient selection or graft preparation. Furthermore, the use of fetal tissue raised considerable logistical and ethical issues that have prevented the development of this approach for routine use in larger sets of patients.

To find a renewable source of midbrain dopamine neurons, several potential stem cell types were pursued starting around the mid-1990s. On a personal note, I got involved in this endeavor after working with Dr. Christian Spenger, a young neurosurgeon at the University of Bern, to drive the first human clinical study in Switzerland using human fetal dopamine neurons in 1995. This experience set me on a journey to explore the potential of neural stem cells as a possible source of dopamine neurons. Neural stem cells seemed to be an obvious choice as they are, unlike embryonic stem cells, already committed to a neural fate, and the remaining challenge is "only" generating the correct neuron subtype. Indeed, we were able

to demonstrate the production of dopamine neurons from dividing midbrain rat neural precursors and their successful engraftment in a rat model of PD.[14] However, the extent of in vitro expansion via proliferation of the midbrain stem/precursor compartment was relatively modest and did not enable a truly unlimited source of dopamine neurons. Therefore, I got involved in a parallel effort using mouse embryonic stem cells (ESCs) that provided the first true proof-of-concept for generating potentially unlimited numbers of midbrain dopamine neurons, and one of the very first examples of generating a specific neuron subtype from a pluripotent stem cell source.[15] The use of pluripotent stem cells such as mouse ESCs resolved two major challenges, first, the ability to generate near unlimited numbers of differentiated cell types because ESCs (unlike midbrain stem cells) can be extensively expanded without losing fate potential. Second, the differentiation of ESCs offered access to the earliest stages of neural development. This turned out to be critical, as the key factors determining ventral midbrain identity such as FGF8 and SHH act during a very early and a very narrow temporal window that precedes the developmental stage when midbrain neural stem or precursor cells are isolated. Accordingly, the fate of the ESC-derived neural lineages can be readily directed to derive neurons specific to any brain region, while neural stem cells are largely restricted regarding their regional identity, even though they are still capable of committing to neuronal versus glial fate at this stage.

Pluripotent stem cells as a renewable source of dopamine neurons

The rapid progress in directing the differentiation of mouse ESCs was illustrated in multiple follow-up papers in the early 2000s that applied the same technology to many ESC lines, including ESCs derived via nuclear transfer[16,17] or by performing more and more in-depth studies *in vivo* to demonstrate rescue of Parkinsonian symptoms in various models of PD.[18] Given such rapid progress, there was the expectation that within just a few years, similar results would be obtained using human pluripotent stem cells (hPSCs) that became available upon the isolation of human ESCs19 and subsequently human iPSCs, following the seminal work of Shinya Yamanaka.[20,21] However, it would take more than 10 years to develop differentiation protocols in hPSCs to generate midbrain dopamine neurons capable of efficient engraftment in mouse, rat, and primate models of PD.[22] In a series of papers between 2009 to 2011, we were able to greatly simplify and accelerate the differentiation of human ESC or iPSCs into neural cells using only two small molecule inhibitors to drive neural

specification, a widely used protocol termed dual-SMAD inhibition.[23] We had to combine dual-SMAD inhibition with the insight that dopamine neurons are derived from FOXA2+ floor plate precursors24 and the finding that activation of WNT signaling is critical for driving midbrain specification and neurogenic conversion of floor plate precursors to ultimately enable this breakthrough in human dopamine neuron generation.[22] We and many other groups went on to show that dopamine neurons derived via our floor plate protocol can restore motor deficits across various PD models, including drug-induced rotation behavior or measures of aberrant movement initiation. We further demonstrated how dopamine neurons achieve functional benefit in the host brain using an optogenetic switch in the grafted neurons. This switch allowed us to selectively turn off activity in grafted dopamine neurons without impacting neurons in the host brain. These studies showed that the functional benefit from the grafted cells is completely dependent on their neuronal activity and activity-dependent dopamine release. We further demonstrated that grafted neurons modulate synaptic input onto the striatum in a manner highly reminiscent of healthy endogenous midbrain dopamine neurons.[25] Given those promising proof-of-concept preclinical and mechanistic studies, there was a great impetus to translate those findings from animal studies towards actual clinical trials in human PD patients.

From proof-of-concept to clinical trial

It would take nearly another 10 years to move from the proof-of-concept study[22] to a first-in-human clinical trial. A key step on this long journey includes the further optimization of the differentiation protocol under conditions that are suitable for clinical translation. In addition to using clinical grade reagents and cell lines, we further optimized the differentiation protocol to achieve better "on target" and "off target" performance.26 For "on target" performance we tracked makers such as engrailed-1, a transcription factor expressed in most dopamine neurons in vivo, but that was inconsistently expressed in human PSC-derived dopamine neurons. In addition, we optimized "off target" performance by avoiding lineages that were present in fetal preparations such as serotonergic neurons, implicated as the potential culprit in triggering graft-induced dyskinesia.[27] Other "off target" cell types that we can largely avoid using our clinical grade protocol include perivascular fibroblast and choroid plexus cell contaminants that have been reported in other dopamine neuron differentiation protocols as potential "off target" cell types.

After optimizing the differentiation protocol, the next step was the production of large batches of dopamine neurons to develop an "off-the-shelf" product. This means that we needed to produce very large numbers of dopamine neurons and define conditions suitable for cryopreservation to be ready for 'off-the-shelf' use in preclinical studies and for the actual clinical trial. In fact, we produced nearly 10 billion cells under clinical grade conditions in a series of 4 replicate differentiation batches. Each batch had to pass a set of detailed release criteria that determined the purity and viability of the dopamine neuron preparations. For example, we showed that nearly all the final cells express the marker FOXA2 (Figure 3). FOXA2 is expressed in the dopamine lineage both at the precursors stage and in postmitotic cells. Furthermore, we demonstrated the lack of any remaining pluripotent cells in the final preparation.

Beyond qualifying the in vitro product, it was essential to test the safety and performance of those cells in vivo. We performed detailed good laboratory practice (GLP)-grade studies on tumorigenicity, biodistribution and toxicity across hundreds of mice at an outside, independent contract research organization (CRO). This is required by the regulatory agencies as to assure that results are reported in an unbiased manner by an organization that has no personal or financial gain from the outcome of the study.

In addition, we performed efficacy studies in a cohort of 48 Parkinsonian rats to show that grafting the cryopreserved dopamine neurons fully restores some of the motor symptoms in this model such as drug-induced rotation behavior (Figure 4). Finally, we performed targeting studies in a

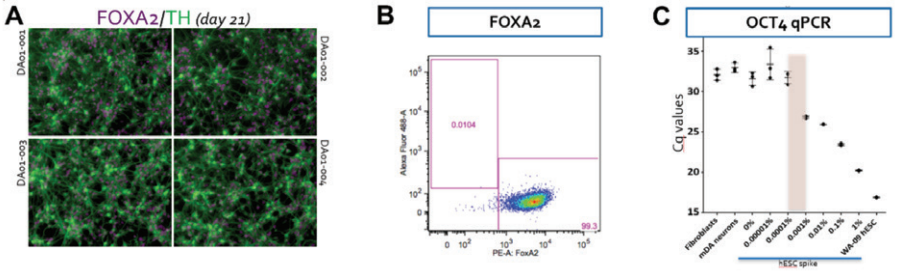

Figure 3. Examples of QC assays assessing the purity of clinical grade dopamine neurons. A) Midbrain dopamine neurons were characterized for the co-expression of TH and FOXA2 across each of the 4 clinical grade batches. B) Purity of FOXA2+ cell compartment measured by flow cytometry C) Lack of any detectable OCT4 (POU5F1) transcript to demonstrate the lack of any remaining undifferentiated hPSCs.

small number of non-human primates to assure that the clinical injection device proposed for human application is suitable to deliver the cells and to provide appropriate cell doses to the brain. All those studies supported the notion that our product is safe, does not form any tumors upon long-term engraftment or other signs of toxicity and reliably induces functional recovery.[28]

In parallel, we developed the clinical protocol for our phase I/IIa human study as detailed on www.clinicaltrials.gov (NCT#04802733). We proposed to enroll 12 patients: 5 patients at a lower dose and 7 patients at a higher dose level. The appropriate dose was calculated based on our preclinical studies and is aimed at replacing 100,000 versus 300,000 dopamine neurons respectively on each side of the patient's brain. We expect that the lower dose is the minimum number of dopamine neurons required to achieve clinical benefit, while the larger dose is closer to providing a

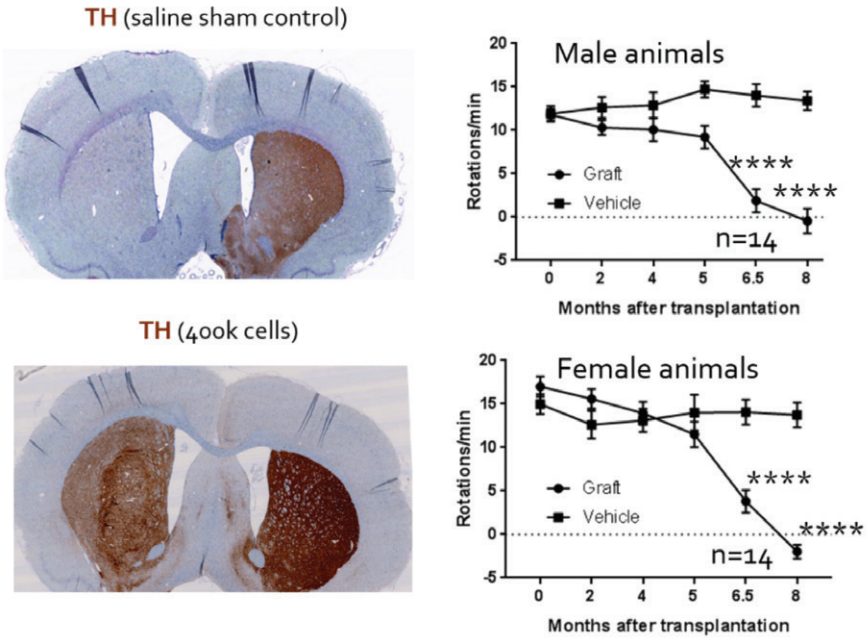

Figure 4. Transplantation of cryopreserved dopamine neurons in rat model of PD.28 The left panels show the unilateral loss of dopamine by histology in sham treated animal, while the animal treated with dopamine neurons shows restoration of the TH signal, a surrogate marker of dopamine in the brain. Right panels: Dopamine neuron transplantation rescued the rotational asymmetry in both male and female rats.

patient with a full complement of new dopamine neurons. The target patient population is moderate to severe PD. It includes patients that cannot be properly treated with conventional therapy such as L-Dopa treatment, but do not exhibit major cognitive deficits or psychiatric comorbidities. Patients have to undergo transient immunosuppression as this is an allogenic, off-the-shelf, product. However, we think that no long-term immunosuppression is required, based on the immune status of the brain and the experience with fetal dopamine neuron grafting that showed survival of dopamine neurons for up to 24 years after only transient immunosuppression.[9]

The surgical targeting involves the injection of cells along three tracts with three deposits each on both sides of the brain (Figure 5). The target region is the post-commissural putamen, a brain region that is part of the

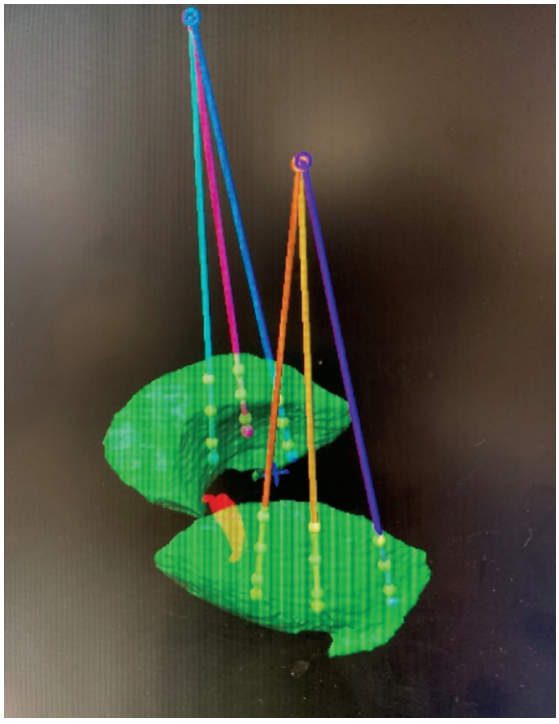

Figure 5. Surgical targeting of the brain using MRI guided stereotactic injection of the cells with a total of 3 tracts with 3 deposits on each side of the brain. Image was kindly provided by Dr. Viviane Tabar, Chair of Neurosurgery at Memorial Sloan Kettering Center, a long-term partner, and collaborator on this project.

striatum and commonly represents the most severely, dopamine-depleted brain region in PD patients. We perform the surgery under intraoperative MRI guidance which allows for exquisite control of targeting the desired brain region and helps minimize risks associated with stereotactic injections, such as risk of causing bleeding by avoiding regions with high densities of blood vessels.

After our protocol was cleared by the FDA in late 2020 and by Health Canada in 2021, the trial started with the lower dose cohort of 5 patients who were all dosed by late 2021. Injections of the higher dose cohort of 7 patients started in early 2022 and is expected to be completed in 2022. The main endpoints of the study are safety and feasibility as typical for a Phase I study. In addition, we will look for early signs of efficacy based on clinical rating scales including UPDRS part III and based on F-Dopa PET imaging to look for evidence of graft survival and function at the 1-year and 2-year marks. While we are awaiting the results from this first clinical study, questions remain about optimal patient selection and cell dose. For example, even within the current study, there were slight differences in patient selection criteria among the two surgical sites. While Health Canada followed our initial proposal of targeting moderate to severe PD patients that are older than 50 years of age, clearance by the FDA required us to limit the study to severe PD patients only and individuals who are at least 60 years of age. This is just one example of how different regulatory bodies can impact clinical development in a manner that is difficult to predict and points to fact that both regulatory bodies and investigators have to learn how to best proceed with this new class of therapies.

Other ongoing trials and efforts to coordinate efforts across the globe

The PD cell therapy community has made a significant effort to coordinate efforts among the various groups involved across the globe. To this end, in 2014 we founded G-Force PD, a global organization with members in Europe, North America and Asia that is aimed at streamlining preclinical and clinical development of PD cell therapies.29 For example, G-Force has developed guidelines for clinical trial design that have been adopted by several groups.[30] Among the G-force members, two groups have already started clinical studies prior to our current trial. In the US, a single patient was injected using autologous iPSC-derived dopamine neurons in a study led by Kwang-Soo Kim and colleagues.[31] The resulting data showed feasibility of the approach, albeit the results were not conclusive regarding graft survival or evidence of graft function. The group

in Kyoto by Jun Takahashi started their trial even earlier using human PSC-derived dopamine neurons[32] from a single iPSC line donor, that is HLA homozygous, and expected to match about 16% of the Japanese population. However, the trial is not restricted to those individuals and all the grafted patients will receive transient immunosuppression similar to that used in our ongoing trial. Neither of those two trials were based on the use of a cryo-preserved, "off the shelf" product, which complicated the QC of the product and led to a protracted timing of those trials. While our current trial is expected to dose all 12 patients within a 12-month period, the Kyoto study has been ongoing for several years with the goal of completing all (n=7) patients.

Next steps – beyond Phase I

Some of the next steps include the production of a commercial-grade dopamine neuron product. This effort is currently ongoing at BlueRock Therapeutics, the company I co-founded in 2017 and that was recently acquired by Bayer. For a commercial-grade product, it is important to further increase lot size by at least 1 to 2 logs in cell number, while defining robust in vitro functional assays that are required by the FDA for qualification of a commercial cell product. Other next steps include the development of a simplified surgical injection device to make loading and cell delivery foolproof for any practicing neurosurgeon. Another important step is the planning of a pivotal phase IIB/phase III study that could lead to the clinical approval of the product for broader use in PD if successful.

In addition, there are also remaining scientific challenges. The current product is suitable for clinical use, but there is the possibility of genetically engineering the human PSC line as to prevent any immune response, and thereby avoiding the need for transient immunosuppression. Further engineering could include modifications to better protect the grafted dopamine neurons from the ongoing disease process such as by lowering alpha-synuclein levels. Another open question is whether cell identity or composition can be further improved to achieve maximal potency and clinical benefit with minimal clinical risk. Yet another interesting challenge is to better understand the in vivo engraftment process and the factors that determine initial graft survival. It is estimated that only about 10% of the dopamine neurons injected ultimately survive long-term in the host brain and the factors limiting survival remain largely unknown. Finally, there is the option of developing a clinical cell replacement strategy that involves not only the striatal target region but places the cells directly into the sub-

stantia nigra to achieve a more complete integration of the grafted neurons. All those areas for further improvement could yield the versions 2.0 or 3.0 of the dopamine neuron grafting paradigm in the future to come.

Conclusions and outlook

The idea of dopamine neuron replacement has been pursued for more than 30 years. Today we have access to technologies that allows the production of nearly unlimited dopamine neurons, and there is a consensus that human PSCs are the currently most appropriate choice for those efforts. While our current trial is ongoing and additional clinical trials are expected to start soon, there are already several lessons from that 30-year journey. First, there is a critical and continuing need for basic research to understand the developmental biology underlying human dopamine neuron differentiation and subtype specification and the factors controlling in vivo engraftment. Further refinements of the dopamine neuron product will likely take advantage of our increasing ability to profile dopamine neurons,[33,34] as well as all the other lineages of the human body,[35,36] in detail using single cell-based approaches. Another lesson is the need to understand and streamline the process of translating findings from the bench to the bedside. While this initial effort took nearly 10 years from the first successful proof-of-concept[22] study to the actual use in PD patients, it is likely that future efforts will move more quicky as clinical grade cell production and regulatory processes become increasingly streamlined. Accordingly, the work currently performed with dopamine neurons in PD may pave the way for other cell-based therapies in the future. Finally, while it is exciting that regenerative approaches for PD are becoming a reality, it will be important to develop strategies that go beyond the standard paradigm of replacing dopamine neurons. There is a need to think outside the box and to develop the next generation of cell-based strategies that can tackle even more challenging problems such as treating the feared cognitive symptoms in late-stage PD, where a breakthrough could herald treatments for other major neurodegenerative disorders such as Alzheimer's disease. Just a few decades ago, the now imminent prospect of cell therapy for PD seemed to be science fiction rather than science. Over this coming decades, the community should strive to develop cell-based approaches that can combine cell replacement with genetic engineering to tackle many additional neural disorders and to bring novel treatment options to the millions of patients suffering from those currently intractable disorders.

Declaration of interests: L.S. is a scientific co-founder and paid consultant of BlueRock Therapeutics, a biotech company recently acquired by Bayer that sponsors the ongoing clinical trial of dopamine neuron grafting in PD.

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EMBRYONIC DEVELOPMENT

Stem Cells, Embryos and Embryo Models

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Background

The study of early embryonic development in mammals has provided fundamental information on how the fertilized egg transitions through successive developmental stages to form a complex organism such as ourselves. It is a remarkable journey that has fascinated me since my days as a graduate student in Cambridge. The mouse embryo has been my main study system of choice but this has always been in the service of understanding human development and improving human pregnancy outcomes. The Marshall Lab at Cambridge was also the research base for Bob Edwards when I was a student. So I was keenly aware of the direct translational implications of work from mouse embryo culture to human IVF. Working with Richard Gardner, I helped establish the mouse blastocyst as a model system to study lineage decision-making in development and identified that the epiblast cells of the inner cell mass were the original pluripotent cells (Gardner and Rossant, 1979).

The blastocyst in both the mouse and the human contains three distinct lineages by the time of implantation in the uterus. There is an outer

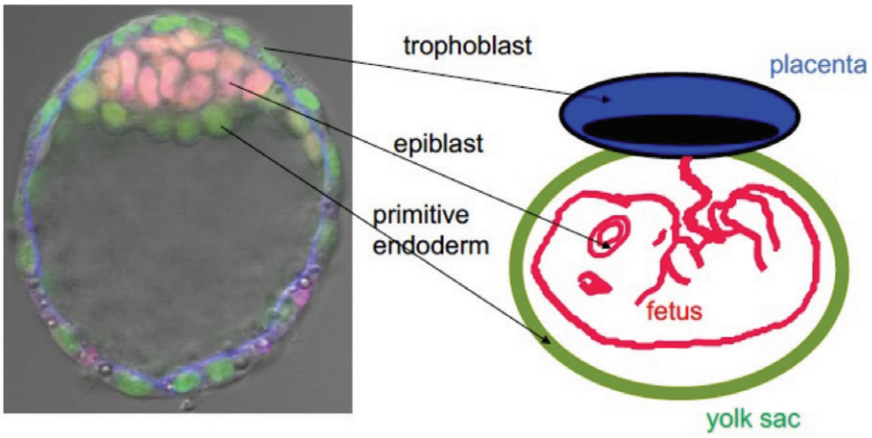

Figure 1. Lineages from the mouse blastocyst.

polarized epithelium, the trophectoderm (TE), which gives rise to the trophoblast cells in the placenta. The trophectoderm encloses a blastocoelic cavity, at one end of which is a group of cells called the inner cell mass (ICM). The ICM consists of the pluripotent epiblast which contributes to all cells and tissues of the embryo proper as well as some extraembryonic membranes such as the amnion. The layer of primitive endoderm (PrE) on the blastocoelic surface of the ICM gives rise primarily to extraembryonic endoderm of the yolk sac. Thus, of the three lineages of the blastocyst, only the epiblast can be considered to be truly pluripotent. However, it is not totipotent as it has lost the capacity to generate TE and PrE of the conceptus. The fate and potential of the different lineages has been determined by ever-increasingly sophisticated chimera and lineage tracing experiments and the signaling pathways and transcription factors involved in specifying cell fate are fairly well understood (reviewed (Rossant, 2018)).

The beginnings of pluripotent stem cell research also date back to the 1970s. Embryonal carcinoma cell lines with some properties similar to the ICM had been isolated from mouse teratocarcinomas (Cronmiller and Mintz, 1978; Martin and Evans, 1974). They could contribute to some normal tissues in chimeras, just like epiblast cells, but the resulting chimeras succumbed to growth of tumors derived from the cell lines (Papaioannou et al., 1978). The concept that it might be possible to capture the pluripotent state of the epiblast by directly culturing blastocysts in vitro was clearly in the wind. Many groups tried to derive such cell lines and two groups, Martin Evans and Matt Kaufman (Evans and Kaufman, 1981), and Gail Martin (Martin, 1981), succeeded in 1981. The derivation of mouse embryonic stem (ES) cells marked the beginning of a revolution in mouse genetics and the foundation of all future pluripotent stem cell research and applications. It was not until 1998, however, that Jamie Thomson first reported the derivation of human embryonic stem cells from excess IVF blastocysts (Thomson et al., 1998). The ethical concerns raised by the use of human embryos for this research was a definite concern for many inside and outside the field. It was the discovery of induced pluripotent stem cells (iPSC) by Yamanaka and colleagues in 2006/7 (Takahashi et al., 2007; Takahashi and Yamanaka, 2006) that really led to an exponential surge of interest in pluripotent stem cell research and its potential applications to understanding and treating human disease.

The correlation between the properties of the embryo itself and its derived stem cells is of ongoing interest and certainly generates some controversy.

Different mouse stem cell states and their relationship to the early embryo

Mouse ES and iPSC grown under so-called 'naïve' conditions (Ying et al., 2008) show gene expression, epigenetic profiles and X-inactivation status that are highly similar to the epiblast of the blastocyst itself. Importantly they also behave like epiblast cells when reintroduced into the early embryo, contributing to all lineages of the fetus itself, including the germ line. It is actually possible to produce entirely ES-derived mice by aggregating ES cells with tetraploid embryos – the so-called 'tetraploid complementation assay' (Nagy et al., 1993). In fact, carefully maintained mouse ES cells can routinely generate complete mice when introduced into diploid pre-compaction 8-cell embryos (Poueymirou et al., 2007), a process that has allowed rapid phenotypic screening of genetic alterations (Cox et al., 2010).

What about the other lineages of the blastocyst? Can they produce stem cell lines in vitro? My lab derived permanent cell lines from both the TE and the PrE of the mouse blastocyst and showed that they could self-renew, differentiate appropriately in culture and contribute to the expected lineages in vivo in chimeras.

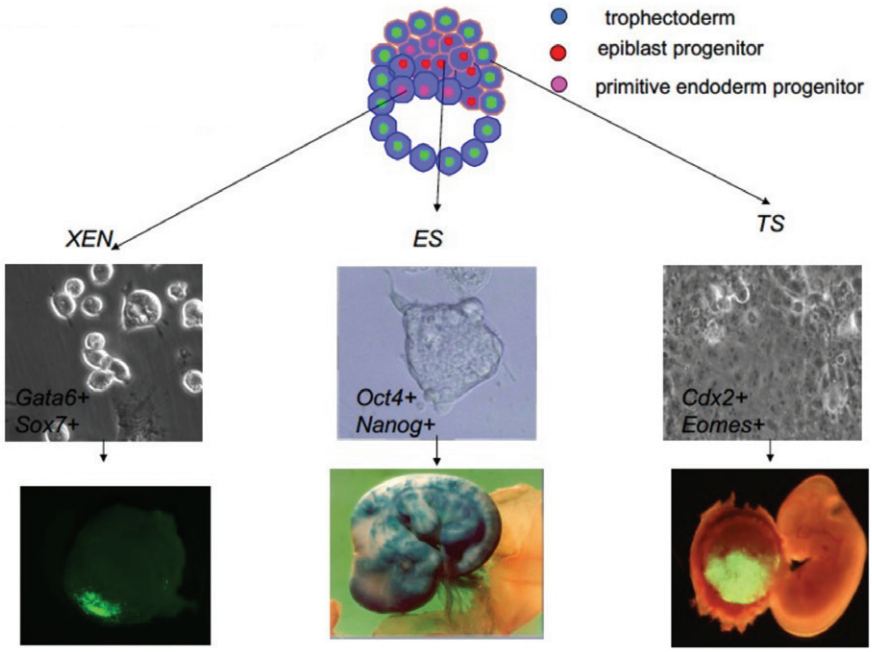

Figure 2. Three lineage-specific progenitor cell lines from the mouse blastocyst.

ES cells, trophoblast stem (TS) cells (Tanaka et al., 1998) and Extraembryonic endoderm (XEN) cells (Kunath et al., 2005) are derived under conditions that reflect the known growth factor requirements within the embryo itself. Most notably, different levels of FGF/ERK signaling act to specify cell fate (EPI vs PrE) or maintain proliferation (postimplantation TE) in the embryo itself. Other pathways, in particular the Hippo signaling pathway (Cockburn et al., 2013), also play key roles in establishing cell fate in the embryo itself. There are still many open questions on the details of the process of lineage commitment in the mouse embryo but the overall correlation between lineage behavior in the embryo and in its derived stem cells holds firm.

Recent work has improved the derivation of TS cells (Lee et al., 2019) and XEN cells (Ohinata et al., 2022) to resemble even more closely their progenitors at the blastocyst stage.

Capturing different cell states in stem cell lines *in vitro* has been extended to other stages of development. It is obviously of interest to ask whether it is actually possible to capture the earlier totipotent state in culture. A stem cell state that reflects some of the properties of the 2-cell stage of development, the 2C-like cell, has been reported to arise spontaneously in standard ES cell cultures and has been proposed to represent the totipo-

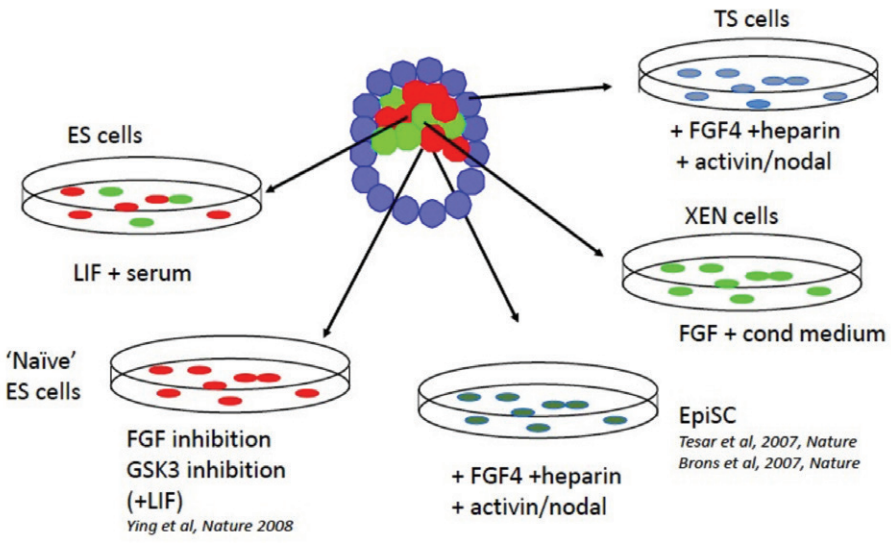

Figure 2. FGF/ERK signaling levels determine different stem cell states in mouse.

tent state of the 2-cell embryo (Macfarlan et al., 2012). In the mouse, the two-cell stage is the time of the major activation of the zygotic genome (ZGA), which is marked by transient activation of retroposons and a set of genes only found at this stage of development. 2C-like cells share many of these gene profiles and may well represent a useful model to study the mechanisms of ZGA. However, they cannot be maintained in a stable state indefinitely in culture (Genet and Torres-Padilla, 2020) and their ability to contribute to all lineages in later development is not well documented (Macfarlan et al., 2012).

There have been a number of studies claiming to derive pluripotent cell lines with extended potential to generate both ICM and trophectoderm derivatives, usually beginning with ES cells. Although many of these do show altered properties from standard ES cells and some gene expression typical of earlier stages, most of them do not show highly convincing capacity to generate TE either *in vitro* or *in vivo*. We reassessed the capacity of two of the best documented cell lines from the Liu (Yang et al., 2017b) and Deng (Yang et al., 2017b) labs for their chimeric potential and showed that, although the cells could occasionally be found in the TE lineages, they were not fully transformed into trophoblast and continued to express ES markers (Posfai et al., 2021). Two more recent papers have used spliceosome inhibition (Shen et al., 2021) and chemical-induced chromatin remodeling (Yang et al., 2022) respectively to shift the cellular state of ES cells towards a more stable totipotent blastomere-like cell. While closer to the blastomere state, the complex chemical interference needed to cause this shift in potential still requires to be understood in terms of its relationship to the progress of totipotency to pluripotency in the embryo itself.

The epiblast cells of the blastocyst undergo further morphological and gene expression changes as the embryo implants in the uterus, leading up to the major germ layer specification events of gastrulation. They do retain full pluripotency during these transition stages but undergo various epithelial reorganizations to form the egg cylinder stage. Epiblast stem cells (EpiSC) can be derived from early postimplantation embryos in the presence of FGF (Brons et al., 2007; Tesar et al., 2007) (see Fig 3) and represent the pre-gastrulation stage epiblast, the so-called primed state. They do not respond to induction of germ cell fate and they cannot contribute to normal development after injection into the blastocyst. More recently the Smith group has proposed that there is also a formative stage of the epiblast in the early post-implantation period during which the epiblast cells exit from the naïve pluripotent state, gain responsiveness to germ cell induc-

tion and are prepared for later lineage responsiveness at the gastrulation stage (Kalkan and Smith, 2014; Smith, 2017). Several groups have isolated stem cell lines with some properties of the formative state (reviewed [Pera and Rossant, 2021]). The relationship between these various cell lines and the stages of epiblast development in the embryo is by no means entirely clear. Pluripotency seems to be a somewhat flexible state of being!

Human development and stem cell states

When human ES cells and iPSC were first derived, they were grown under conditions that included FGF and ERK activation, leading to the general conclusion that they were closer to mouse EpiSC than the naïve pluripotent state. It took time and considerable effort to develop culture conditions that could transform human ES cells to a stable naïve state, but this has now been achieved in a number of labs (Takashima et al., 2014; Theunissen et al., 2014). Expression profiling confirms that these cells are closer to the early ICM of the human blastocyst, including in their X-inactivation status, although this state seems to be relatively transient in the embryo itself. As the embryo implants there is a fairly extended period of period of 4-5 days where the post-implantation epiblast persists in a relatively stable state of gene expression (Nakamura et al., 2016) whilst undergoing expansion in numbers. Both formative and primed ES cells in humans can be considered to represent different phases of this transition. At this stage, as in the mouse, the exact identities of the different cell states and their comparison to the embryo itself are unclear (reviewed [Pera and Rossant, 2021]).

It is becoming clear, however, that human naïve stem cells do have a broader lineage potential than their mouse equivalents. Several groups have shown that naïve hES cells retain some potential to differentiate down the TE pathway, depending on appropriate culture conditions (Cinkornpumin et al., 2020; Dong et al., 2020; Guo et al., 2021; Io et al., 2021). This parallels experimental data showing that isolated ICMs from the mature human blastocyst can still generate TE in outgrowth culture (Guo et al., 2021). This is in contrast to results from the mouse where ICM cells clearly lose TE potential after initiation of blastocyst formation (Posfai et al., 2017).

Recently two groups have reported that it is possible to identify a subset of cells in naïve hES cultures that express many of the properties of the 8-cell blastomere stage of development, which marks the time of major ZGA in the human embryo (Mazid et al., 2022; Taubenschmid-Stowers et al., 2022). Similar to the mouse 2C-like cells, these 8C-like cells express features of zygotic genome activation and share common markers of ZGA

like HERV-L and Trpx1. These cells may be useful for studying human ZGA but, like the mouse, this is not a stable state that can be used to promote totipotent development.

What about stable stem cell lines from the TE and hypoblast in human? Again, such lines have been derived but not with as much ease as from the mouse blastocyst. Human TS cells have been derived from both blastocysts and early villus biopsies (Okae et al., 2018). They seem to have the lineage potential of the early postimplantation cytotrophoblast, rather than the TE of the blastocyst. There have been two reports of isolating extraembryonic endoderm-like cells (Linneberg-Agerholm et al., 2019) or yolk sac-like cells (YSLC) (Mackinlay et al., 2021) from human ES cells, which may have some properties of the hypoblast.

More details on current understanding of human embryo development and its relationship to stem cell states is found in Rossant and Tam (Rossant and Tam, 2022).

Stem-cell derived blastocyst models from mouse to human

There are still many gaps in our knowledge of the molecular processes of blastocyst formation in humans and there are several groups working to fill those gaps with direct data from human embryos in culture. However, use of human embryos for research is limited by regulation or legislation in many jurisdictions and, even where permissible, the supply is limited. As with other approaches to studying human development using stem cell-derived organoids, there is considerable interest in using stem cells to model early development. Here I focus on the production of so called blastoids as models of the blastocyst itself. The Rivron lab generated structures resembling blastocysts by controlled aggregation of mouse ES cells and TS cells (Rivron et al., 2018). The primitive endoderm lineage was not well represented in these original blastoids. They could cause a decidual response in the uterus but did not develop further.

Blastoids with more primitive endoderm cells were produced when mouse extended potential ES cells (Yang et al., 2017a; Yang et al., 2017b), were either combined with TS cells (Sozen et al., 2019) or cultured in suspension culture alone (Li et al., 2019). Although these blastoids were closer in morphology and expression profiles to the blastocyst, they still failed to show embryo development after implantation. In the case of blastoids derived entirely from extended potential ES cells (Li et al., 2019), our re-analysis of the gene expression profiles did not support a bona fide TE identity for all of the putative TE cells in the blastoids (Posfai et al., 2021).

Clearly even in the mouse, the blastoid is not yet really equivalent to the blastocyst. In the mouse one always has the gold standard of the embryo to fall back on when trying to validate the system. When generating human stem cell-based embryo models, it is still very important to be able to compare with the embryo itself, even though there is a limitation on the temporal extent of study. Thus, the potential to be able to generate large numbers of human embryo models from stem cells is very attractive. The logical place to start with generation of human blastoids might be expected to be combinations of human blastocyst-derived stem cells, in a similar manner to the mouse. However, there have been no published reports on successful generation of human blastoids from combining human ES and TS cells. Instead, there have been a series of reports claiming to generate blastoids directly from human ES cells by various culture manipulations without addition of any specific extraembryonic cell types (Fan et al., 2021; Kagawa et al., 2022; Liu et al., 2021; Sozen et al., 2021; Yanagida et al., 2021; Yu et al., 2021). One study claimed to produce iblastoids as an intermediate during the process of reprogramming adult cells to iPSC (Liu et al., 2021), while most other studies began with human ES cells in a putative naïve state. Given the reports that ES cells in the naïve state retain some TE potential (Cinkornpumin et al., 2020; Dong et al., 2020; Guo et al., 2021; Io et al., 2021), these groups claim to have revealed this potential in a relatively controlled manner so as to produce blastoids at reasonable efficiency. Some of the reported human blastoids do show quite striking resemblance to the blastocyst itself in both morphology and gene expression, but in-depth comparisons of the gene expression profile of the cell types in the blastoid with the embryo itself always need to be made. Formation of an epithelial cyst with enclosed pluripotent cells is not sufficient to confirm a functional TE phenotype. Our reanalysis of the published data shows that all human blastoids contain undefined cell types at various proportions (Zhao et al., BioRxiv). Further, the claimed TE lineage in the blastoids derived during reprogramming is closer in expression profile to amnion than TE. Clearly it is early days for generating reproducible, homogeneous human blastoids that can mimic early developmental stages in vitro. More refinement of culture conditions and careful comparison with normal blastocysts and early post-implantation stages will be needed to validate these potentially powerful experimental models.

It has been suggested that broader use of stem cell-derived blastoids could avoid some of the regulatory and ethical issues of human embryo research. A blastoid may resemble the products of conception but is not derived by the union of egg and sperm. Its genotype reflects the diploid

genotype of its founding cell line. However, blastoids do come with their own legal and ethical concerns. Although currently blastoids, even in the mouse, are clearly not functional embryo equivalents, would further improvements bring them closer to such capacity? When would a stem cell model be considered to have crossed the line and become an embryo? In some jurisdictions such as Australia, blastoids are already considered as requiring the same regulatory oversight as embryos themselves (Matthews and Morali, 2020) and the US NIH is not currently funding such research. The recently revised ISSCR Stem Cell Guidelines took these issues into consideration and proposed that integrated stem cell models like blastoids (with cell types potentially able to generate a functional placenta and embryo) should be subject to special oversight and restricted to short term culture (Lovell-Badge et al., 2021), while still recognizing that they are not embryo equivalents. The guidelines also specifically prohibit transfer of any stem cell-based embryo model (including blastoids, gastruloids and other models) to the uterus of a human or animal host.

Conclusions

Many years of research on mouse embryo development and differentiation have provided the tools, the fundamental knowledge and the practical applications that underlie the current excitement about exploring human development more directly. The advent of single cell genomics and transcriptomics, in vivo live imaging, CRISPR gene editing has provided new insights into understanding lineage development in both mouse and human embryos. The development of stem-cell based integrated and non-integrated embryo models (Rossant and Tam, 2021; Weatherbee et al., 2021) provides new avenues for human embryology research but with a clear need to be validated against normal embryonic processes. Overall, increased scientific knowledge of the mechanisms of embryogenesis will help us understand the basis of both genetic developmental anomalies and non-genetic congenital diseases, as well as shedding light on the causes of early embryo loss and pregnancy disorders. Embryo and stem cell research have always been closely intertwined and there is no doubt that will continue in the future to great effect.

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Hematopoietic Cell Therapies from Human Induced Pluripotent Stem Cells

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As universal precursors for any cell type in the human body, human induced pluripotent stem cells (iPSCs) hold great promise for cell replacement therapies and regenerative medicine. Similar to embryonic stem (ES) cells, but avoiding ethical controversy and regulatory burdens, iPSCs can be expanded indefinitely and induced to differentiate into multiple cell types. With genetic manipulation to ensure immune compatibility, iPSCs can in principle serve as an unlimited source for "off-the-shelf" therapies (Lanza et al., 2019). Among the multitude of cell types that could be used to treat human disease, iPSC-derived blood cells are arguably the most amenable to clinical use, as human hematopoietic stem and progenitor cells (HSPCs), red blood cells, and platelets have been used in all corners of the globe to treat blood malignancies, genetic disorders, and cytopenias for decades with great success (Appelbaum, 2007). Bone marrow, red cell and platelet procurement entail a cumbersome and expensive blood donation system that critically depends on donor altruism and availability, and can be disrupted by unpredictability, supply chain inefficiency, and shortages and is subject to pathogen contamination (viral, parasitic, and bacterial). Large-scale manufacture of HSPCs, red blood cells, platelets, lymphoid and myeloid lineages from iPSCs provides an appealing alternative source for bone marrow transplantation and various hematopoietic therapies, and consequently has great therapeutic importance. Efficient *in vitro* differentiation of iPSCs into HSPCs and other blood lineages relies on lessons learned from developmental studies of embryonic hematopoiesis. In mammals, blood cells are produced in "waves" in temporally and anatomically distinct sites that support the emergence, maintenance, or proliferation of HSPCs. The first wave of hematopoiesis arises in the mammalian yolk sac, an extra-embryonic tissue that produces so-called "primitive" erythroid cells (Palis et al., 1999; Whitelaw et al., 1990), macrophages (Takahashi et al., 1989), and megakaryocytes (Tober et al., 2007). The multipotent erythromyeloid progenitors (EMPs) and HSCs capable of supporting lifelong production of

all mature blood lineages emerge later during the "definitive" wave of hematopoiesis in the aorta-gonad-mesonephros (AGM) region of embryonic mesoderm (Medvinsky and Dzierzak, 1996). It has been shown in multiple vertebrate animal models, from zebrafish to human, that definitive hematopoiesis happens through a highly conserved trans-differentiation process known as endothelial-to-hematopoietic transition (EHT), during which a subset of endothelial cells with hemogenic (blood-forming) potential differentiate and egress from the ventral wall of the aorta to enter the circulation as multipotential EMPs as well as a cohort of *bone fide* HSCs (Jaffredo et al., 1998; Kissa and Herbomel, 2010; Zovein et al., 2008). The HSCs generated from EHT later colonize fetal liver to further mature and expand significantly before they seed the bone marrow to maintain life-long adult hematopoiesis (Houssaint, 1981; Kieusseian et al., 2012).

A detailed roadmap of the underlying mechanisms of embryonic blood ontogeny and the emergence of definitive hematopoiesis has been summarized in depth in several recent reviews (Dzierzak and Bigas, 2018; Liggett and Sankaran, 2020; Sugden and North, 2021). Employing principles of morphogen-induced mesodermal patterning and fate-sustaining cytokines, multiple groups have established protocols that direct the differentiation of human iPSCs into HSPCs *in vitro* that can give rise to a variety of more terminally differentiated blood lineages. In the first such study, stromal cell lines derived from mouse hematopoietic tissues were used to support the differentiation of human ES cells into progenitor cells that formed hematopoietic colonies (Kaufman et al., 2001). Subsequently, culture systems employing the spontaneous differentiation of ES cells into embryoid bodies (EBs) enabled stromal-free differentiation of ES cells into HSPCs (Chadwick et al., 2003; Zambidis et al., 2005). Most early *in vitro* differentiation protocols recapitulated the primitive wave of hematopoiesis, though subsequent methods enabled the generation of CD34+ cells with lymphoid potential (Kyba et al., 2002; Vodyanik et al., 2005; Wang et al., 2005). More recent studies have used T cell differentiation as an indication of definitive multilineage potential, and have established that activation of Wnt/β-catenin signaling combined with inhibition of Activin/Nodal signaling can pattern mesoderm towards definitive hematopoiesis (Kennedy et al., 2012; Sturgeon et al., 2014). Small molecule-based manipulation of these signaling pathways during the EB stage of differentiation enables the generation of definitive hemogenic endothelium (HE; defined as CD34+ KDR+ CD184- CD73-) from human pluripotent stem cells (PSCs). In the presence of a cocktail of cytokines and chemicals that pro-

mote hematopoiesis, these HE cells can go through a Notch-dependent EHT-like conversion to form CD34+ CD45+ HSPCs that are capable of giving rise to erythroid, myeloid, and lymphoid cells (Ditadi et al., 2015). Such a strategy has also been applied to human iPS cells (Kennedy *et al.*, 2012). Chemical inhibition of ActivinA/TGFβ pathway and aryl hydrocarbon receptor (AHR) facilitates the generation of iPSC-derived HSPCs that produce erythroid cells expressing adult globin proteins (Leung et al., 2018). Despite considerable effort, to date no group has succeeded in deriving HSCs with long-term, self-renewing, multi-lineage hematopoietic reconstitution potential in lethally-irradiated murine hosts, considered the cardinal definition of the HSC. However, two groups including our own have leveraged transcription-factor driven conversion of embryoid-body derived hematopoietic progenitors into engrafting cells with durable multi-lineage differentiation potential, albeit with low efficiency (Sugimura et al., 2017; Tan et al., 2018). These studies produce iPSC-derived HSPCs that are capable of multilineage engraftment of primary and secondary mouse recipients, and establish that PSCs indeed hold promise as a source for HSPC transplantation. Nevertheless, such a system requires the expression of transgenes, which is not optimal for clinical translation. To date, *in vitro* protocols appear to lack key developmental and microenvironmental cues to promote proper differentiation of engraftable HSPCs. Key deficiencies are likely to include exposure to the proper dose and duration of retinoids, which are well known to pattern developmental processes (Luff et al., 2022), as well as biomechanical forces, as it is has been established that longitudinal shear and circumferential stress forces trigger the emergence of HSPCs from the HE of the aorta (Adamo et al., 2009; Diaz et al., 2015; Kim et al., 2015; Lundin et al., 2020; North et al., 2009).

Although the generation of *bona fide* HSC from iPSCs without genetic manipulation has not yet been achieved, considerably more success has been had by several groups in producing definitive HSPCs with the capacity to differentiate further into cells of the lymphoid lineage (Demirci et al., 2020; Kennedy et al., 2012; Park et al., 2018; Sturgeon et al., 2014). Given that chimeric antigen receptor (CAR) T cell therapy has shown remarkable efficacy against several types of blood cancer (June et al., 2018), there is growing interest in the prospect that iPSC-derived lymphoid cells could become a more facile and readily available source for adoptive immunotherapies. Current CAR-T therapies utilize patient-derived T cells that are collected via apheresis, cultured in the lab and engineered to express CARs that can specifically recognize tumor antigens. After expan-

sion, autologous CAR T cells can be infused back to the patient to target tumor cells, with a recent study showing that CAR T cells can persist and lead to decade-long leukemia remissions (Melenhorst et al., 2022). Despite success against CD19-positive lymphoid leukemia and lymphoma and BCMA-positive multiple myeloma, and much promise for targeting a wider array of solid tumors, adoptive immunotherapy is currently limited by its dependence on autologous T cells, which renders the manufacturing process cumbersome, time-consuming, and expensive (Fesnak et al., 2016). iPSC-derived T cells provide an appealing alternative source for the production of CAR T cells, which if "cloaked" to reduce immunogenicity may enable precisely manufactured off-the-shelf adoptive T cells therapies. Compared to other blood lineages, production of T cells from iPSCs has proven challenging because T cell maturation relies on signaling via the Notch receptor pathway normally provided by thymic epithelial cells (De Smedt et al., 2005). As a surrogate, engineered mouse stromal cells that express Notch ligands, such as OP9-DL1/DL4, have been included in co-culture with HSPCs to support in vitro T cell differentiation from various stem cell types (Schmitt and Zúñiga-Pflücker, 2002; Timmermans et al., 2009). Exploiting such an approach, iPSCs harboring tumor-specific T cell receptors (TCR) or expressing CARs have been used to generate tumor antigen-specific T cells or CAR T cells showing antitumor activities *in vitro* and in animal models (Themeli et al., 2013; Vizcardo et al., 2013). These studies provide proof-of-concept that iPSC-derived T cells have the potential for clinical applications such as cancer immunotherapy. However, in the first studies, molecular characterizations of the iPSC-derived CAR T cells revealed a transcriptional signature more akin to innate-like T cells that express γδ T Cell Receptors (TCR) rather than mature T Cells that express αβ TCR. Moreover, the first reported iPSC-CAR T cells predominantly expressed CD8αα homodimer, which does not engage major histocompatibility complex (MHC) as efficiently as the CD8 αβ co-receptors found on more mature αβ TCR-expressing T cells that circulate in our blood (Van Kaer et al., 2014). As a result of these innate-like features, iPSC-CAR T cells produced by these early methods were not as functionally robust as clinical grade CAR T cells derived from peripheral blood mononuclear cells (PBMC) (Themeli et al., 2013). More recent studies have explored new strategies to generate iPSC-T cells via three-dimensional organoid-like culture systems, and have shown enhanced efficiency in producing αβ TCR+ CD8+ T cells from human iPSCs (Montel-Hagen et al., 2019; Seet et al., 2017). Studies to date on these enhanced *in vitro*

T cell culture systems have included only limited transcriptional analysis at the single cell level, thereby hindering comparison to PBMC-derived mature T cells. Moreover, the translational potential of some protocols remains limited by the usage of mouse stromal cells. To overcome these obstacles, the Daley lab has been using immobilized Notch ligands instead of stromal cells to support T cell differentiation from iPSCs. Similar stroma-free protocols have been used to generate T cells from iPSCs that are reprogrammed from T cells with pre-rearranged TCRs (Iriguchi et al., 2021; Shukla et al., 2017). We have further shown that *in vitro* stroma-free differentiation can recapitulate normal TCR rearrangement and yield T cells expressing a highly diverse TCR repertoire. Additionally, previous studies from the Daley lab have identified transcription factors and epigenetic modulators that act as important regulators for multilineage blood potential and lymphoid commitment (Doulatov et al., 2013; Vo et al., 2018). Leveraging this information, we have combined the stroma-free T cell differentiation protocol with epigenetic factor-mediated reprogramming to further facilitate T cell differentiation from iPSCs. As a result, we have generated iPSC-T cells that are more developmentally mature and functionally robust. These iPSC-derived T cells exhibit a molecular signature that resembles mature αβ TCR+ T cells from PBMC, and when engineered to express tumor-specific CARs, display enhanced efficacy against tumor cells in xenograft mouse models. Such a strategy is compatible with large-scale production of iPSC-derived T cells and is highly amendable to immune-cloaking techniques that would be needed to realize "off-the-shelf" adoptive T cell therapies.

In addition to T cells, immunotherapies using NK cells have likewise shown great promise in the treatment of both hematopoietic and solid tumors (Basar et al., 2020; Wrona et al., 2021). Unlike T cells, NK cell cytotoxicity is not restricted to specific MHC molecules but depends on a more versatile regulation of activating vs. inhibitory signals (Fauriat et al., 2010). As a result, NK cells have advantages in targeting MHC-downregulated tumor cells that can escape from T-mediated antitumor responses. Moreover, CAR NK cells are less likely to cause graft-versus-host disease (GVHD) and CAR T cell-associated toxicities such as cytokine release syndrome (Liu et al., 2020; Ruggeri et al., 2002). Multiple protocols have been developed to generate CD3-CD56+ NK cells from pluripotent stem cells for the production of CAR NK cells (Knorr et al., 2013; Woll et al., 2009; Zeng et al., 2017). Current studies on iPSC-derived NK cells are focusing on further engineering the NK cells to promote expansion

capacity or enhance antitumor activities (Cichocki et al., 2020; Li et al., 2018; Zhu et al., 2020). We have shown that a set of epigenetic modulators acts during early lymphoid commitment to regulate the NK cell vs. T cell fate decision. Using various genetic editing tools, we are able to temporally manipulate the expression of these epigenetic regulators and fine-tune the *in vitro* differentiation of iPSCs to produce mature NK cells. Compared to control iPSC-derived NK cells, these epigenetically reprogrammed NK cells exhibit elevated surface expression of CD16 and other activation receptors that are essential for the NK cell-mediated killing response. Whether such developmentally mature NK cells show enhanced anti-tumor activity remains to be proven.

In summary (Figure 1), iPSCs represent a compelling source for the generation of hematopoietic cells for applications in research and clinical medicine. Advances in genetic editing of iPSCs holds additional value for producing cell products with defined antigenic and receptor profiles (e.g., universal donor O-Red Blood Cells, PLA1-negative platelets, and TCR-deficient T cells), while advances in bioprocess engineering are re-

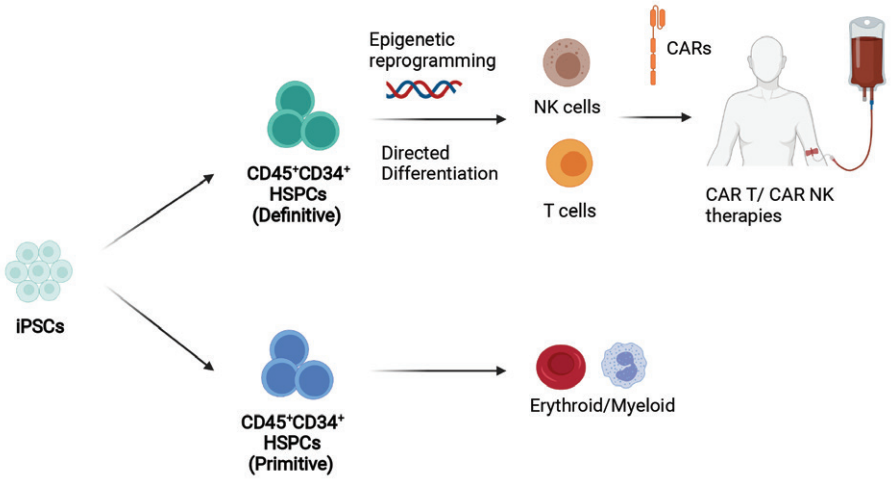

Figure 1. Generating blood cells from iPSCs. Human iPSCs can be induced to differentiate into primitive or definitive HSPCs. Primitive HSPCs are lineage-restricted and can only give rise to erythroid/myeloid cells, while definitive HSPCs can be used to generated NK and T cells of the lymphoid lineage. Epigenetic reprogramming during lymphoid differentiation allows the production of more mature, functional NK/T cells. These cells can be further engineered with tumor-specific CARs to produce CAR NK/T cells that are being clinically deployed for adoptive cancer immunotherapies.

quired to achieve efficient and cost-effective cell manufacture at clinical scale for truly off-the-shelf therapeutics. Clinical trials have already commenced for iPSC-derived platelets (Nakamura et al., 2021) and NK cells (Cichocki et al., 2020) with the expectation that T cells, red blood cells, and HSCs anticipated to follow, ushering in a future where cells become living medicines.

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HOW STUDYING STEM CELLS IN MODEL SYSTEMS CAN ADVANCE REGENERATIVE MEDICINE

Understanding the Sources of Regeneration in Animals

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Ever since antiquity the ideas of immortality, resistance to bodily injuries and the restoration of missing body parts have populated the myths of many civilizations. The ancient Greeks, for example, gave us the myth of Tithonus, immortal but still capable of aging; of Achilles who could not be injured – except at the proverbial heel – by either sword or spear; and Prometheus – bearer of fire – condemned by Zeus to regenerate his liver to perpetually feed voracious vultures. In America, Aztec records and even older Mayan mythology encompassed by the Popol Vuh are both abundantly imbued with the concept of perpetual regeneration (Bazzett, 2018). Irrespective of which civilization one may consider, it is highly improbable that such shared human beliefs were not inspired in some fashion by nature, which we know today has been a cauldron of biological invention for nearly 4 billion years. Thus, it is also extremely implausible that we have learned from Nature all that there is for us to know. On the contrary, we have but barely scratched the surface of biology. In fact, we do not know what is already possible. The sheer number of species out there waiting

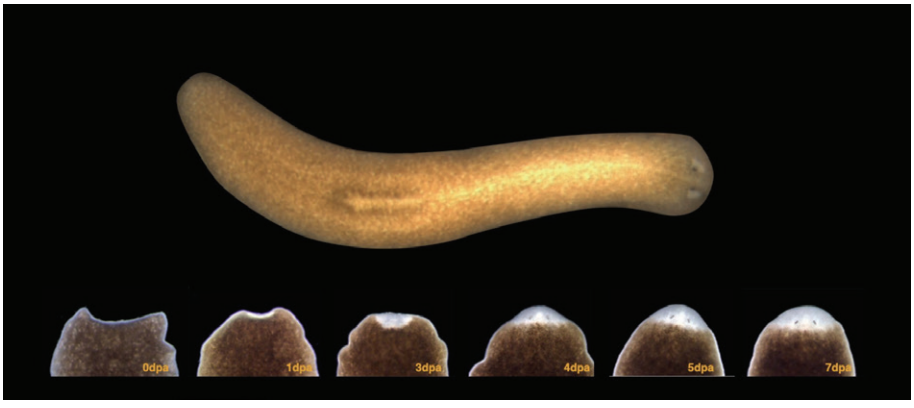

Figure 1. The planarian *Schmidtea mediterranea* (top). Bottom, a planarian regenerating its head in 7 days after decapitation.

to show us what is indeed biologically possible is staggering. Equally remarkable is the fact that our species has the necessary tools to decode and understand them all if we so wished.

For the past 20 years, my laboratory has exploited the diversity of animal life to address the problem of regeneration. The central question for us is: why is regeneration so broadly but unevenly distributed in the animal kingdom? Likewise, in those animals with robust regenerative capacities: do they share common mechanisms to restore missing body parts or did unique mechanisms emerge independently in every species? Both are fundamental questions awaiting satisfactory mechanistic explanations. In an effort to carry out a rigorous molecular dissection of regeneration, we chose to study an organism with extraordinary regenerative capacities, the free-living, fresh-water flatworm planaria, *Schmidtea mediterranea* (Figure 1). We reasoned that if the wild type phenotypes were already extraordinary, i.e., regenerating a complete animal from a random body fragment, perturbing such biology should yield even more extraordinary phenotypes that would help illuminate the mechanisms underpinning regeneration.

RNA-mediated genetic interference (RNAi) provided us with a tool to perturb gene function (Sánchez Alvarado and Newmark, 1999) and regeneration screens were performed which identified hundreds of genes involved in regeneration (Reddien et al., 2005) and uncovered novel functions for key embryonic genes in an adult context (Arnold et al., 2019; Arnold et al., 2021; Gurley et al., 2008; Rink et al., 2009).

A key source of these animals' regeneration prowess is a group of abundant, adult stem cells known as neoblasts. These cells are the only known cells to proliferate in adult asexual planarians and thus can be completely eliminated by exposing the animals to ionizing radiation (Bardeen and Baetjer, 1904). When the stem cells are thus abrogated, animals survive for 3-4 weeks on the virtue of their differentiated cells, but as these turnover, the animals eventually lose structural integrity and die. We devised methods to purify planarian stem cells, defined their expression profiles by bulk and single-cell RNA sequencing, and discovered a remarkable diversity of transcriptional states associated with these stem cells (Zeng et al., 2018). We also were able to prospectively isolate the pluripotent neoblasts from this cell population and demonstrated that when a single isolated cell was injected into animals devoid of stem cells – and thus destined to perish – such cells could rescue the viability of the stem cell-deficient animals and restore all the animal's functions and properties, including their capacity to regenerate (Figure 2).

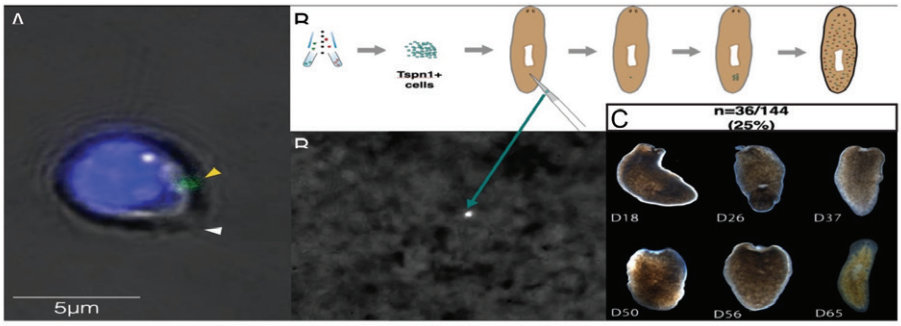

Figure 2. Planarian neoblasts are adult pluripotent cells. Using single-cell RNA sequencing 12 clusters of neoblasts were identified. One of them, referred to as NB2, contained pluripotent neoblasts which could be purified using antibodies. A single, antibody purified cell (A) can be injected into animals in which all stem cells were eliminated using irradiation (B and B') 25% of all single cell injections were capable of both rescuing lethally irradiated animals and restoring the regenerative capacity of the host (C). Modified from Zeng et al., 2018.

Intriguingly, when we studied these cells under three different biological contexts (tissue homeostasis, in vivo clonal expansion and regeneration), we were surprised to see that the same cell type displayed significantly different expression profiles depending on the context in which they were operating (Figure 3A). Hence, we have shown these cells to be remarkably dynamic, constantly occupying diverse states of continuous fate determination. Because pluripotent stem cells are generally assumed to primarily exist transiently in early embryogenesis, and can only be perpetuated artificially in vitro, our findings that pluripotent stem cells can be maintained in adult animals despite showing distinct transcriptional changes dictated by either physiological homeostasis and/or injury, are all the more provocative. This led us to propose a probabilistic model of stem cells to explain the plasticity of genomic output (transcriptome) displayed by these cells (Figure 3B). In this model, self-renewal becomes a conceptual property not permanently possessed by a discrete population, but transiently held by a small number of cells and arising probabilistically depending on the demands of the animal (Adler and Sánchez Alvarado, 2015). If these stem cells stochastically express progenitor markers for specific organs, perhaps injury induces changes in the frequency or periodicity of expression, resulting in altered differentiation of stem cell progeny. Such a model allows us to frame the remarkable plasticity of planarian in terms of dynamic cell states rather than statically-defined cell types.

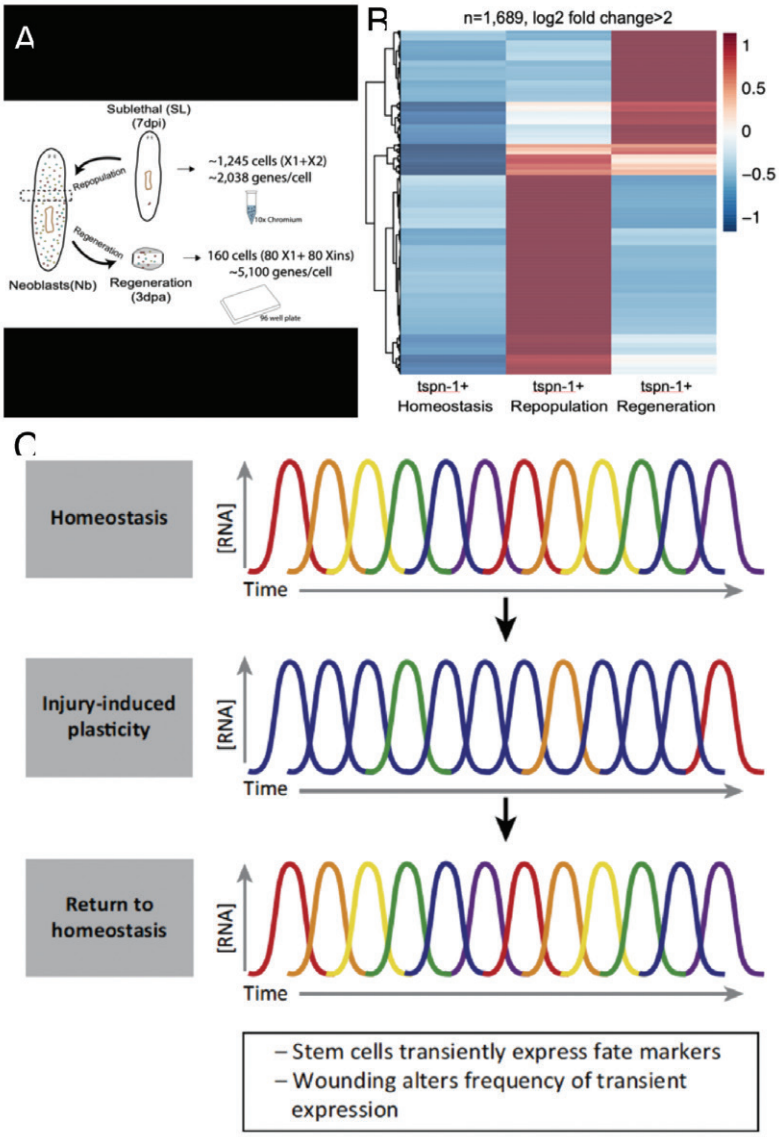

Figure 3. Pluripotent neoblasts display multiple transcriptional states. (A) Three different conditions were explored to measure the transcriptional profiles of pluripotent neoblasts: homeostasis, *in vivo* clonal expansion, and regeneration. (B) Three different profiles were detected for each condition from the same tetraspanin-1 (tspn-1) positive cells. Modified from Zeng et al., 2018. (C) Probabilistic model for transcriptional states of cell types. Each graph represents two different cells across time change the chorot and frequency of expression of genes in probabilistic rather than deterministic terms. Adapted from Adler et al., 2015.

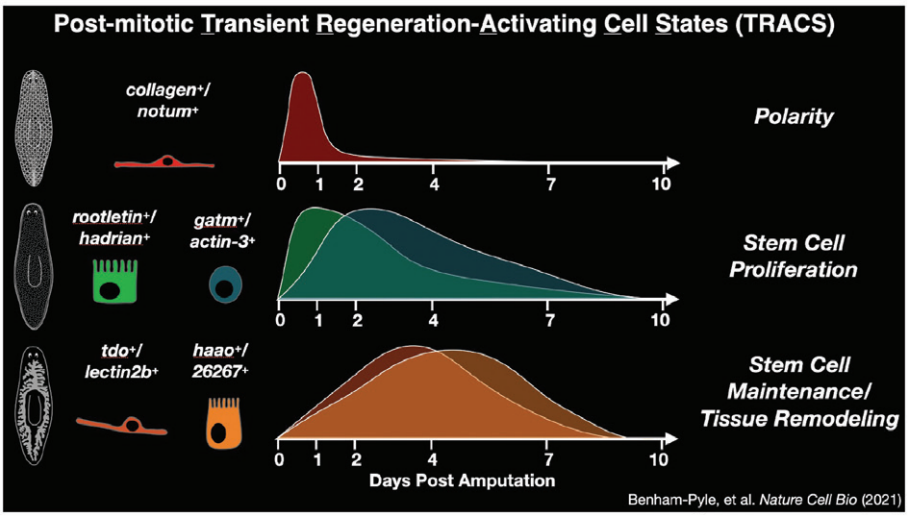

Figure 4. Discovery of post-mitotic Transient Regeneration-Activating Cell States (TRACS). Shown are the expression kinetics of TRACS in adult derivatives of mesoderm (muscle), ectoderm (epidermis) and endoderm (gut). Image by Blair Benham-Pyle.

We then wondered whether the ability of stem cells to occupy multiple transcriptional states would be a property shared by postmitotic differentiated cells. To address this problem, we generated a comprehensive atlas of whole-body regeneration in *S. mediterranea* (Benham-Pyle et al., 2021). This work revealed the existence of wound-induced cell states. An analysis of ~300,000 single-cell transcriptomes captured from regeneration-competent and regeneration-incompetent tissues identified transient regeneration-activated cell states (TRACS) in the muscle, epidermis and intestine. We also found that TRACS occurred only in post-mitotic cells and that their manifestation did not require cell division per se. TRACS were also characterized by distinct spatiotemporal distributions, and RNAi depletion of TRACS-enriched genes produced specific regeneration defects, depending on the tissues targeted. Muscle TRACS were found to be essential for tissue polarity, while epidermal TRACS were important for stem cell proliferation and endodermal (gut) TRACS were found to regulate stem cell proliferation and tissue remodeling (Figure 4). Our results uncovered that regenerative ability can emerge from coordinated transcriptional plasticity across adult derivatives of all three germ layers (Benham-Pyle et al., 2021).

Concluding Remarks

Our work on regeneration and in planarians has revealed previously unsuspected properties of both stem and postmitotic, differentiated cells. First, our work provides strong evidence that adult undifferentiated and differentiated cells possess unappreciated plasticity and can exist in multiple transcriptional states. In light of the current extensive reliance on induced pluripotent stem cells (iPSCs) to produce differentiated cells for therapeutic interventions in regenerative medicine it is appropriate to consider whether or not these reprogrammed cells possess the ability to occupy different transcriptional states.

Second, the extensive adoption and growing use of single cell RNA sequencing is providing a strong body of evidence for the existence of reproducible transcriptional states elicited by different biological conditions. As such, it is also appropriate to ask how homogeneous or not are the transcriptional profiles of differentiated iPSCs after their transplantation and integration into host tissues. Given that it should be possible to label iPSCs and thus follow their fates in host tissues, it would be interesting to purify these cells after they have been functioning in host tissues for a reasonable period of time and determine their single cell transcriptional profiles. Will differentiated iPSCs possess more, less, or no transcriptional plasticity? What would those profiles look like when the host tissues are challenged by conditions in which injury or disease are introduced? Understanding whether the iPSC postmitotic differentiated progeny behave distinguishably or indistinguishably from host differentiated cells should help us better understand whether unsuspected limitations of iPSCs may exist that may limit their long-term utility as therapeutic agents.

Third, our data provide strong evidence for us to question what is meant in adult organisms by "terminal differentiation". Given the discovery of TRACS, that these transient transcriptional changes occur in postmitotic cells, and that they have essential roles in promoting regeneration, would it not be more accurate to think of "terminal differentiation" as "stable differentiation" instead? The implications here for regeneration biology and regenerative medicine are that if some cells are more stably differentiated than others, then their ability to regenerate or be restored may be more difficult. One corollary would be to ask whether differentiated iPSCs integrated in tissues are "ultra-stable" or possess limited transcriptional plasticity and therefore are less fit than wild type adult cells to respond to environmental insults, for example.

Finally, one important conclusion from our work is that revealing the mechanisms promoting and suppressing stable differentiation states will likely be essential to better understand the differentiated state of adult cells. This has two important implications. First, a clear mechanistic understanding of how stable differentiation is promoted and suppressed will shed light on why regenerative capacities are broadly but unevenly distributed across animals, including humans. And second, it may help us identify better ways to generate mature, differentiated, postmitotic iPSCs for regenerative medicine.

Acknowledgments

I would like to thank all members of my laboratory, past and present, for their dedication, invaluable insights and scientific accomplishment in helping us advance our understanding of animal regeneration. I would also like to acknowledge Blair Benham-Pyle for providing Figure 4. ASA is an investigator of the Howard Hughes Medical Institute and the Stowers Institute for Medical Research.

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The Salamander Limb – An Exemplary Model of Natural Regeneration Relies on Positional Memories and Cellular Interactions

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Salamander appendage regeneration was discovered in the mid 1700s by Spallanzani, who first documented appendage regeneration in frogs and salamanders (Spallanzani, 1768). This and the work of others at this time demonstrating regeneration in hydra and worms showed that regeneration is a phenomenon widespread in nature, an observation which contributed substantially to the debate on whether organism development occurs from pre-existing germs (preformation) or by the unfolding of a series of events (epigenesis) (Bodemer, 1964). Since then, biologists have been fascinated by the concepts, rules and molecules that govern this remarkable process. The high degree of conservation among tetrapods is reflected in the

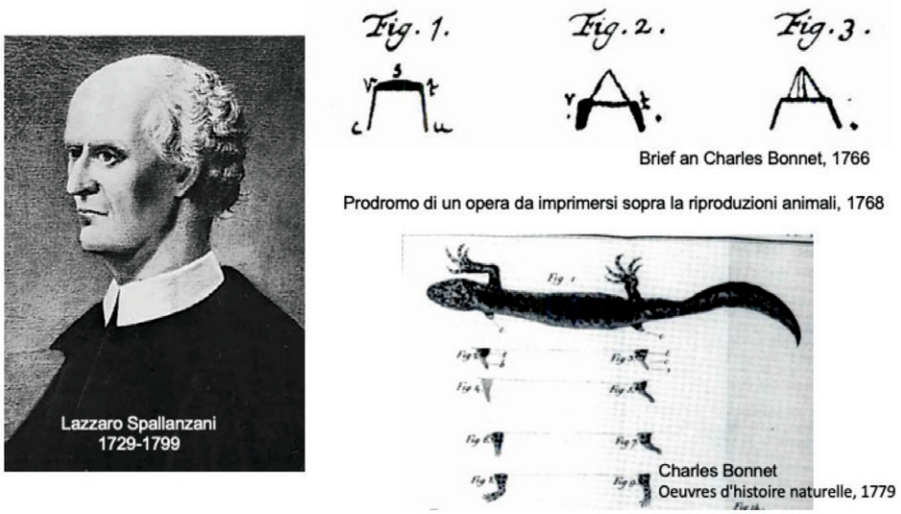

Figure 1. Lazzaro Spallanzani discovered salamander appendage regeneration. Sketches of tail and limb regeneration from his correspondences and writings.

similarity of molecular programs governing limb development between salamanders and mammals. Given this similarity, this unique limb regeneration system provides a guiding light for what kind of tissue organization and injury response is necessary to achieve functional regeneration, which may be emulated in the future to induce regeneration in mammals.

Regenerating the correct part of the limb

Positional memories are at the basis of appropriate regeneration of the missing part. When a salamander arm is amputated at the wrist, only a hand is regenerated, while when the limb is amputated in the upper arm, the elbow, lower arm and hand are regenerated. Butler and others provided key insights into the nature of this problem (Butler, 1955). He was able to generate inversely oriented limbs by amputating a limb at the wrist and then suturing it back into the body. Blood vessels and presumably nerves repopulated the structure so that when the "circularized" limb was amputated in the upper arm, a normal stump as well as an inversely oriented stump was generated. The normal stump regenerated the lower arm and hand as expected. Interestingly, the inversely oriented limb similarly regenerated lower arm and hand.

These results revealed that the regenerating limb does not read directionality of limb tissue but rather the cells at the amputation plane have a memory of their positional identity, and newly formed blastema cells change their identity to more distal (direction fingertip) identities. This conclusion gave rise to the concept of "The rule of distal transformation".

The limb contains many different tissue types including epidermis, dermis, muscle nerve, Schwann cells, ligaments, tendons, bone and an interesting question is whether all tissues harbor this positional identity or only some tissues. To test this question, tissue-specific transplantation of GFP-expressing hand cells into the upper arm, followed by amputation

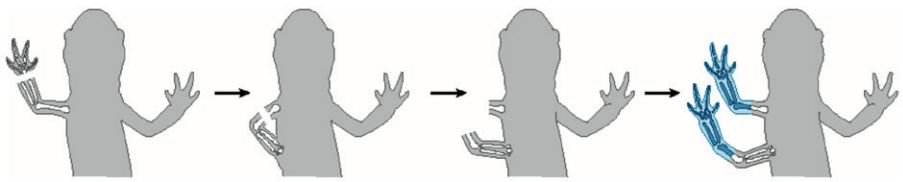

Figure 2. Grafting experiments that gave rise to the "Rule of Distal Transformation" by which it was concluded that cells at the amputation plane have a memory of their location in the limb, and cells in the blastema acquire more distal identities Image taken from (Nacu and Tanaka, 2011).

was implemented (Kragl et al., 2009; Nacu et al., 2013). The prediction of this experiment was that any tissue with positional memory would only contribute to the regenerated hand. Such work found that only cells from the lateral plate mesoderm lineage (connective tissue, namely dermis, tendons, ligaments, bone) showed hand determination. This meant that any molecular system controlling positional memory in the mature limb would be present in the connective tissue cells.

We investigated molecular correlates of positional memory. Upper arm, lower arm and hand development are strongly influenced by two clusters of genes called the HoxA and HoxD complexes. The genes HoxA/D9, HoxA/D11, HoxA/D13 are expressed sequentially as the upper arm, lower arm and hand, respectively are specified. We compared the expression characteristics of the HoxA proteins during upper arm versus hand regeneration (Roensch et al., 2013). During upper arm regeneration, the blastema cells initially at 6 days express HoxA9 and then HoxA13 is expressed at the tip of the blastema by day 8. In contrast in a hand blastema, the HoxA13 protein and HoxA9 protein are simultaneously expressed already at day 6. This strongly suggested that upper arm and hand cells start with a different setpoint for initiating HoxA gene expression, and suggests that HoxA proteins may be functionally involved in positional memory. Our current work examining the chromatin organization in upper versus lower arm cells indeed shows that mature upper arm and hand cells have differential chromatin organization at the HoxA locus (Kawaguchi, Wang and Tanaka, unpublished).

Which tissues and processes distalize the identity of cells at the amputation plane has been a topic of long speculation and models. Some models suggested that the wound epidermis may be the most distal identity, while others proposed a series of cellular transformations based on cell interactions (Maden, 1977; Slack, 1980). Interestingly, many of the early models hypothesized that the first blastema cells generated had the most distal identity, with subsequent intercalation of values, based on averaging identities as cell proliferation produced an increasing number of cells (Bryant et al., 1977; Maden, 1977). In contrast, Meinhardt proposed a model in which a border generated between anterior and posterior cells resulted in the generation of a signaling center that supported growth of cells that recapitulated the progressive sequence of upper arm, lower arm and then hand regeneration as seen in development rather than by intercalation (Meinhardt, 1983). Our experiments observing HoxA protein expression during upper arm regeneration strongly support this progressive mode of

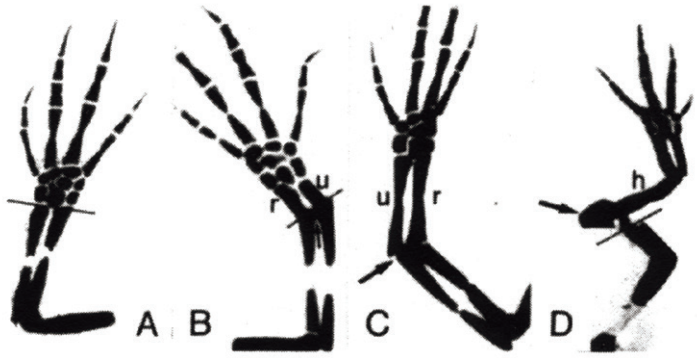

Figure 3. Treatment of regenerating limb blastemas with retinoic acid causes resetting of positional identity whereby wrist determined cells can regenerate an entire arm. Taken from (Maden, 1982).

upper arm, lower arm, and hand sequence of distalization rather than the intercalation model (Roensch et al., 2013).

The molecular identity of factors that distalize blastema cells is not fully understood. Based on limb development studies, it may be expected that fibroblast growth factors and possibly bone morphogenetic factors are involved, yet they seem insufficient alone to yield cell identity distalization and some models have suggested that the lower arm to hand transition occurs cell autonomously, yet nonetheless, this transition must occur spatially at the tip of the blastema (Capdevila et al., 1999; Mercader et al., 2000; Rosello-Diez et al., 2014). How this occurs in this spatially defined domain is not fully understood.

Interestingly, positional identity can be experimentally reset during regeneration, by exposing the blastema to retinoic acid (Maden, 1982). Upon generation of a hand blastema, if the animal is exposed to retinoic acid, a complete arm is regenerated from the wrist. Molecular studies showed that this effect is mediated by the Meis transcription factor, whose function during development is to specify upper arm development (Mercader et al., 2005).

Specificity of regeneration to amputation – the role of anterior/posterior positional memory

Why does regeneration occur upon appendage amputation and not simply from wounding? The answer to this question also ultimately rests on the existence of positional memory in limb cells. Remarkably, trans-

plantation of left blastemas to right limb stumps, results in the regeneration of two additional, ectopic limbs (for references see (Bryant et al., 1977; Nacu and Tanaka, 2011). These results were interpreted to mean that anterior and posterior cells in the mature limb harbor separate memories of their positional identity. Upon blastema formation, as anterior and posterior cells enter the blastema, it was hypothesized that either their interface or communication between anterior and posterior cells is required for limb outgrowth. The presence of both anteriorly and posteriorly-derived cells only occurs upon limb amputation and therefore explains why regeneration does not happen upon simple limb wounding.

Lheureux (Lheureux, 1977) followed by others, and most recently Endo and Gardiner (Endo et al., 2004), demonstrated this concept by generation of ectopic limbs. Ectopic limbs could be elicited by deviating nerves to an anterior lateral wound site in the upper limb, together with grafting of posterior full thickness skin to generate an ectopic anterior/posterior interface at that site. Nerve deviation alone resulted in formation of a blastema-like structure, but this tissue regressed after three weeks. With the combination of nerve and anterior/posterior skin interface a fully patterned limb grew from that site, demonstrating the central role of anterior/posterior interfaces in sustaining limb regeneration.

We recently defined the molecular nature of the anterior and posterior requirement for regeneration. Sonic hedgehog is a morphogen localized to the posterior limb bud required for limb development and is re-expressed in the regeneration limb blastema (Nacu et al., 2016). We hypothesized that the capability to express sonic hedgehog represents the posterior tissue requirement in regeneration. To test this hypothesis, we

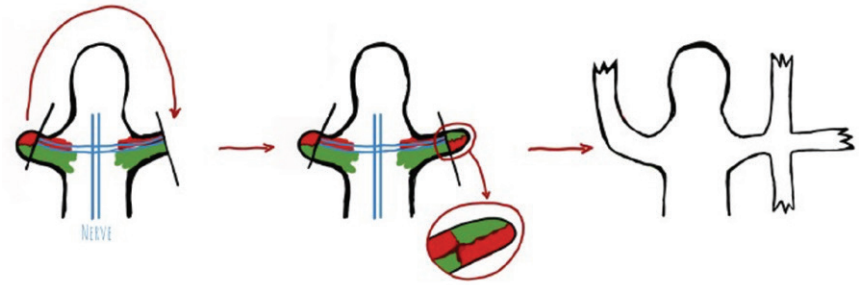

Figure 4. Positional memory in anterior and posterior limb tissue defines amputation specificity of limb regeneration. Transplantation of left blastema onto right limb stump produces two ectopic anterior/posterior interfaces yielding regeneration of two ectopic limbs.

deviated nerves to the anterior side and treated animals with smoothened agonist to activate sonic hedgehog signalling in place of posterior skin. This treatment was sufficient to induce an ectopic limb on the anterior surface. Given the known developmental positive feedback loop between sonic hedgehog and Fgf8 in limb development, we asked whether anteriorly localized Fgf8 represents the anterior component for limb regeneration. To test this hypothesis, we deviated nerves to the posterior upper limb surface and then induced Fgf8 expression via baculoviral induction. This expression was sufficient to induce a limb-like outgrowth from the posterior surface.

Based on these results, the current model is that cells in the adult posterior limb have potential to express sonic hedgehog while cells in the adult anterior limb have the potential to express Fgf8. Amputation of the limb results in anterior and posterior cells migrating to the limb tip and forming blastema cells that then launch the expression of these developmental regulators. This initiates a positive feedback interaction between anterior and posterior cells reinforcing expression of sonic hedgehog and Fgf8 which are essential for outgrowth of the limb. An important future direction is how the genome and chromatin are organized differently in anterior and posterior cells to restrict the potential to express sonic hedgehog only posteriorly and Fgf8 only anteriorly.

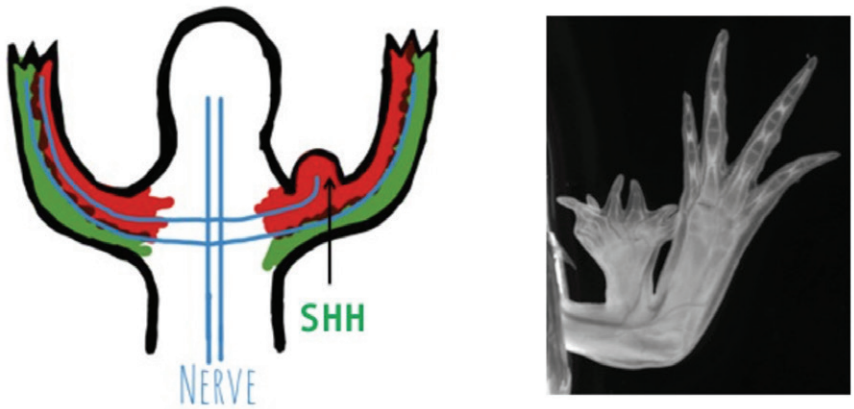

Figure 5. Sonic hedgehog signalling can replace posterior limb tissue in the Accessory Limb Model. Lheureux (1977) and Endo et al. (2004) showed that anterior nerve deviation plus posterior tissue can induce an ectopic limb. Nacu et al. demonstrated that nerve-deviation plus Sonic Hedgehog pathway agonist can induce a limb. Panel on right from Nacu et al. (2016).

Tissue interactions required for regeneration

Regeneration involves the coordination of many different cell types, and these interactions occur dynamically to promote changes in cell differentiation, migration, proliferation, positional identity and maturation. Immediately after amputation, the blood clots at the wound site followed by migration of epithelial keratinocytes over the limb stump surface. The detection of injury by epithelial cells likely relies on changes in osmolarity, and the epithelial migration process appears to be controlled in part by TGFB signalling (For references, see (Bassat and Tanaka, 2021)). Subsequently, underlying connective tissue cells migrate to the amputation surface in response to Platelet-Derived Growth Factor, presumably from the clotted blood and later from blastema cells that express the ligand themselves (Currie et al., 2016).

These connective tissue cells form the majority of the blastema and direct the positioning and patterning events of limb regeneration described above. Cells are stimulated to undergo their first proliferative division by MARCKS-Like Protein released from the epidermis (Sugiura et al., 2016). This special wound epidermis also secretes a number of other factors such as Wnt3 Wnt5, Anterior Gradient and retinoic acid and is essential for the process of regeneration (See (Bassat and Tanaka, 2021)).

Interestingly, recent work suggests that infiltrating macrophages expressing Midkine, and nerve fibers are required for the wound epithelium to acquire its full properties (Tsai et al., 2020). Nerves release not only Anterior Gradient but also BMPs and FGFs that are important for the initial growth of the blastema cells (Kumar et al., 2007; Makanae et al., 2014). Finally, the anterior Fgf8 and posterior sonic hedgehog feedback loop is initiated in the blastema which sustains growth and patterning.

Conclusions and outlook

This is an exciting time in stem cell biology where our ability to study organogenesis and recapitulate it in vitro is pointing our ambitions to elicit in vivo regeneration. So far, stem cell transplantation approaches have not taken the spatial map of a tissue very much into account. The salamander, which regenerates its limb with exquisite precision and functionality has shown that cellular memory states in many different parts of the limb are used as a template and starting place for appropriate regeneration. These observations point to the need to establish such landmarks in human scenarios of regeneration.

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HOW STEM CELLS COPE WITH CHANGING TISSUE ENVIRONMENTS AND ASSAULTS

Stem Cells: Coping with Stress

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As defined by the United Nations Framework Convention on Climate Change (Farber and Carlarne, 2017), climate change is an unnatural change to our climate that is attributed directly or indirectly to human activity that alters our world's atmosphere (Farber and Carlarne, 2017) (Farber DA, Carlarne CP. Climate change law. Ohio St Pub Law Work Paper. 2017;419). Plants are altering the pollens and allergens they generate. Air pollutants are becoming more complex and numerous. The ozone layer is thinning. Existing pathogens and microbes are shifting territories (Hauser et al., 2021). New pathogens and microbes are emerging that we've never seen before. As exemplified by SarsCoV2, we are all painfully aware of the consequences. Indeed, inflammatory disorders and cancers of the epithelia of the skin, the intestine and the lung are on the rise. This is because, bearing the brunt of these environmental changes are our epithelial tissues – they form the cellular barrier between our body and the outside world.

In studying the stem cells of the skin epithelium over the course of my career, I've learned that when epithelial stem cells malfunction, either in making a proper barrier or in communicating with the immune system upon a barrier breach, chronic inflammation occurs. Our first realization came back when I was just embarking on my academic career at The University of Chicago. I had just cloned and characterized the human skin keratins, which form an extensive infrastructure of filaments (cytoskeleton) that protects the epidermal stem cells and their differentiated progeny from mechanical stress. Given that our skin epidermis is at the body surface, this mechanical framework is essential. Indeed, as we showed, patients that lack the stem cell keratin network, composed of keratins 5 and 14, have a blistering skin disorder known as epidermolysis bullosa simplex (Coulombe et al., 1991; Fuchs and Green, 1980; Fuchs et al., 1981; Vassar et al., 1991). The disorder is rooted in the fact that when the stem cells lack this network, they are prone to rupturing upon rubbing the skin – in severe cases, even washing the face, or walking. By contrast, patients with mutations in keratins 1 and 10, which are only expressed in the differen-

tiating progeny generated by the stem cells, have thickened, crusty skin, but are prone to bacterial infections and cancer (squamous cell carcinoma) (Cheng et al., 1992; Fuchs et al., 1992; Fuchs and Green, 1980). Seeking the underlying reasons for this, we learned that the healthy stem cells, recognizing that the skin barrier they made isn't right, respond in a futile attempt, by proliferating to create excess layers of cells to try to patch the barrier. In turn, since the barrier is defective, pathogens can enter, triggering a hyperproliferative response.

In more recent work, we examined another structural protein of the terminally differentiating cells of the epidermis (Fig. 1). Filaggrin has long been known to be expressed by the late-stage terminally differentiating cells of the epidermis. So-called granular cells because of the presence of electron dense granules in their cytoplasm, these epidermal cells represent the last transcriptionally active progeny of the stem cells. Soon after they form, all nuclei and organelles are lost as the granular cells flatten out to form the dead cells that are sloughed from our body surface, continually replaced by inner layer cells moving outward. In studying the human disorder, atopic dermatitis, affecting up to 3% of the world population,

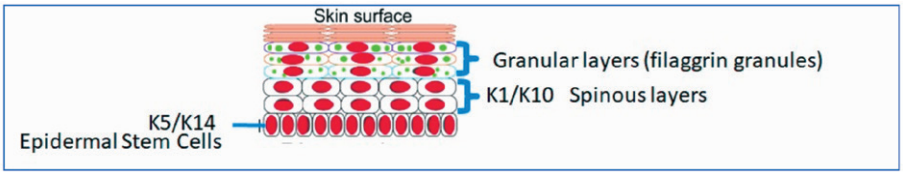

Figure 1. Structure of the epidermis. The epidermis is a stratified squamous epithelium comprised of an innermost (basal) layer of progenitors (referred here as stem cells) that periodically commit to differentiate, creating progeny that depart from the basal layer and move outward to the skin surface. During this program, the stem cells cease to express keratins 5 and 14, and switch to the expression of keratins 1 and 10. These 'spinous layers' are typified by a robust network of keratin filaments that attach to specialized cadherin-mediated cell-cell junctions, called desmosomes. During differentiation, another protein, filaggrin, is expressed, but it must accumulate to a critical concentration before it undergoes a conformational change, forming oil-like protein aggregates, referred to as keratohyalin granules (green). As the protein concentration of filaggrin continues to rise, the granules put mechanical force on the nuclei and organelles, a process thought to contribute to their eventual loss, leading to the dead flattened squames that form the skin's barrier to exclude harmful microbes and retain essential body fluids (Quiroz et al., 2000). In skin homeostasis, the squames are sloughed from the skin surface, replaced by a continual flux of inner layer cells differentiating and moving outward. In humans, mutations in keratins 1/10 and filaggrin cause mechanical fragility, leading to breaches in the skin barrier, particularly at flexural joints (Fuchs et al., 1992; Cheng et al., 1992; Palmer et al., 2006). The result is often hyperproliferation of the stem cells to rescue the barrier breach and increased susceptibility of chronic inflammation and increased risk of skin cancer.

researchers identified mutations in filaggrin (Palmer et al., 2006). At the time, it was thought that this disorder was strictly an immune disorder, based upon mutations in immune cell genes (Kaltoft et al., 1994; Kawashima et al., 1998; Nishio et al., 2001; Osawa et al., 2007; van der Stoep et al., 1993). There has been skepticism as to whether filaggrin mutations even manifest the disorder (Spidale et al., 2020). Indeed, it is a big protein made up of unstructured protein repeat units, and the mutations are scattered throughout the protein, with no apparent consequence to the physiology. Recently, however, we learned that filaggrin undergoes conformational changes that are pH-sensitive and thermal sensitive (Quiroz et al., 2020). In epidermal cells, filaggrin protein begins to be made as epidermal stem cells give rise to differentiating progeny. As the protein accumulates in differentiating cells, it reaches its critical concentration sufficient to induce a conformational change. This change results in the protein transitioning to an oil-like granule which becomes more viscous as the epidermal cells differentiate and the protein accumulates. The granules also interact with K1 and K10 filaments, whose carboxy and amino terminal domains also undergo these liquid phase transitions. The result is a dense viscous network that puts mechanical pressure on the nuclei and organelles, contributing to their loss to form the barrier. The patient mutations create truncated filaggrins that fail to accumulate a sufficient concentration to undergo these liquid phase transitions, and the result is the retention of organelles and the failure to make a proper skin barrier (Quiroz et al., 2020). These data have brought clarity to our understanding of how the skin barrier is formed, and add further evidence that structural defects in the skin barrier can result in chronic inflammatory disorders and increased susceptibility to cancer. Currently AD patients are given immunosuppressive drugs, which have unwanted side effects, and often do not fix the problem. By investigating the basic science of skin stem cells, we hope to be able to uncover biology that will lead to improved therapeutics for disorders like AD.

Our studies of the skin and its stem cells have led us deeper into the biology of chronic inflammatory disorders, which as a cohort, include not only psoriasis, atopic dermatology and chronic wound healing disorders, but also inflammatory bowel disease and asthma. These are all disorders of barrier epithelia at the interface between the body and the external environment (Niec et al., 2021). A common feature of these disorders is that the epithelial hyperproliferation often occurs at flexural regions (elbows, knees for skin disorder, for example), and it typically comes and goes. Upon the next assault, it often occurs in the same spots and with increasing

severity. And curiously, the secondary trigger need not be the same as the initial irritant. The first stimulus might be poison ivy, while the next one might be a pathogen or other irritant.

In 2017, we decided to probe deeper into the biology that underlies these curious phenotypes. In watching how wounds heal, we noticed that if the skin of animals had been exposed briefly to an irritant that triggers an immune response known as a "Th17" inflammatory response, and then wounded a month after the skin pathology had returned to normal, the skin always healed its wound faster if it had been pre-exposed to inflammation (Fig. 2). Even 6 months after the initial inflammatory stimulus, the skin still responded more quickly to heal its wounds faster. We then exposed naïve skin to a yeast infection, a "Th2" inflammatory response, and a wound as primary and secondary stimulus. Each time, inflammation conditioned the skin to heal wounds better (Naik et al., 2017).

We thought at first that immune cells would be involved. Indeed, B and T cells can permanently rearrange their receptors so that they can recognize a pathogen the next time they encounter it. Indeed, this is how vaccines work. However, when we repeated the experiment on mice that lacked all B and T lymphocytes, we again found that after we exposed the skin to an inflammatory stimulus, let the pathology return to normal and then wounded the skin, it always healed the wound faster. We also looked at whether innate immune cells, including macrophages, might be involved. However, after eliminating these immune cells, we began to wonder whether the epidermal stem cells themselves might be the ones to harbor this memory. Indeed, this turned out to be the case (Naik et al., 2017).

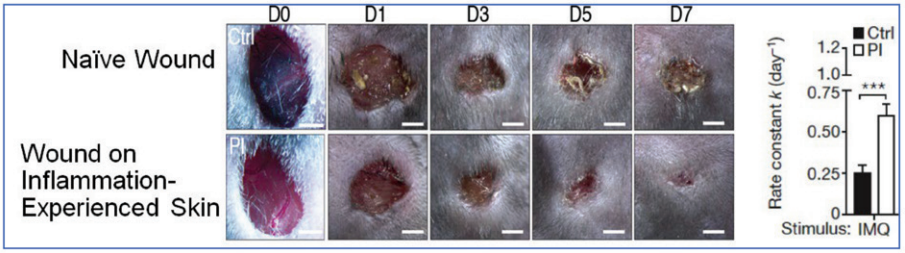

Figure 2. The effects of inflammation on wound repair. In our studies, we exposed mice to an inflammatory stimulus, imiquimod, which induces a Th17 immune response. After withdrawal of the stimulus, the pathology returned to normal within 30bdays. If we then subjected the mice to a mild skin wound, we discovered that the wound always healed faster than if naïve mice were wounded (Naik et al., 2017). This ability was rooted in the ability of the epidermal stem cells to retain a memory of their inflammatory experience.

First, we looked at the transcriptional profile of the epidermal stem cells before treatment, at the height of inflammation and after the inflammation had resolved. Very few genes were changed at the transcriptional level. However, when we turned to chromatin, we found a different story. While >10,000 chromatin sites became open soon after the inflammatory stimulus was administered, >1000 of these sites – mostly in gene regulatory regions known as enhancers – remained open long after the stimulus was withdrawn. In testing these open sites for activity, we learned that they harbored inflammation-sensing activity, and in mice, had the capacity to activate a reporter gene following an inflammatory stimulus (Naik et al., 2017) (Fig. 3). Moreover, the genes associated with these "inflammation sensors" were rapidly activated upon a secondary stimulus, e.g., wounding.

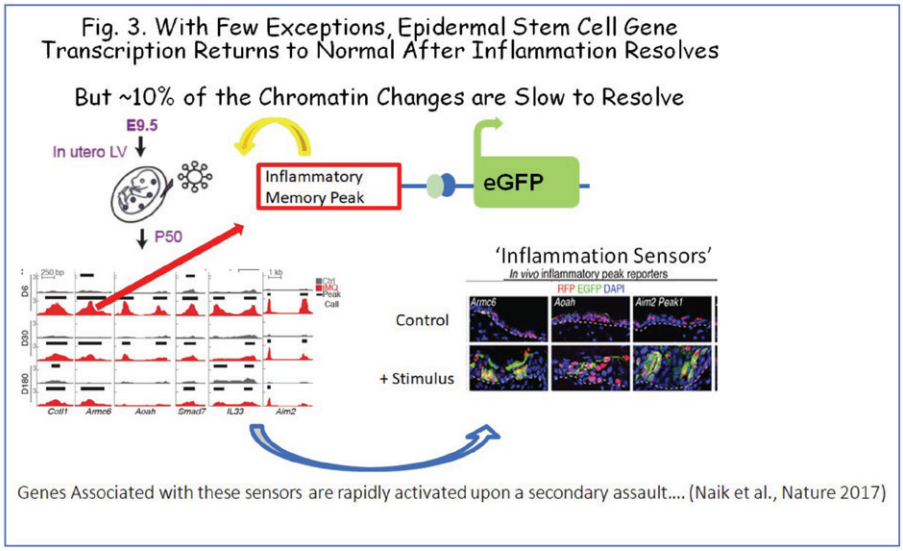

Figure 3. The memory of inflammation in barrier epithelial cells is harbored within the chromatin and retained for months after the inflammation has resolved. Performing ATAC-seq (Assay for Transposase-Accessible Chromatin with high-throughput sequencing), we learned that ›10,000 domains (red peaks) opened in the epidermal stem cell chromatin after imiquimod treatment (grey, naïve chromatin), and while most resolved following withdrawal of the stimulus, ›1000 peaks remained open at least 6 months after the inflammatory experience (Naik et al., 2017). To test if these peaks have inflammation sensing activity, we excised the peaks and used them as enhancers to drive expression of the fluorescent reporter protein eGFP in mice. Using our powerful lentiviral delivery method to target mouse skin, we tested three reporters. Without inflammatory stimulation, they had no activity, but once imiquimod was administered, the inflammation memory peaks drove eGFP expression. The genes associated with these inflammation sensors were rapidly turned out upon exposure to a secondary assault (Naik et al., 2017).

In the past several years, we've now addressed three major questions: How is this memory established? How is memory retained? And how is memory recalled? In the first set of experiments, we simply scanned the transcription factor motif frequency of these inflammatory sensors and compared it to the motif frequency of the many genes that closed back following inflammation and the many genes whose expression was insensitive to inflammation (Larsen et al., 2021). Stat3 and Fos:Jun (AP1) sites were markedly enriched in these memory domains. Looking at inflammation, we learned that Stat3 is phosphorylated and rapidly activated following Th17 inflammation and FOS is rapidly induced as well. FOS's obligatory heterodimerizing partner, Jun, was already present in homeostatic epidermal stem cells. Using "CUT and RUN" technology to map whether these transcription factors bind to the DNA, we discovered that the inflammation-sensing chromatin is silent in homeostatic skin, but rapidly opened upon the Th17 response. pSTAT3 and FOS:JUN bind to these sites. By ablating these transcription factors in the skin epidermis, we learned that pSTAT3 is required to act as a "pioneer factor" in opening the chromatin at these sites, while FOS:JUN is necessary to remodel the chromatin to recruit RNA polymerase and transcribe the genes (Larsen et al., 2021).

What then happens after inflammation, when STAT3 and FOS are no longer there? Without FOS, transcription of the genes associated with memory domains shuts off. However, once the chromatin was open, not only JUN but also several other stem cell transcription factors gain access to the chromatin and bind. In addition, one histone modification in particular, H3K4me1, also gained access to the chromatin, and in contrast to H3K27ac, this mark persisted in the memory state. Hence the memory is retained because there are stem cell factors and histone modifiers that bind and remain bound to the memory domain once inflammatory transcription factors had opened it.

How is memory recalled? In this case, since the chromatin is open, STAT3 is no longer required, and if we ablate Stat3 after memory has been established, it does not prevent transcriptional activation following stress. However, in order to activate transcription at these sites, FOS must be induced. FOS, however is induced in response to a wide variety of different stresses. Within 4 hours after a general stressor, FOS is induced, and binds to the memory domains, chromatin is remodeled and the associated genes are transcribed (Larsen et al., 2021). Moreover, when we re-analyzed all the published data on inflammatory memory to look for parallels to this mechanism we unearthed, we found a remarkable conservation – in all cases,

AP1 factors, FOS/JUN appear to be integral to memory establishment and recall, while the initial transcription factor involved in opening chromatin appears to differ – Stat1, Stat4 or even NFkB. These factors appear to be integral in choosing what genes will be associated with a particular memory, whereas FOS/JUN appear to be general stress-induced factors crucial to remodel the chromatin in memory establishment and memory recall.

These findings begin to answer many long-standing puzzles. Since the 1930s it has been known that plants that survive one pathogen are often resistant to other pathogens that they've never seen before. Infants have long been vaccinated against Bacille Calmette-Guérin (BCG) acquiring resistance against tuberculosis. Epigenetic memory applies the lessons learned from one experience towards a new experience. Memory has an evolutionary advantage in enhancing protection against harmful microbes and also wound repair, as all stem cells must be mobilized to repair tissue injury. (However, it can also be maladaptive as in the case of chronic inflammation).

Since our original publication reporting the existence of epigenetic memory of inflammation in a stem cell, it has now been shown that hematopoietic stem cells have memory (de Laval et al., 2020) and multipotent hematopoietic progenitors possess inflammatory memory (Christ et al., 2018; Mitroulis et al., 2018). It has also been shown that BCG educates cells against tuberculosis (Kaufmann et al., 2018). Importantly, however, we now know that memory extends to other types of epithelial barrier cells: airway epithelia bear epigenetic memory of asthma (Ordovas-Montanes et al., 2018) and intestinal epithelia bear epigenetic memory of gut pathogens (Lim et al., 2021). In addition, epigenetic memory can be inherited. If a mom mouse eats a bacterial pathogen, the inflammation can be transmitted to the fetus through the circulation, and the offspring then carry the epigenetic memory into adulthood (Lim et al., 2021). In the case of pancreatic epithelium, exposure to inflammation predisposes the tissue to acinar ductal metaplasia, increasing the risk of pancreatic cancer (Del Poggetto et al., 2021; Quiroz et al., 2020).

These findings raise many questions: are there different kinds of memories? Are memories cumulative? In the last year, we addressed these questions by generating a wound model in mice in which we specifically mobilized hair follicle stem cells to exit their niche, migrate upward, confront the wound and associated inflammation, undergo a fate change to epidermal stem cells and repair the missing epidermis. Thereafter the hair follicle-derived stem cells behave as epidermal stem cells. Using chromatin landscaping at high throughput level, we showed that at each stem along

this journey, the hair follicle stem cells retain epigenetic memories of their experiences: Memories that they used to be hair follicle stem cells and are now epidermal stem cells; memories that they migrated; memories that they encountered inflammation and signs of epigenetic adaptation indicative that the stem cells now have a different set of tasks than they had before (Gonzales et al., 2021). These memories have profound consequences. I've already discussed the consequences of inflammatory memory. But the stem cells also harbor memories that they used to be hair follicle stem cells. This memory confers increased plasticity to the stem cells: when challenged, they can make both hair and epidermis, while naïve epidermal stem cells only make epidermis. They also bear memories of migration. When challenged, the wound-memory stem cells can migrate much faster than their naïve counterparts.

In closing, since our studies in 2017, the field has exploded, as each day, new memories are uncovered in new cell populations. How long do these memories last? We've followed memory for up to 6 months in the skin stem cells of mice (equivalent of 5-6 years in humans). With the ability to accumulate memories, and the longevity of stem cells, these findings raise concern for our future. Neurons are long-lived and non-dividing – the prime situation for harboring memories. Increasingly it is becoming clear that neurodegenerative disorders such as Alzheimer's Disease are associated with inflammation in the brain, raising the question as to whether inflammatory memory in neurons may be at the roots of these disorders. Epigenetic memory of inflammation also raises potential consequences in aging. Will we show increased susceptibility to inflammatory stimuli that we've never encountered before? Could epigenetic memory explain why the COVID19 response has often be more severe in aging individuals? And what happens in a world where allergens, pollens, pollutants and other irritants are ever on the rise? Our next challenge will be to come up with therapeutic strategies to erase the bad memories and keep the good ones.

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Leptin Receptor$^+$ Stromal Cells are Critical Regulators of the Adult Bone Marrow Environment, Controlling Hematopoiesis and Osteogenesis Through Several Mechanisms

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Two physiologically important processes occur throughout adult life in the bone marrow: hematopoiesis (the production of blood and immune cells) and osteogenesis (the production of bone cells). Consistent with this, the bone marrow contains two types of stem cells – hematopoietic stem cells (HSCs) and skeletal stem cells (SSCs, often also called mesenchymal stem cells). A major focus of our laboratory has been to identify the cells that create the HSC niche – the specialized microenvironment that maintains HSCs by producing the factors they require. We discovered the Leptin Receptor-expressing (LepR$^+$) stromal cells that are a key element of the HSC niche: they are the major source of known factors for the maintenance of hematopoietic stem and progenitor cells in the bone marrow. LepR$^+$ cells also include the SSCs that are the major source of osteoblasts and adipocytes in adult bone marrow. Beyond these functions, LepR$^+$ cells regulate the bone marrow environment through several other mechanisms, including sensing and mediating the effects of load-bearing exercise on osteogenesis and lymphopoiesis, promoting vascular regeneration after myeloablation, and suppressing bone marrow inflammation. LepR$^+$ cells thus sustain HSCs and modulate adult osteogenesis through several different kinds of mechanisms.

The HSC niche: In 2005 we discovered that HSCs reside immediately adjacent to sinusoidal blood vessels in hematopoietic tissues, including in the bone marrow and spleen (Kiel et al., 2005). Sinusoids are a specialized form of venuole that is only present in hematopoietic tissues. Based on that observation, we proposed the existence of a perivascular niche for HSCs around the sinusoids. At the time, this was very controversial as the leading

(osteoblastic niche) model was quite different. During the subsequent 10 years we critically tested both models, ultimately proving that HSCs do reside in perisinusoidal niches (Figure 1) and disproving several key elements of the osteoblastic niche model (Morrison and Scadden, 2014).

One important step toward the identification of the HSC niche was identifying the stromal cells that are the critical source of factors for HSC maintenance. We discovered the LepR$^+$ mesenchymal stromal cells that surround sinusoids and arterioles throughout the bone marrow and that are the main source of known factors that are required for the maintenance of HSCs, including Stem Cell Factor (SCF) and the chemokine Cxcl12. To test whether LepR$^+$ cells were functionally important sources of factors for the maintenance of HSCs, we conditionally deleted *Scf* (Ding et al., 2012), *Cxcl12* (Ding and Morrison, 2013; Greenbaum et al., 2013) and other proposed niche factors (Zhou et al., 2015) from LepR$^+$ cells as well as all of the other candidate niche cell populations that had been proposed by other labs. This analysis showed that LepR$^+$ cells and endothelial cells (which expressed much lower levels of these factors) were functionally important sources of factors for the maintenance of HSCs. When we deleted *Scf* from LepR$^+$ cells and endothelial cells, all of the quiescent and serially transplantable HSCs disappeared from adult bone marrow, demonstrating that all

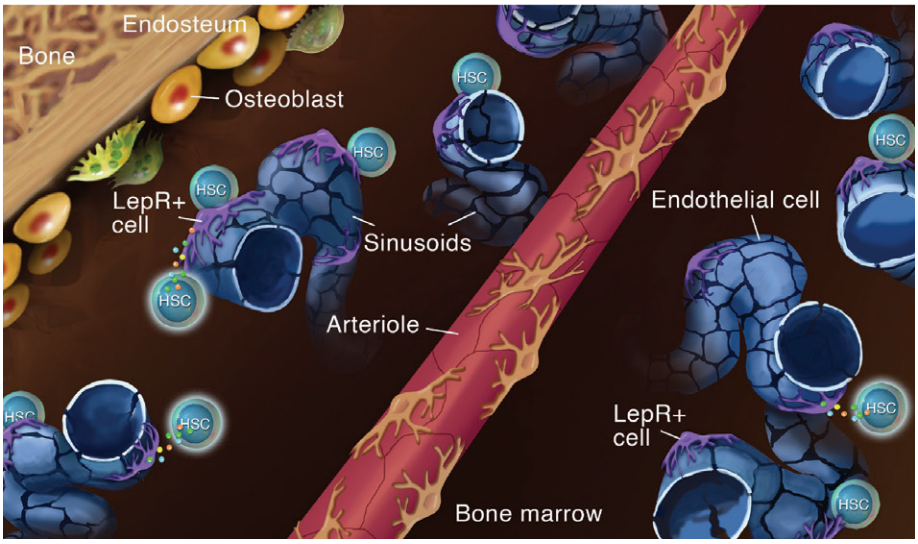

Figure 1. HSCs reside in perivascular niches in which factors required for HSC maintenance are produced by LepR+ cells and endothelial cells.

quiescent bone marrow HSCs rely upon perivascular niches for their maintenance (Oguro et al., 2013). Conversely, other cell populations that had been proposed as potential niche cells, such as osteoblasts, expressed little or none of these niche factors. Conditional deletion of these factors from these candidate niche cells had no effect on HSCs in adult bone marrow.

HSCs are adjacent to sinusoidal blood vessels (blue) throughout the marrow (Acar et al., 2015; Kiel et al., 2005), where LepR$^+$ cells and endothelial cells maintain HSCs by producing SCF (Ding et al., 2012), CXCL12 (Ding and Morrison, 2013; Greenbaum et al., 2013), and other factors (Fang et al., 2020; Himburg et al., 2018).

We went on to show that restricted progenitors reside in cellularly and spatially distinct niches as compared to HSCs. For example, a subset of common lymphoid progenitors reside in osteoblastic niches, at the endosteum (the interface of bone and bone marrow) where they derive factors for their maintenance from osteoblasts (Ding and Morrison, 2013; Greenbaum et al., 2013). LepR$^+$ cells also synthesize SCF for the maintenance of many restricted hematopoietic progenitors, including early myeloid, lymphoid, and erythroid progenitors (Comazzetto et al., 2019; Cordeiro Gomes et al., 2016). At least some of these restricted progenitors localize adjacent to sinusoidal blood vessels, like HSCs (Comazzetto et al., 2019).

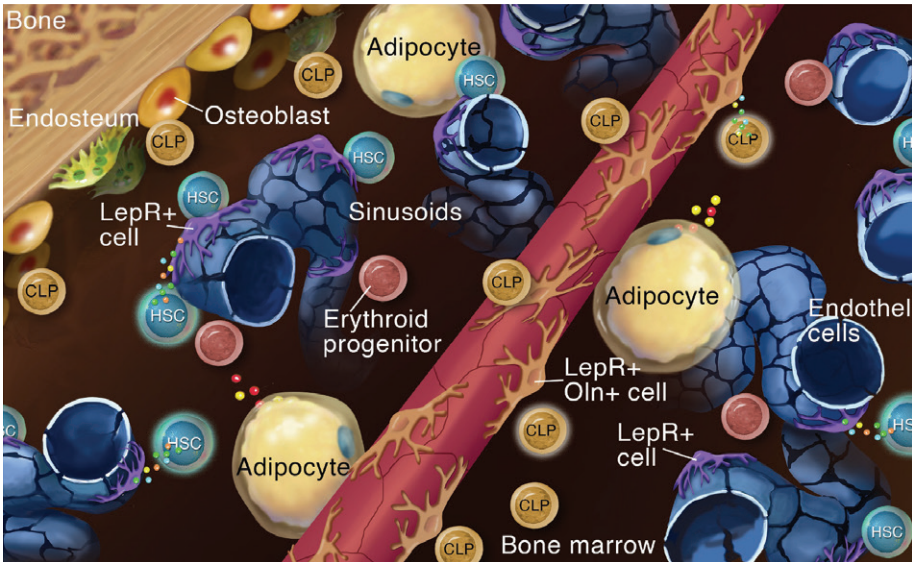

Figure 2. Distinct niches for HSCs and restricted progenitors in the bone marrow.

Daniel Lucas' laboratory extended these results by showing that there are likely to be distinct domains along the sinusoids in which different kinds of myeloid progenitors reside within distinct perisinusoidal neighborhoods that are specialized for cells at different stages of myeloid differentiation (Zhang et al., 2021). This suggests there are different kinds of $LepR^+$ cells that are specialized to create different kinds of niches in distinct locations within the bone marrow (Comazzetto et al., 2021).While HSCs and erythroid progenitors are sustained in peri-sinusoidal niches (blue) by factors from $LepR^+$ cells and endothelial cells (Comazzetto et al., 2019; Ding and Morrison, 2013; Ding et al., 2012) (colored dots), early lymphoid progenitors (CLPs) are sustained in peri-arteriolar niches (red blood vessel) by SCF from $Osteolectin^+LepR^+$ cells that proliferate in response to mechanical activation of Piezo1 (Shen et al., 2021). Other CLPs are sustained in endosteal niches by CXCL12 from osteoblasts (Ding and Morrison, 2013; Greenbaum et al., 2013). Adipocytes promote HSC regeneration by producing SCF and other factors (Zhou et al., 2017).

Consistent with these conclusions, recent single cell RNA sequencing studies of bone marrow stromal cells have confirmed that $LepR^+$ cells express the highest levels of niche factors within the bone marrow but that these cells are quite heterogeneous (Baccin et al., 2020; Baryawno et al., 2019; Tikhonova et al., 2019).

Skeletal stem cells (SSCs) in adult bone marrow: $LepR^+$ cells include the SSCs that give rise to all of the adipocytes and osteoblasts that form in adult bone marrow (Zhou et al., 2014). When bone marrow cells are cultured adherently to form mesenchymal stem cells, these mesenchymal stem cells arise from the $LepR^+$ cells (Zhou et al., 2014). The physiological function of the $LepR^+$ SSCs in the bone marrow is to give rise to the osteoblasts that contribute to the maintenance and repair of the adult skeleton (Zhou et al., 2014) and to form the adipocytes that accumulate during aging or after myeloablation (Zhou et al., 2017). $LepR^+$ cells, therefore, not only regulate HSC maintenance and hematopoiesis but are also a critical source of osteoblasts and adipocytes in adult bone marrow.

Peri-sinusoidal $LepR^+$ cells create niches for HSCs and certain restricted progenitors, including erythroid progenitors (Comazzetto et al., 2019). The adipocytes formed by adipocyte progenitors promote HSC maintenance and hematopoietic regeneration by synthesizing SCF (Zhou et al., 2017). The $LepR^+Osteolectin^+$ progenitors create a peri-arteriolar niche for early lymphoid progenitors as well as giving rise to osteoblasts that maintain the adult skeleton (Shen et al., 2021). Osteoblasts create a distinct

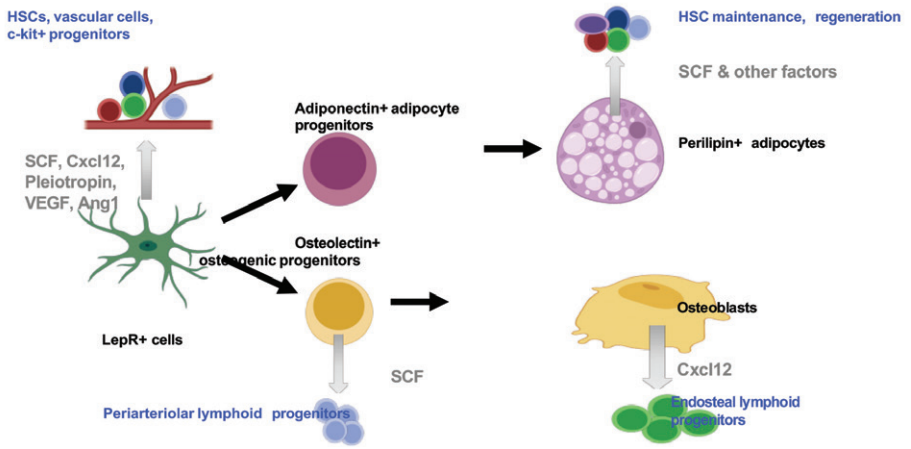

Figure 3. LepR+ cells in adult bone marrow give rise to adipocyte progenitors and osteogenic progenitors (Shen et al., 2021; Zhou et al., 2017; Zhou et al., 2014).

endosteal niche for early lymphoid progenitors at the interface of bone and bone marrow (Ding and Morrison, 2013; Greenbaum et al., 2013).

LepR$^+$ cells are also a critical source of growth factors that regulate osteogenesis. In an effort to identify new growth factors, we performed RNA sequencing on bone marrow LepR$^+$ cells. We looked for transcripts that are preferentially expressed by these cells and predicted to encode growth factor-like secreted proteins whose function had not been studied *in vivo*. This led to the identification of a new bone-forming growth factor that we named *Osteolectin*. Osteolectin promotes the maintenance of adult skeletal bone mass by acting on LepR$^+$ cells to promote their differentiation into osteoblasts (Yue et al., 2016).

Osteolectin is a C-type lectin domain protein, originally named CLEC11a (Bannwarth et al., 1998; Mio et al., 1998) that is expressed by a subset of LepR$^+$ cells as well as by osteoblasts, osteocytes, and chondrocytes (Yue et al., 2016). Others had detected Osteolectin/CLEC11a expression in the bone marrow and presumed it to be a hematopoietic growth factor (Hiraoka et al., 1997). We generated Osteolectin-deficient mice and found they are developmentally normal, with normal hematopoiesis (Yue et al., 2016); however, they exhibit accelerated bone loss throughout adulthood and delayed fracture healing. Osteolectin-deficient bone marrow stromal cells show impaired osteogenic differentiation, but normal adipogenic and chondrogenic differentiation. Recombinant Osteolectin promotes osteo-

genesis by LepR$^+$ cells in culture and injection of the recombinant protein into mice systemically increases bone formation.

Osteolectin thus maintains the adult skeleton by promoting the differentiation of LepR$^+$ cells and other mesenchymal progenitors into mature osteoblasts.

We identified α11β1 integrin as the Osteolectin receptor (Shen et al., 2019). α11 integrin is highly restricted in its expression to osteogenic cells, including LepR$^+$ cells and osteoblasts. α11β1 binds *Osteolectin* with nanomolar affinity and is required for the osteogenic response to Osteolectin (Shen et al., 2019). Like Osteolectin-deficient mice, *Lepr-cre*; *α11fl/fl* mice are grossly normal but exhibit reduced osteogenesis and accelerated bone loss during adulthood (Shen et al., 2019). Osteolectin binding to α11β1 promotes Wnt pathway activation, which is necessary for the osteogenic response to Osteolectin.

Osteolectin appears to mediate much of the osteogenic effect of parathyroid hormone (PTH), an agent widely used to treat osteoporosis (Zhang et al., 2021). PTH promotes Osteolectin expression by bone marrow stromal cells and increases serum Osteolectin levels in mice and humans. Osteolectin deficiency attenuates Wnt pathway activation by PTH in bone marrow stromal cells and substantially reduces the osteogenic response to PTH *in vitro* and *in vivo*. The identification of Osteolectin, and its receptor α11β1, thus revealed a new mechanism that is necessary for the maintenance of adult skeletal bone mass, fracture repair, and the response to an anabolic factor that is used to treat osteoporosis.

LepR$^+$ cells mediate the effects of the bone marrow mechanical environment on osteogenesis and lymphopoiesis: One of the primary recommendations to people with osteoporosis is to engage in load-bearing exercise, because this increases the production of bone cells, thickening and strengthening of bones. However, the mechanism by which load-bearing exercise increases bone formation has been unclear.

We generated Osteolectin reporter mice to identify the LepR$^+$ cells in the bone marrow that synthesize Osteolectin. We found that Osteolectin expression distinguishes peri-arteriolar LepR$^+$ cells poised to undergo osteogenesis from peri-sinusoidal LepR$^+$ cells poised to undergo adipogenesis (Shen et al., 2021). We found that peri-arteriolar LepR$^+$Osteolectin$^+$ cells are rapidly-dividing, short-lived, osteogenic progenitors that increase in number after fracture and are depleted during aging (Figure 3). *Scf* deletion from peri-arteriolar Osteolectin$^+$ cells does not affect the maintenance of HSCs or most restricted hematopoietic progenitors but it depletes common

lymphoid progenitors, demonstrating the existence of a periarteriolar niche for lymphoid progenitors created by Osteolectin$^+$ cells. Deletion of *Scf* from these Osteolectin$^+$ cells broadly depletes lymphoid progenitors, impairing lymphopoiesis and survival after acute bacterial infection (Shen et al., 2021).

Remarkably, voluntary running increases, and hindlimb unloading decreases, the frequencies of peri-arteriolar Osteolectin$^+$ cells and lymphoid progenitors in the bone marrow, demonstrating mechanical regulation of periarteriolar niches. Our results suggest that mechanical forces are transmitted along arterioles, from bone surfaces into the marrow, where they are sensed by LepR$^+$Osteolectin$^+$ cells, increasing the division of these cells and expanding the numbers of osteogenic and lymphoid progenitors in the periarteriolar niche. The mechanism by which Osteolectin$^+$ cells sense these mechanical signals involves opening of the Piezo1 mechanically-regulated ion channel. Deletion of Piezo1 from Osteolectin$^+$ cells depletes Osteolectin$^+$ cells and lymphoid progenitors around arterioles. This identified a new mechanism by which load-bearing exercise promotes osteogenesis and immune function. LepR$^+$ cells thus sense the mechanical environment in the bone marrow and transduce mechanical signals into niche factor expression in a manner that regulates both osteogenesis and lymphopoiesis.

LepR$^+$ cells regulate vascular regeneration in the bone marrow: Irradiation and chemotherapy not only deplete HSCs but also disrupt their niche in the bone marrow, particularly damaging the sinusoids (Hooper et al., 2009; Knospe et al., 1966; Kopp et al., 2005; Li et al., 2008).

Regeneration of the perivascular niche after injury, including endothelial and LepR$^+$ cells, is necessary for the regeneration of HSCs and hematopoiesis after myeloablation (Hooper et al., 2009; Kopp et al., 2005). After 5-fluorouracil treatment, Tie2 signaling (which is regulated by its ligands Angpt1, Angpt2, and possibly Angpt3 (also known as Angptl1) (Augustin et al., 2009; Eklund and Saharinen, 2013; Fagiani and Christofori, 2013; Thomson et al., 2014)) regulates the remodeling of blood vessels in the bone marrow (Kopp et al., 2005).

We systematically assessed the expression and function of *Angiopoietin-1* (*Angpt1*) in bone marrow (Zhou et al., 2015). *Angpt1* was not expressed by osteoblasts. *Angpt1* was most highly expressed by HSCs, and at lower levels by c-kit$^+$ hematopoietic progenitors, megakaryocytes, and LepR$^+$ stromal cells. Global conditional deletion of *Angpt1,* or deletion from osteoblasts, LepR$^+$ cells, *Nestin-cre*-expressing cells, megakaryocytes, endothelial cells or hematopoietic cells in normal mice did not affect hematopoiesis, HSC maintenance, or HSC quiescence.

Deletion of *Angpt1* from hematopoietic cells and LepR$^+$ cells had little effect on vasculature or HSC frequency under steady-state conditions but accelerated vascular and hematopoietic recovery after irradiation while increasing vascular leakiness. Hematopoietic stem/progenitor cells and LepR$^+$ stromal cells thus regulate vascular and niche regeneration by secreting Angpt1, reducing vascular leakiness but slowing niche recovery. LepR$^+$ cells and endothelial cells also promote the regeneration of sinusoids after myeloablation by synthesizing VEGF-C (Fang et al., 2020).

LepR$^+$ cells and adipocytes maintain quiescent HSCs by suppressing inflammation: Adiponectin is a circulating factor that suppresses inflammation (Berg et al., 2001; Fruebis et al., 2001; Yamauchi et al., 2001). It is synthesized by adipocytes throughout the body (Hu et al., 1996; Maeda et al., 1996; Nakano et al., 1996; Scherer et al., 1995) as well as by LepR$^+$ cells and adipocytes in the bone marrow (Baccin et al., 2020; Baryawno et al., 2019; Tikhonova et al., 2019. Adiponectin suppresses the activation of macrophages (Ohashi et al., 2010; Yamaguchi et al., 2005), NK cells (Wilk et al., 2013), and T cells (Surendar et al., 2019) through multiple mechanisms, reducing their production of inflammatory factors, including IFNy (Surendar et al., 2019) and TNF (Maeda et al., 2002; Masamoto et al., 2016; Ohashi et al., 2010). Adiponectin deficiency has been reported to have no effect on HSCs or hematopoiesis in the bone marrow of specific pathogen free mice but after bacterial infection, adiponectin promotes hematopoietic progenitor proliferation by suppressing TNF expression (Masamoto et al., 2016).

The bone marrow becomes more inflammatory during aging (Chambers et al., 2007; Ergen et al., 2012; Henry et al., 2015; Valletta et al., 2020; Yamashita and Passegue, 2019; Young et al., 2021). Inflammatory factors promote HSC activation and chronic inflammation promotes HSC depletion (Baldridge et al., 2010; Essers et al., 2009; Matatall et al., 2016; Pietras et al., 2016). However, HSCs remain mainly quiescent (Pietras et al., 2011) and increase in number with age in most mouse strains (Morrison et al., 1996). This suggests the existence of mechanisms to protect HSCs from chronic inflammation in adult bone marrow. Regulatory T cells protect HSCs from immune cells after allogeneic transplantation (Fujisaki et al., 2011; Hirata et al., 2018), raising the question of whether there are factors that protect HSCs from immune cells and sustain HSC quiescence in normal adult bone marrow.

Adiponectin binds two receptors, AdipoR1 and AdipoR2, which have ceramidase activity that increases upon adiponectin binding. We found adiponectin receptors are non-cell-autonomously required in hematopoie-

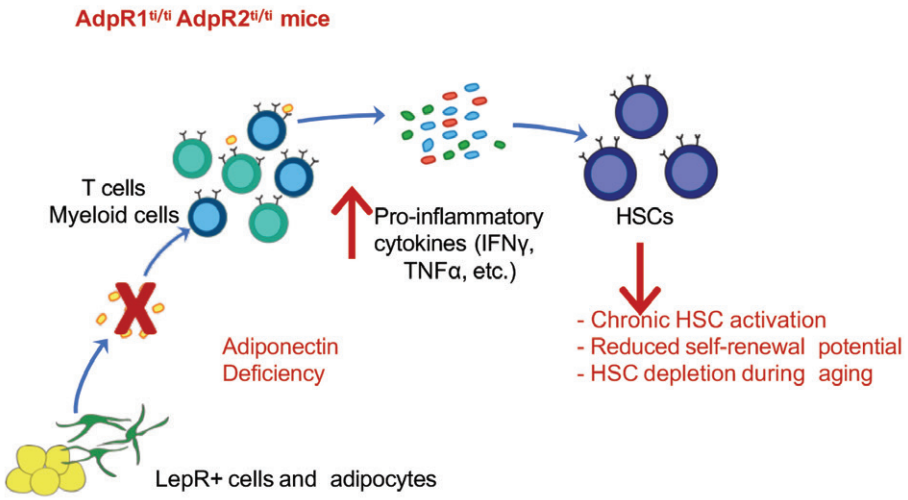

Figure 4. A complex combination of cell-intrinsic and cell-extrinsic mechanisms sustain adult HSCs by suppressing inflammation, including adiponectin expression by adipocytes and LepR+ cells.

tic cells to promote HSC quiescence and self-renewal (Meacham et al., 2022). Adiponectin receptor signaling suppresses inflammatory cytokine expression by myeloid cells and T cells, including interferon gamma (IFNy) and tumor necrosis factor (TNF). Without adiponectin receptors, the levels of these factors increase, chronically activating HSCs, reducing their self-renewal potential, and depleting them during aging. Pathogen infection accelerates this loss of HSC self-renewal potential. Blocking IFNy or TNF signaling partially rescues these effects. Adiponectin receptors are thus required in immune cells to sustain HSC quiescence and to prevent premature HSC depletion by reducing inflammation.

In adiponectin or adiponectin receptor deficient mice, myeloid cells and T cells secrete inflammatory cytokines, chronically activating HSCs, reducing their self-renewal potential, and depleting them during aging. Niche cells thus sustain adult HSCs by suppressing inflammation by immune effector cells.

Conclusions

LepR$^+$ stromal cells regulate hematopoiesis and osteogenesis in the bone marrow through several different kinds of mechanisms:

1. They are the main source of factors required for the maintenance of

HSCs and several early restricted progenitors, including SCF and Cxcl12 (Ding and Morrison, 2013; Ding et al., 2012).
2. They regulate vascular regeneration after myeloablation by synthesizing Angpt1 (Zhou et al., 2015) and VEGF-C (Fang et al., 2020).
3. A subset of LepR$^+$ cells are the SSCs that give rise to the osteoblasts and adipocytes that form in adult bone marrow (Zhou et al., 2014). LepR$^+$ cells are the bone marrow cells that give rise to mesenchymal stem cells in culture. The osteoblasts formed by LepR$^+$ cells contribute the maintenance of adult skeletal bone mass and the repair of certain kinds of bone injuries. The adipocytes formed by LepR$^+$ cells promote the regeneration of HSCs and hematopoiesis after myeloablation (Zhou et al., 2017).
4. LepR$^+$ cells secrete bone-forming growth factors that are necessary for the maintenance of adult skeletal bone mass, including Osteolectin (Shen et al., 2019; Yue et al., 2016).
5. LepR$^+$ cells mediate the effects of load-bearing exercise on osteogenesis and lymphopoiesis by sensing mechanical forces transmitted along arterioles in the bone marrow as a result of opening of the Piezo1 mechanically activated cation channel (Shen et al., 2021).
6. LepR$^+$ cells and adipocytes suppress inflammation in adult bone marrow by secreting adiponectin, which suppresses the expression of inflammatory cytokines by immune effector cells (Meacham et al., 2022). This is necessary for the maintenance of quiescent HSCs throughout adult life.

Acknowledgements

This review summarizes a lecture given at a Pontifical Academy of Sciences Workshop on "Stem Cells and Their Promise for Regenerative Medicine" in Vatican City in May 2022. S.J.M. is a Howard Hughes Medical Institute (HHMI) Investigator, the Mary McDermott Cook Chair in Pediatric Genetics, the Kathryn and Gene Bishop Distinguished Chair in Pediatric Research, the director of the Hamon Laboratory for Stem Cells and Cancer, and a Cancer Prevention and Research Institute of Texas Scholar. This work was supported by the National Institutes of Health (DK118745), the Moody Medical Research Institute, the Josephine Hughes Sterling Foundation, and the Robert J. Kleberg, Jr. and Helen C. Kleberg Foundation (all to S.J.M).

C.E.M. performed some of the work described in this review and helped to write the review. Her work was supported by a Postdoctoral Fellowship from the American Cancer Society (PF-13-245-01-LIB).

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Innate Allergy and Intestinal Cell Fate

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Immune cells infiltrate all organs of the body. Traditionally identified by roles in host defense, tissue immune cells are now known to contribute to homeostasis of stem cell niches in organs throughout life. Positioned during embryogenesis, innate immune cells derived from yolk sac and fetal liver are gradually replaced by cells derived from adult bone marrow, creating cellular layers through ontogeny that interact to respond to local perturbations ranging from circadian to metabolic to pathologic events. Helper lymphocytes elaborate cytokines using canonical modules that orchestrate interactions among lymphoid, myeloid and non-hematopoietic tissue cells. Although lacking antigen receptors, innate helper lymphocytes, or ILCs, are abundant in small intestine, where allergy-associated ILC2s contribute to small intestine physiology by altering epithelial cell fate after feeding using a pathway hijacked by intestinal helminths. Uncovering immune pathways involved in organ homeostasis may lead to understanding diseases of increasing prevalence, such as food allergy, and might be exploited to enhance healthspan.

The foundations of immunology were advanced by recognition of the fatal infectious complications accompanying genetic loss of rearranging T and B cell antigen receptors that pioneered bone marrow, hematopoietic stem cell, and gene replacement therapies that are covered by others in this Symposium. Successes drove the focus of immunology towards understanding the processes of adaptive immunity that generate the rearranged receptors necessary to protect from environmental pathogens, and that remain the goal of successful vaccines. A prescient contribution by Charlie Janeway from a 1989 Cold Spring Harbor Symposium addressed immunologists' 'dirty little secret' that successful immunization required administration of the antigen with an 'adjuvant', usually containing microbial products from mycobacteria or pertussis with alum (1). Janeway pointed out that randomly generated receptors could not alone discriminate 'non-self' from 'self' antigens and reasoned that evolutionarily encoded receptors that recognized conserved molecules from infectious organisms likely occur at the time of antigen receptor engagement, thus guiding the T cell towards meaningful commitment and avoidance of autoimmuni-

ty. Janeway's insights accounted for the adjuvant requirement and led to discovery of what was later designated 'signal 2' required for successful T cell signaling. Later studies from his lab and others led to the discovery of Toll-like receptors, a family of leucine-rich repeat proteins that decorate macrophages, dendritic cells and B cells, which constitute the major cells that present peptide antigens to helper T cells. Toll-like receptors (TLRs), deeply rooted in evolution and named for Drosophila Toll involved in dorsal-ventral patterning and (shown later) host defense, sparked a frenzy of research, resulting in not only Nobel Prizes but also discovery of families of Pattern Recognition Receptors (PRRs) arrayed across the surface, cytosol and endosomal vacuoles of human cells, including 10 TLRs, ~15 C-type lectin, ~15 nucleotide oligomerization domain (NOD)-like and ~15 RIG-I-like and AIM2-like nucleic acid receptors, that engage the spectrum of constituents from bacteria, fungi and viruses, and lead to elaboration of signal 2 required for optimal T cell activation. Modifying mRNA to bypass PRR engagement was a critical engineering feat in developing RNA vaccines against SARS-CoV-2 as recognized by the 2021 Lasker Award to Kariko and Weissman (2).

Seminal studies by Mossman and Coffman in the late 1980s from DNAX Institute at Schering-Plough identified subsets of T helper cells, designated type 1 and type 2, as distinguished by the groups of cytokines they produced (3). T helper 1 (Th1) cells secreted interferon-gamma (IFNg), involved in classical activation of myeloid cells for host defense, whereas T helper 2 (Th2) cells secreted IL-4, IL-5 and IL-13 involved in allergic immunity, including the alternative activation of macrophages, recruitment of eosinophils, and B cell switching to IgE antibodies that arm mast cells and basophils. Two decades later, Cua and Kastelein from DNAX pioneered studies leading to discovery of the third T cell subset and further work quickly elucidated the factors important for establishing and maintaining Th17 cells in mice and humans (4). Th17 cells produce IL-17 cytokines, which mediate neutrophil accumulation associated with clearance of cell debris and microbes during acute injury, and IL-22, which induces epithelial cell proliferation and secretion of antimicrobial peptides.

The discovery of subsets of T helper cells pushed identification of the transcriptional programs that establish the three effector cytokine modules, eventually resulting in the epigenetic model of T helper cell differentiation by which naïve CD4 helper T cells are instructed by dendritic cells and PRR-elicited signals to mature into effector T cells that migrate to tissues to elaborate their respective cytokines. Further study revealed that not only

adaptive CD4 T cells selected by peptides embedded in classical MHC molecules, but also unconventional CD4 T cells, including NKT cells, MAIT cells and gd T cells expressing receptors selected on noncanonical MHC molecules, could be grouped according to the three helper cell subsets (5). Finally, a group of innate lymphoid cells, later designated ILCs (6), which lack expression of antigen receptors altogether, was recognized over years of study, culminating with the discovery of 'allergic-like' ILC2s in 2010, to complete the three canonical groups based on core transcription factor dependence and cytokine outputs that mirrored those of adaptive and unconventional T cell subsets. Thus, three stereotyped outputs characterize effector mechanisms by which helper lymphocytes confront perturbations in body tissues (here, we won't consider regulatory and follicular T cells, which mainly communicate with other lymphocytes). The lack of antigen receptors on ILCs creates the opportunity to uncover tissue signals that activate these pathways and align with the demands of basal homeostasis (7).

Outputs from tissue resident helper lymphocytes organize acute response to injury (type 3 associated with IL-17/IL-22-associated immunity) and recall (type 1 associated with IFNg and TNFa), and can be conceptualized respectively as acute neutrophilic responses, whether sterile or infectious, and memory, by which host tissues respond more quickly to a second challenge as designated by 'trained' immunity (8). Type 3 and type 1 responses are initiated by PRRs arrayed on multiple cell types, including at epithelial barriers, which induce inflammatory cytokines to activate these distinct immune modules in innate and adaptive immune cells. The third output, associated with allergy, is less obviously associated with host health, and is proposed to organize aversive responses (e.g.; itch, cough, vomiting, diarrhea, etc.) associated with noxious environmental insults but also epithelial adaptations (e.g.; increased mucus) (9). Type 2 responses are initiated by a group of cytokines designated 'alarmins', that are expressed from distinct types of sentinel cells in response to perturbations of homeostasis. Receptors for alarmin cytokines are expressed constitutively on tissue resident ILC2s, positioning these cells as integrated sensors of tissue disruption. Multiple functional and GWAS studies have implicated alarmin cytokines like IL-33, TSLP, IL-25 and IL-18 or their receptors in allergic pathology, and therapeutics targeting alarmins are active against allergic diseases.

Innate lymphoid cells emerge from fetal liver around E14 in the mouse and at comparable periods in human fetal development around the time of villus initiation in the small intestine. Like macrophages, ILC2s expand in overlapping waves during fetal, postnatal and adult life, enter tissues, prolif-

erate and activate tissue-specific transcriptomes before adopting a predominantly tissue-resident state (10). Largely maintained by locally deposited and self-renewing precursors, turnover from adult-derived ILC2 precursors occurs with variable kinetics that generally reflect epithelial turnover in the tissue of residence, with more rapid turnover in intestine and skin and slower turnover in adipose and lung. Upon perturbations that stimulate continued activation, ILC2s proliferate and enter the blood, resulting in circulating ILC2s and cytokines that mediate effects at distal tissues (11). The potent cytokine potential of ILC2s has implicated these cells in pathologic allergic states while increasing studies have begun to implicate these cells in tissue homeostasis (12). Numbers of tissue ILC2s are relatively unaffected in germfree mice and fetal human small intestine contains IL-13+ ILC2s, suggesting that tissue residency and cytokine profiles are independent of the microbiota.

Our interest in the role of ILC2s in small intestine physiology began with investigations of eosinophil circadian variation, which was driven by metabolic rather than circadian cues (13). Activated ILC2s produce the eosinophil survival factor, IL-5, and IL-13, which induces release of eosinophilic chemotactic factors by stromal cells, suggesting that activation of intestinal ILC2s with feeding underpinned the oscillations by which blood eosinophils diminish with feeding and increase with fasting. Indeed, fasting was associated with diminished IL-5 production by lamina propria ILC2s, which increased and was accompanied by IL-13 production with feeding, driven in part by induction of the neuropeptide VIP. Thus, feeding induced small intestinal ILC2 activation, production of IL-5 and IL-13, and entry of blood eosinophils into gut tissue, consistent with the decrease in circulating eosinophils in response to nutrient intake. Intestinal eosinophils may be important in tissue remodeling in response to luminal perturbations through release of proteases and growth factors, and further research is needed to understand the basic biology of these cells and their involvement in normal gut physiology.

Although ILC2s integrate multiple synergistic signals from tissues to activate and produce cytokines, the alarmin cytokines IL-33, IL-25 and TSLP constitute key contributors; in the absence of all 3 signaling pathways, activation of both innate ILC2s and adaptive Th2 cells is greatly attenuated in most tissues (14). Single-cell sequencing studies revealed high expression of the IL-25 receptor on small intestine lamina propria ILC2s (15), and epithelial tuft cells were subsequently identified as the unexpected source of IL-25 (16,17). Tuft cells and goblet cells expand remarkably

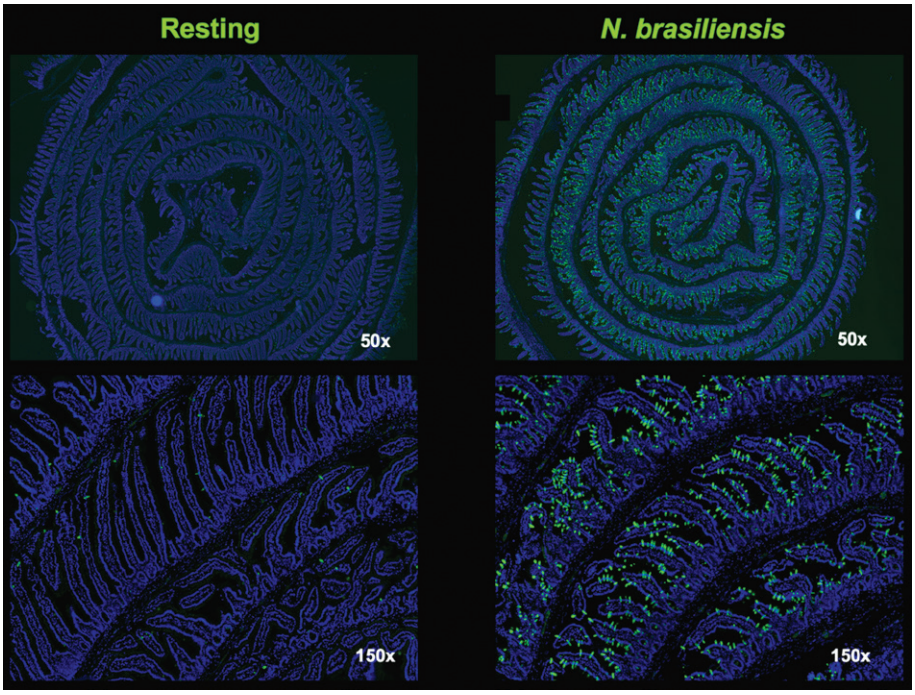

Figure 1. Coiled small intestinal 'Swiss' rolls from uninfected mice (Resting) or mice 7 days after infection with a parasitic nematode, *Nippostrongylus brasiliensis*, with tuft cells (green) delineated along the villi.

after challenge with parasitic helminths, which elicits proliferation and activation of small intestinal ILC2s (Fig. 1). Recognized over 60 years ago by their blunt, long apical microvilli extending into the hollow lumen, tuft cells are rare chemosensory cells in most mucosal epithelia of vertebrate organs, including upper and lower respiratory tract, gastrointestinal and parts of the genitourinary tract, and within endodermal-derived medullary epithelial cells of the thymus. Intestinal tuft cells are post-mitotic cells derived from columnar crypt stem cells and typically turnover during the normal 3-5 days of villus transit from crypt to apex. Tuft cells in other tissues are long-lived, reflecting slower epithelial turnover in organs like lung or gallbladder. Tuft cells use taste-associated signaling from upstream GPCRs via the Ca++-activated cation channel TRPM5 to depolarize and activate canonical effector outputs, including IL-25, eicosanoids including leukotrienes and prostaglandin D2, ATP and acetylcholine, reflecting unique outputs among epithelial cells (18).

Although historically given distinct names in different tissues, such as tuft cells in gut, brush cells in trachea, and microvillus cells in the oronasal epithelia, all tuft cells depend on expression of the transcription factor Pou2F3, suggesting that epithelial cells that depend on Pou2F3 and express typical taste cell signaling pathways and these canonical outputs can all be considered members of the tuft cell family, a designation that includes type 2 taste cells which sense umami, sweet and bitter (19). Expression of various taste and vomeronasal receptors, as well as multiple other GPCRs, suggests that tuft cells are chemosensory cells arrayed to detect luminal signals and transfer information to host cells, which in small intestine serves to change the epithelial boundary by altering cell fate among transit amplifying cells.

Constitutive expression of IL-25 in tuft cells suggests that ILC2 activation initiates a feed-forward circuit to increase tuft and goblet cell numbers until the luminal signal becomes attenuated. ILC2 activation is driven both by IL-25 from the increased numbers of tuft cells but also by release of cysteinyl leukotrienes from tuft cells that synergize to drive proliferation and cytokine production. The resultant epithelial alterations account for the increased goblet cell and mucus response that accompany intestinal helminth infection. These effects were genetically traced to IL-13 generated by ILC2s downstream of IL-25 in tuft cells and upstream of direct effects of IL-13 on epithelia, as shown the ability of exogenous IL-13 to increase tuft cell numbers in organoids that was dependent on epithelial expression of IL-4Ra, the signaling component of the IL-13 receptor. Deleting the ubiquitin-modifying enzyme A20 (TNFAIP3), a negative regulator of IL-25 signal transduction, in ILC2s caused spontaneous IL-25-mediated small intestinal adaptation in mice characterized by lengthening and increased muscle mass with elevated numbers of ILC2s and tuft cells, thus attesting to the constitutive activity of this pathway *in situ* (20). These small intestinal adaptations were stable over time and associated with persistent alterations in tuft cell and ILC2 numbers. The affected mice were resistant to subsequent helminth infection explaining an observation termed 'concomitant' immunity by which intestinal helminth infection establishes a resistant state that impedes maturation of new eggs or larvae from the same or even different helminths. Intriguingly, resistance to injury at distal mucosal sites, such as lung and conjunctivae, also occurs, reflecting the circulation in blood of ILC2s and their cytokines that accompany proliferation and egress from perturbed tissues (11,21).

The appearance of mice with increased numbers of tuft cells in several academic research facilities led to identification of infection by unsuspect-

ed cecal *Tritrichomonas muris*, a parabasalid protist widespread in feral animals, that, like helminths, induces the small intestinal tuft cell-ILC2 circuit (22). *T. muris* are obligate anaerobes with an array of enzymes that degrade complex plant polysaccharides; related Tritrichomonads constitute the cellulose-degrading organisms found in various species of termites. Rather than mitochondria, protists use hydrogenosomes, which lack the electron transport chain, to generate ATP via decarboxylation and oxidation of pyruvate resulting in production of the metabolites acetate and succinate. Small intestine tuft cells prominently express GPR91, the succinate receptor, and succinate was sufficient to induce tuft cell IL-25, ILC2 activation and initiation of circuit amplification that was lost after deletion of tuft cells, TRPM5 or IL-25, establishing a role for small intestine tuft cells in luminal succinate sensing (20). Additional GPCRs are being discovered that define the chemosensory spectra of not only intestinal tuft cells, but also tuft cells in other organs that express diverse GPCRs that establish the repertoire by which these epithelial sensors deconvolute their microenvironment.

Helminth parasites and protists, widespread in the animal kingdom, likely evolved to induce the adapted intestinal state to facilitate generation of eggs and larvae while avoiding immune attack and generating a host niche resistant to further colonization. While intestinal parasitism has decreased, the rising prevalence of diseases like food allergy in Westernized cultures is ascribed to the 'hygiene hypothesis', which proposes aberrant immune deviation driven by exposure to microbiota and nutrients that deviate from developmental programs initiated during embryogenesis and early perinatal life as established earlier in evolutionary history (23). Food allergy, an immune attack on ingested nutrients resulting in symptoms ranging from bloating and hives to life-threatening anaphylaxis, impacts almost 10% of children in the United States at great economic cost. Animal models and human studies implicate type 2 immunity in food allergy, often driven by IgE-mediated recognition of harmless ingredients shared by common food groups. The rising prevalence of food allergy raises the possibility that this pathologic response reflects dysregulation of a physiologic role for type 2 immunity in monitoring food quality, perhaps driven by metabolites or unnatural chemical constituents in modern processed diets (24). In this model, positive food qualities are sensed by enterocytes and enteroendocrine cells whereas negative food qualities are sensed by chemosensory cells like tuft cells and serotonin- and histamine-secreting enterochromaffin cells; both become 'coded' and reinforced by neural circuitry. Whereas some ingestants can be directly sensed by genetically encoded

receptors like GPR91 for succinate or bitter receptors on type 2 taste cells, akin to PRRs in type 1 and type 3 immunity, others become 'associated' with negative outcomes during ingestion and 'tagged' through induction of adaptive type 2 immunity, leading to IgE and the arming of mucosal mast cells that degranulate and mediate aversive responses upon subsequent exposure. Thresholds for detection of high-quality nutrients or noxious ingestants likely differ, with higher sensitivity for the latter necessary to protect the host from harmful entities hidden in food. As such, the ILC2-tuft cell feed-forward circuit would represent a food-triggered amplification system enacted to increase detection of potentially toxic constituents once the threshold for ingestion has occurred. Intestinal pathobionts have hi-jacked the process to drive intestinal adaptation and enhance host resilience in support of their reproductive niche. Indeed, intestinal parasitic infection promoted colonization resistance from bacterial pathogens and attenuated inflammatory bowel disease pathology (25,26).

An additional component of the small intestinal feeding response consists of anticipatory propagation of information via hormone and neural circuitry to the distal small intestine to coordinate the response to food intake. A second ILC2 population in the small intestine resides in the muscularis mucosa embedded within the dense network of nerves and macrophages constituting the enteric nervous system. Expression of the IL-13 receptor on populations of enteric neurons (27) and macrophages, which communicate to regulate intestinal physiology, raises the possibility that the second ILC2 population plays a role in propagation of information to the enteric nervous system. In contrast to lamina propria ILC2s, which express the IL-25 receptor, muscularis ILC2s express the IL-33 receptor and produce IL-5 and IL-13 in response to this alarmin cytokine. Intestinal IL-33 is expressed in multiple cell types near the crypt base, including fibroblastic reticular cells and lymphatic endothelial cells in proximity to the enteric plexus. While we continue to refine this area of research, our working hypothesis suggests a three-part process by which luminal information regarding food quality is transferred to ILC2s and crypt transit-amplifying cells to ensure optimal detection and handling of both positive- and negative-quality ingestants by the small intestine, revealing an intrinsic system commandeered by luminal helminths to enable parasitism (Fig. 2).

In summary, an unusual subset of innate lymphocytes that produce cytokines typically associated with allergic diseases populates the small intestine in the lamina propria and muscularis during development and

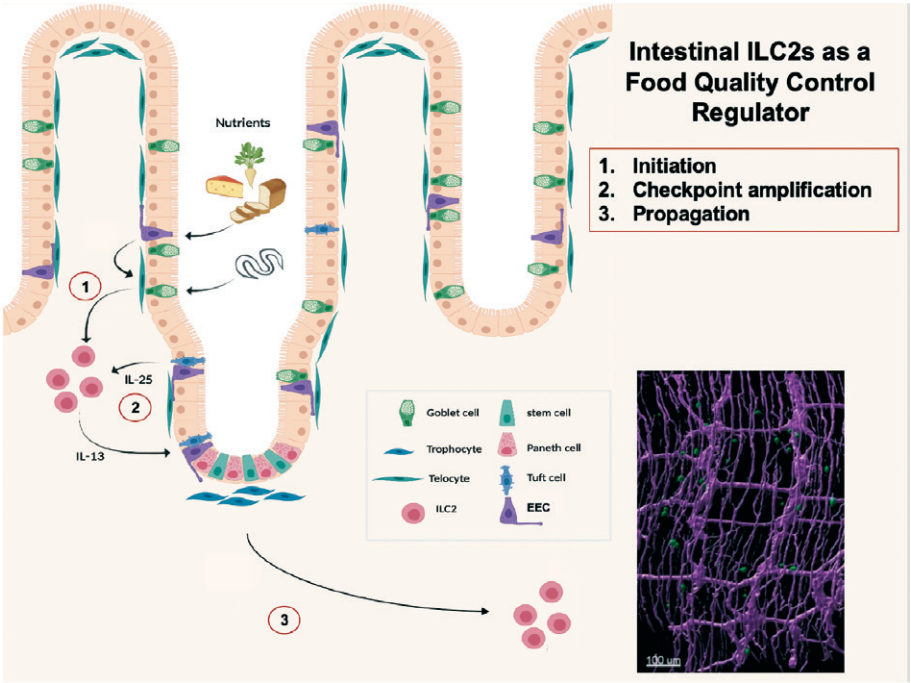

Figure 2. A potential signaling cascade activated by food in the small intestine. Parasites bypass the cascade and directly activate tuft cells to drive the circuit.
1. Intestinal nutrients signal through epithelial and submucosal lamina propria cells to activate ILC2s.
2. IL-13 initiates the IL-25 feed-forward tuft cell circuit.
3. Crypt-associated signals activate muscularis ILC2s (insert: green, ILC2s; purple, neurons) in the enteric plexus.

becomes activated to secrete cytokines after ingestion of food. Through a pathway that involves epithelial nutrient detection and relay through lamina propria support cells, intestinal ILC2s are activated to mediate a secretory cell bias among transit amplifying cells, thus increasing goblet and tuft cell production by a forward-amplifying circuit. Increased mucus and enhanced surface area and peristalsis impact gut physiology to optimize nutrient extraction while also increasing the detection capacity for ingested toxins and irritants. Further understanding the links between innate immune cells, stem cell outputs and small intestinal physiology promises to reveal insights into intestinal health and disease. Innate tissue-resident immune cells, including macrophages and ILCs, interact with stem cell

niches and affect physiology in many organs (28), and further research defining these pathways are likely to provide substantial opportunities for improving human health.

Acknowledgements

The author thanks lab members and colleagues for discussion, Chang Liao (and BioRender) and Satoshi Koga for help with Fig. 2, and funding from HHMI, the National Institutes of Health and the Sandler Asthma Basic Research Center at UCSF.

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ORGANOIDS

The Use of Organoids to Model Human Disease

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Abstract

When an oocyte is fertilized, it divides and – within days – stem cells arise. These embryonic stem cells (ESCs) build the entire body with all its organs and specialized cells. Consequently, they are said to be 'pluripotent'. Once the body plan is established, the individual organs are maintained and repaired lifelong by much more specialized stem cells, dedicated to the organ in which they reside. These are termed tissue stem cells or adult stem cells (ASCs). Since ASCs produce only a limited number cell types, they are termed 'multipotent'. Together, these two stem cell types hold promise for eternal life in the (probably distant) future. Meanwhile stem cells are rapidly changing the face of biomedical science and are actively being applied in the clinic. Below, I discuss principles of stem cell biology and of the mini-organs that can be grown from stem cells outside the human body, in the lab.

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It was in World War II when stem cells came to prominence. Radiation sickness was first described by the Red Cross Hospital Surgeon Terufumi Sasaki when nuclear bombs were dropped on Hiroshima and Nagasaki. Dr Nagasaki noted the gradual decrease of white blood cells from the blood of patients within weeks after the exposure to radiation. Subsequent observations in the 1950s revealed that bone marrow cells reversed the loss of blood cells when injected intravenously into radiated recipients. The conclusion from these experiments was simple and powerful: rare but powerful cells exist within the bone marrow that protect against the consequences of radiation. These cells produce all known varieties of blood cells: platelets, white cells and red cells. Moreover, they can recreate themselves, a phenomenon called self-renewal. Inspired by these findings, Thomas and coworkers endeavored to develop the transplantation of these bone marrow stem cells for therapeutic purposes. Realizing that it would

not be straightforward to transplant cells between unrelated individuals, their first attempt involved identical twins, one of whom suffered from leukemia and received bone marrow from the healthy sibling.

Bone marrow transplantation then rapidly developed further. An important step involved the replacement of a 'qualitative' phenomenon – the rescue of blood cell production by bone marrow from a donor – by a much more defined procedure. For this, the exact identity of the elusive multipotent hematopoietic stem cell (HSC) needed to be established.

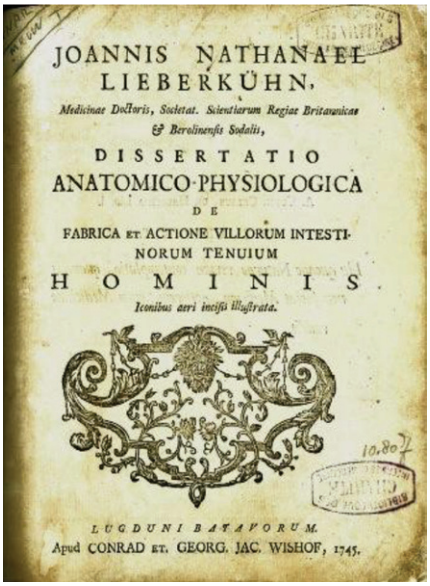
For this, the Toronto-based stem cell scientists Till and McCulloch devised a simple, animal-based lab test allowing to visualize HSCs. This so-called spleen focus-forming assay proved that individual bone marrow cells could indeed generate large numbers of all other blood cell types. Combined with a series of *in vitro* technologies that were developed later, the definitive identification of clonogenic HSCs was accomplished which heralded the start of the still sprawling HSC discipline.

Sixty years beyond these breakthrough discoveries, bone marrow- and cord blood-derived HSC transplantation now represents one of the routine therapeutic modalities used for malignant disease in hematology, but also for certain autoimmune diseases and even for multiple sclerosis.

From decades of studies on HSCs, a generally accepted definition of HSC attributes and of the architecture of the differentiation hierarchy driven by HSCs has arisen.

1) A key definition of an HSC involves the potential to recreate itself, i.e. to self-renew. This attribute is termed *longevity*; 2) A single HSC can produce all blood cell types. This attribute is termed *multipotency*. Direct daughters of HSCs are proliferative and while they increase their numbers, they gradually specialize into one of the different cell type lineages: platelets, red blood cells, lymphocytes, monocytes, granulocytes. This process involves a highly controlled stepwise choreography and is *irreversible*.

In the past few decades, new technologies have allowed the identification of stem cells in a variety of solid organs and tissues. These tissues can crudely be classified into two groups. In most organs (such as prostate, lung, or liver), cells do not divide much – if at all – under normal conditions. Yet, these organs can display vigorous waves of cell division when damaged. Other organs (such as the skin, or the inner lining of esophagus, stomach and gut) show constant cell division. This results in the continuous replacement of the pertinent tissues by healthy, young cells. The bone marrow and the blood cells that are generated from HSCs belong to this second class of tissues.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HOMINIS.
Iconibus æri incisis illustrata.
LUGDUNI BATAVORUM.
Apud CONRAD ET. GEORG. JAC. WISHOF, 1745.

Figure 1. Joannis Nathanael Lieberkühn, thesis 1745. Museum Boerhave, Leiden, the Netherlands.

Research in our lab has focused on the cells that cover the insides of the small bowel. The tissue that they form is called the intestinal epithelium. While studying the intestinal epithelium, we have come to realize that intestinal ASCs display a series of highly unexpected attributes, some of which also define ASCs in other solid organs. The small bowel epithelium is highly compartmentalized in so-called crypt-villus units. Mouse guts comprise about one million of such units while the human small intestine might contain some one billion of these. A villus protrudes from the wall of the short bowel into the central 'channel' or lumen; this architecture dramatically increases the capacity of the intestinal lining to absorb nutrients from the gut lumen. The villus surface consists of a single layer of specialized cells that perform various functions to digest food and absorb nutrients, while keeping unwanted entities such as indigestible food components, bacteria etc., outside the body proper.

Within a villus, blood vessels allow the further transit of nutrients towards the liver. Surrounding the villus base, 8-10 small pits protrude outward into the wall of the gut. These pits were first discovered by a young German scientist, Jonathan Nathanael Lieberkühn (1711-1756), in Leiden in the Netherlands (Fig. 1).

They have since been named crypts of Lieberkühn. For his studies, he injected heated wax into blood vessels of isolated organs to visualize tissue architecture. In the middle of the last century, it was realized that crypts are the site where the most active stem cells of the mammalian body reside. These intestinal stem cells drive a stem cell hierarchy which populates the remainder of the crypt as well as the surface of the villi. Six main cell types can be distinguished (Fig. 2).

Enterocytes represent the most common cell type on the villus and are responsible for the absorption of nutrients and liquids. For this, it carries

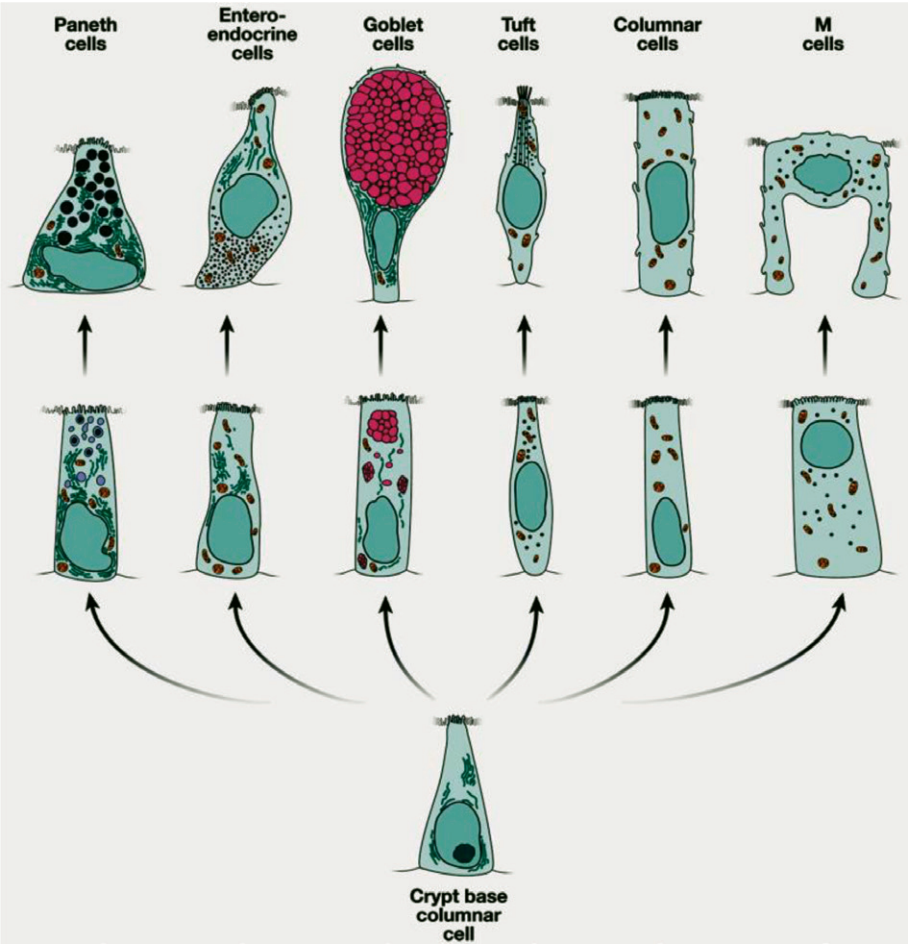

Figure 2. The six lineages of the intestinal epithelium produced from CBC cells.

a large number of tiny folds on its surface, together forming the so-called brush border, again to maximize nutrient absorption. Paneth cells are located at the base of crypts; their main function involves the defense against luminal microbes.

They perform this function through the production and secretion of bactericidal peptides and proteins. The function of Tuft cells remains somewhat elusive but they appear to play a key role in immunity against helminths. Goblet cells are secretory cells which produce mucus to enable smooth transport of the food bolus through the gut lumen and to restrict entry of microbes into the host body. Enteroendocrine cells come in five or six flavors, each producing a unique set of hormones, which control many aspects of metabolism, hunger and satiety.

Lastly, Microfold (M) cells only occur in the intestinal lining that covers specialized lymphoid structure that are termed Peyer's patches. M cells transport small and large antigenic particles from the gut lumen to the underlying lymphoid cells and thus play a key role in establishment and maintenance of mucosal immunity.

Leblond (Fig. 3) and Stevens were the first to describe the kinetics of the physiological behavior of the stem cell hierarchy of the intestinal crypt-vil-

Figure 3. Charles Philippe Leblond (1910-2007) French-Canadian stem cell pioneer.

lus stem cell units. Their landmark study was published in 1947 and was performed on rats. They observed that adult rats constitutively generate new cells in great numbers in their crypts. They also claimed – to the disbelief of many – that the lifespan of a single intestinal epithelial cell would not be much more than a few days. They acknowledged also the consequence of this observation: the dramatic, daily production of cells in crypts had to be in cue with a location elsewhere in the crypt-villus units where the cells – after having enjoyed a brief lifespan – would meet their demise. Leblond and Stevens thus wrote, "...the cells formed in the crypts of Lieberkuhn move upward along the side of the villi to be ejected when they reach the villi tips". An immediate conclusion from these notions would be that the intestinal stem cells that drive the vigorous French-Canadian stem cell pioneer's cell replacement would live at or near the base of the crypts.

In contrast to all other cells of the intestinal epithelium, the stem cells would be defined by two key attributes: they should continuously regenerate themselves (longevity) and they should produce all other cells of the tissue (multipotency).

Formulated more directly: As a laboratory mouse lives about three years, crypt stem cells should be able to persist for three years, and during that time should continuously generate enterocytes, goblet cells, Paneth cells, enteroendocrine cells and M cells. J.P. Leblond initiated the research into the identity of the stem cells of intestinal crypts. Leblond and Cheng first made a key observation: Paneth cells are not the only cell type present at the bottom of the crypt. Intermingled between the large, non-dividing Paneth cells with their eye-catching granules, careful examination using electron-microscopic techniques unearthed the existence of a tiny cell type, consisting of little more than a nucleus and a few organels.

These cells turned out to divide every day for the lifetime of the mouse and based on their columnar morphology, they were termed crypt base columnar (CBC) cells. Joseph Paneth also noted these cells in his study from 1887 in which he described the Paneth cells (Fig. 4).

Much more recently, Nick Barker and others in my lab identified *Lgr5* as an exclusive molecular flag, present uniquely in CBC cells. Nick Barker went on to create a number of knock-in mice targeting the *Lgr5*-locus (Fig. 4). Using these mice lines, we confirmed all essential predictions made originally by Leblond: The CBC cell, which uniquely expresses Lgr5, is the crypt stem cell. It continuously divides and does so lifelong. It generates all other cell types of the epithelium. Paneth cells are a key part of the stem cell niche which supports the vigorous activity of the CBC

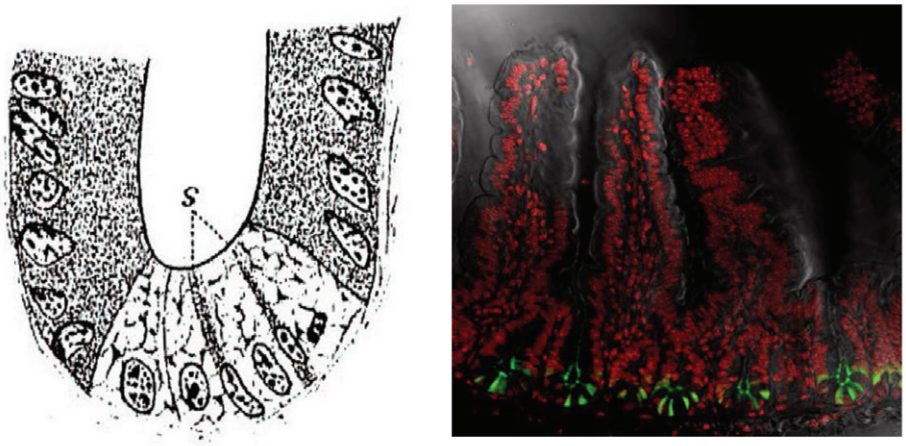

Figure 4. Images of the CBC stem cell in crypts. Left: Paneth, J. (1887). Ueber die secernirenden Zellen des Dünndarm-Epithels. *Archiv f mikrosk Anatomie.* Right: Barker et al. (2007). Identification of stem cells in small intestine and colon by the marker gene LGR5. *Nature*.

stem cell. Non-stem cells display plasticity and can dedifferentiate to become CBC cells, when the original CBC cells are lost.

In the early 2000s it was widely believed that ASCs could not be maintained outside the human or mouse body for more than a few days, let alone that ASCs could be encouraged to increase their numbers in a Petri dish. It is still true that after almost seven decades of the clinical application of bone marrow transplantation, all attempts to amplify HSC numbers in vitro have remained futile. The advantages of such stem cell expansion in vitro are obvious. Currently, it takes HSCs isolated from one donor to treat one patient. If stem cell numbers could be boosted in vitro, multiple patients could be treated with a stem cell isolate obtained from a single donor, or a patient could be treated multiple times using a single stem cell sample. Based on our observation that CBC cells undergo one cell cycle each day, CBCs have gone through one thousand consecutive cell cycles in the gut of an aged lab mouse. We had previously determined which growth signals are key to maintain active CBC cells in a mouse *in vivo*. Based on these observations, Toshiro Sato in our lab designed a 3-dimensional culture system for CBC cells with the intention to amplify their numbers in vitro. The approach is based on a hydrogel consisting of collagen and Laminin (Basement Membrane Extract, or MatrigelR). To the hydrogel,

the Wnt agonist R-spondin1 is added. We later discovered that R-spondins are ligands of the 7-transmembrane Lgr5 receptor. Two other key growth factors are Epithelial Growth factor and Noggin, a BMP-blocking secreted protein. When single CBC cells, sorted from Lgr5-GFP mice, are placed in this hydrogel-based medium, defined structures grew out, rather than the expected lumps of CBC stem cells. Careful analysis of these structures revealed that they contained all cell types of the gut epithelium, in normal ratios and at their normal location: Paneth cells and CBC stem cells at the base of the protruding crypts and all other cell types lining the central lumen. Dr Toshi Sato named these ever-expanding structures "mini-guts". A more scientific term is 'small intestinal epithelial organoids'. The term 'organoid' is now broadly used for structures that are grown from stem cells and that recapitulate key features of the organ of interest in terms of architecture, cell type composition and function.

Thus, "mini-guts" are grown in vitro from a single Lgr5 stem. They faithfully phenocopy central aspects of the physiology of normal gut epithelium. Bud structures that emanate from the periphery of organoids contain resident CBC cells, Paneth cells and rapidly-dividing transit-amplifying cells. These crypt-like buds create a flow of differentiated cells of the various lineages towards the lining of the central lumen. The dynamics of this process closely mimics that of the crypt-villus units of the small intestine: CBC cells generate daughter cells each day. These daughter cells themselves proliferate for a while, after which they mature into any of the prototypic cell types and after some days die and are discarded into the luminal space of the organoids.

A large batch of mini-guts was grown from a single adult colonic CBC cell, isolated from the Lgr5-GFP transgenic mouse line. The resulting organoids were transplanted in the lab of our collaborator Mamoru Watanabe. They were introduced intraluminally into the inflamed colons of several dozen mice treated with the chemical DSS to induce a colitis-like syndrome. The transplanted mini-gut organoids integrated fully into the damaged wall of the DSS-treated colons and maintained functionality for the duration of the experiment. Based on these and subsequent experiments, Watanabe and colleagues have embarked on a project to treat treatment-resistant inflammatory bowel disease patients with autologous colon epithelial organoids.

Since these initial mini-gut studies, we and many others have developed protocols to grow organoids from numerous other mouse and human organs (Fig. 5).

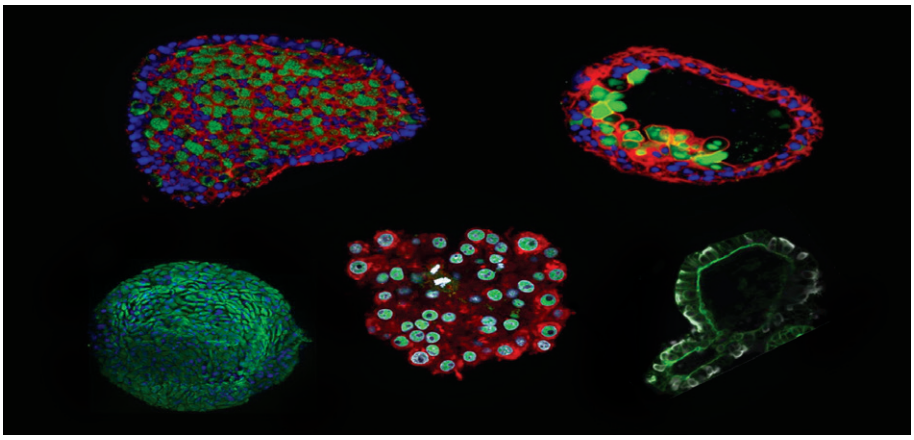

Figure 5. Different organoid types. From top left to bottom right: Airway; airway infected with RSV virus (virus in green); cervix; liver; intestine infected with SARS-CoV2 (white).

These protocols invariably allow the establishment of organoids that capture key characteristics of the organ of interest. Adult stem cell-based organoids simply require a small piece of tissue, obtained from a biopsy or from a surgical resection sample as starting material. A parallel technology starting from pluripotent stem cells was originally pioneered by Dr Yoshiki Sasai, who focused on generating structures of the central nervous system and the retina. ASC-related organoid approaches exploit the maintenance and repair capacity of adult stem cells, which are fully fated towards the organ in which they reside. By contrast, pluripotent stem cell-derived organoids exploit the capacity of ES cells or iPS cells to generate each part of a mammalian body; the organoids are fated in vitro towards the organ of interest by mimicking the developmental journey they would have experienced in the developing embryo. A multitude of technologies, discoveries and applications have emerged around this organoid concept. Breathtaking types of organoids derived from iPS cells are the cerebral organoids (or "mini-brains" of Lancaster and Knoblich), and the mini-kidneys of Melissa Little and colleagues from Melbourne.

Organoids representing human tissues are increasingly embraced by basic biomedical scientists. Organoids derived from healthy human tissues find applications as alternatives to animal experimentation. Human organoids may also better represent diseases: when directly grown from cancers, they appear superior models of human cancer when compared to

'classical' cancer cell lines. Organoids also find applications in other disease areas: In infectiology, organoids allow the study of a variety of pathogens, be it bacteria such as the stomach ulcer-causing Helicobacter; viruses such Noro- and influenza viruses, RSV and the SARS-CoV2 virus, and eukaryotic microbes such as Cryptosporidium.

Organoids model hereditary diseases such as cystic fibrosis. In oncology, organoids can be directly grown from tumors and they are now believed to faithfully recapitulate tumor cells of individual patients.

Organoids are being validated for personalized medicine strategies. Tissue samples taken from patients can be grown as organoids to serve as avatars of the pertinent patient. Drug testing of the avatar-organoid predicts and allows tailoring of drug- or radiation-based treatment of individual patients. Indeed, organoid approaches already allow rapid ex vivo testing of drug responses on tissue samples obtained from individual patients. As a preeminent case study, a minigut-based cystic fibrosis (CF) assay yields a test result within 14 days after obtaining a small biopsy from a given CF individual. The outcome of this test is unambiguous in predicting if the patient will respond clinically to the CF medicine. In the Netherlands, the CF organoid test has been performed for a majority of CF patients. When a positive assay result is obtained, the corresponding CF individual will be prescribed the (expensive) CF medicine. Organoids offer a similar opportunity to cancer patients (see above). A number of clinical studies has already underscored the high predictive value of cancer organoid-based drug sensitivity screening in a personalized health care setting.

Cancer organoid-based assays allow evaluation of multiple therapeutic regimens prior to selecting the most optimal one to be given to the patient. It will however still take some years before this approached is validated well enough to become a routine diagnostic tool. For that, organoid derivation and drug screening will require automation and a significant improvement in terms of speed and cost.

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This manuscript summarizes a lecture given at Vatican City in 2022. It was based on several reviews written by the author.

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Organoid Assays For In Vitro and In Vivo Models of Lung Disease & Cancer

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Chronic and acute lung diseases are amongst the leading causes of morbidity worldwide (Soriano et al., 2020). Due to their prevalence and severity, which is further exacerbated with aging, these diseases require further understanding from a cellular and molecular perspective. The lung encapsulates a variety of cell niches including the trachea, bronchioles, and alveolar space, which are supported by the smooth muscles, fibroblasts, and endothelium. When analyzing the varying lung diseases such as bronchopulmonary dysplasia, cystic fibrosis, chronic obstructive pulmonary disease, pulmonary fibrosis, and SARS-COV-2, one common thread of lung disease is a depletion or dysfunction of lung alveolar epithelial cells, bronchiolar or airway epithelial cells, or both (Figure 1). Therefore, better understanding how lung cell niches protect and repair the epithelial cells may allow us to combat these chronic and acute lung conditions. To a large extent the specialized niches control stem cell self-renewal and differentiation in the lung (Lee et al., 2014). It has been previously shown that lung repair processes are mediated via progenitor cells in the lung. Therefore, these progenitor cells serve an important function in the lung microenvironment. There is an

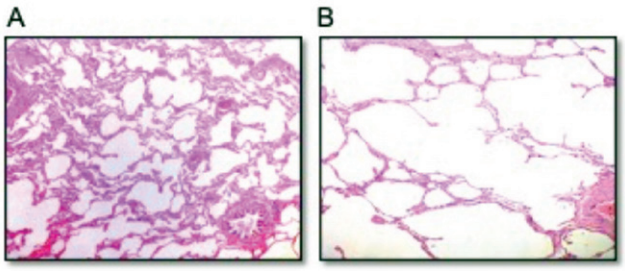

Figure 1. Lung morphology of normal and diseased lung. (A) Morphology of a normal lung. (B) Morphology of a diseased lung. *Modified from American Lung Association, Berkeley Lab, Google images.*

abundance of progenitor cells in the lung, such as basal cells in the trachea, Alveolar type II cells (AT2 cells) in the alveolar space, Club cells in the bronchioles, and many others. In order to better understand lung disease emergence and progression, as well as its potential repair process, it is important to further understand these important progenitor cells.

Previous work in our lab has demonstrated the ability of organoids to model the progenitor cell properties of alveolar and bronchiolar cell types (Lee et al., 2014; Kim et al., 2005). The three-dimensional (3D) organoid co-culture system we developed involves the co-culturing of epithelial EPCAM$^+$ cells (SCA1$^-$ or SCA1$^+$) with supporting lung mesenchymal cells in Matrigel. These 3D culture systems are able to mimic the lung niche and advance the understanding of lung biology. SCA1$^+$ lung epithelial cells give rise to both alveolar and bronchiolar organoids, modeling the progenitor

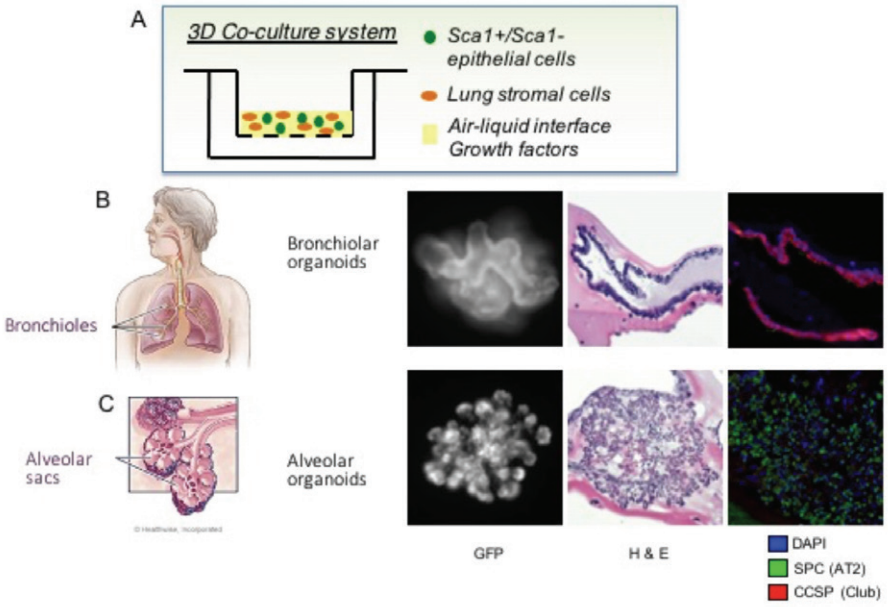

Figure 2. Schematic of bronchiolar and alveolar organoid cultures. (A) Lung 3D Co-culture system utilizing SCA1+ or SCA1- progenitor cells, lung stromal cells and growth factors. (B) Depiction of bronchiolar organoids. From left to right: anatomical diagram of bronchioles, representative IF images of bronchiolar morphology via GFP staining, H & E images, IF staining for blue, DAPI; green, SPC; red, CCSP. (C) Depiction of alveolar organoids. From left to right: anatomical diagram of alveolar sacs, representative IF images of alveolar morphology via GFP staining, H & E images, IF staining of alveolar space for blue, DAPI; green, SPC; red, CCSP. *Lung anatomical illustration adapted from Healthwise Inc.*

cell capacity of multipotent bronchioalveolar stem cells (BASCs), whereas SCA1^{-} lung epithelial cells give rise to only alveolar organoids, modeling the capacity of AT2 cells. In the context of oncogenic Kras expression, AT2 cell organoids mimic tumor progression *in vivo* tumor progression and recapitulate early-stage lung adenocarcinoma in patients (Figure 3)(Dost et al., 2020). This resource could aid in identifying transcriptional and proteomic differences that distinguish normal epithelium from lung cancer. Thus, organoids serve as a useful tool to model numerous lung diseases and understand their mechanisms and progression.

Aging is a predominant risk factor for chronic lung diseases and lung cancer. Despite its significant impact, the impact of aging on lung progenitor cell functions remains largely understudied from a cellular perspective. Previous work has demonstrated lung progenitor population changes, compromised repair, and epigenetic instability as a result of aging in mice (Schneider et al., 2021). Specifically, in our laboratory, we have seen a change in the frequency of lung alveolar progenitor cells with aging. The changes largely show a shift toward significantly fewer AT2 cells and significantly more bronchiolar progenitors including BASCs (Rowbotham et al.,

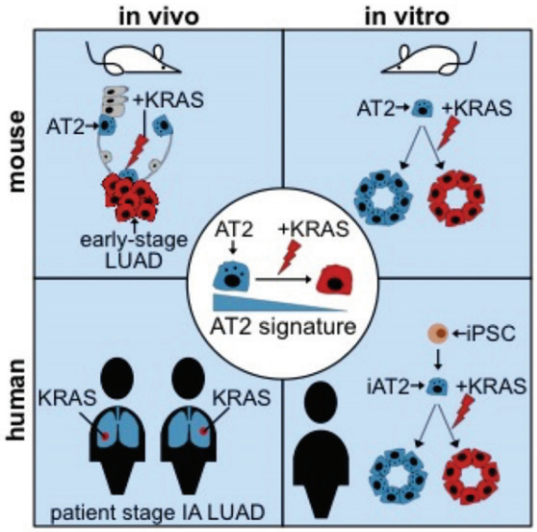

Figure 3. Shared properties of mouse models of lung cancer, mouse AT2 cell organoid cultures, iPSC-derived AT2 cell organoids with samples from patients with KRAS mutant lung cancer. In all 4 contexts, AT2 cells exhibit loss of AT2 identity after expression of oncogenic KRAS. *Modified from Dost and Moye et al., Cell Stem Cell, 2020.*

bioRxiv, 2021). Additionally, changes in organoid-forming efficiency and typology have been demonstrated with age. Organoids grown from SCA1- epithelial cells from old mice yielded a lower alveolar colony-forming efficiency than organoids grown from cells taken from young mice. A similar trend was observed when analyzing organoids grown from SCA1^{+} epithelial cells with old mice yielding a lower alveolar colony-forming efficiency than young mice. However, organoids grown from SCA1^{+} epithelial cells from old mice yielded a higher bronchiolar organoid-forming efficiency.

Besides the effects aging has on lung progenitor cells, we found aging causes epigenetic alterations, such as a decrease in Lysine 9 methylation. This was indicated by H3K9me2 fluorescence data showing a lower fluorescence value for old mice as compared to young mice. Lysine 9 methylation depletion modeling through use of an inhibitor of G9a, the methyltransferase that bi- and tri-methylates lysine 9, reduced alveolar progenitor activity in organoid cultures. Aging has also been shown to increase lung damage post alveolar injury via bleomycin (Hecker et al., 2014). We have recapitulated this phenotype with depletion of Lysine 9 methylation in young mice by G9a inhibitor administration. Bleomycin-injured mice suffered greater persistent lung damage when G9a inhibitors were administered. Interestingly, mice depleted of Lysine 9 methylation showed an expansion of bronchiolar progenitors and a decrease in alveolar progenitors, suggesting that, at the expense of alveolar progenitors, bronchiolar progenitor cell activity is enhanced. All of these differences demonstrate how

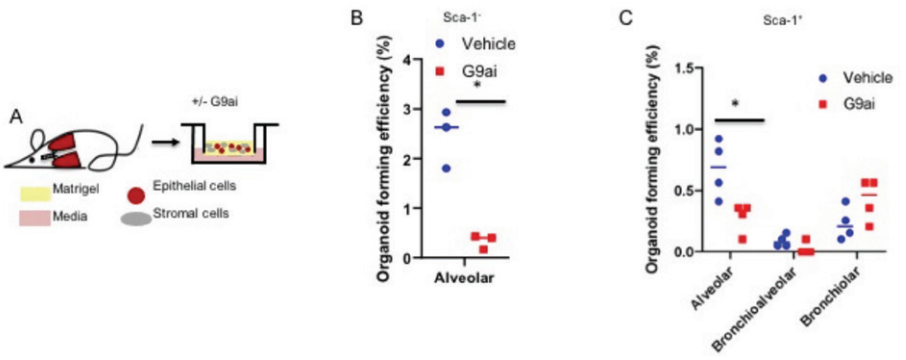

Figure 4. H3K9me2 depletion in young mice impairs alveolar progenitor activity. (A) Schematic depicting *in vitro* G9ai organoid experiments. (B) Quantification of organoid forming efficiencies from SCA1- progenitor cells +/- G9ai. *=p<0.05. (C) Quantification of organoid forming efficiencies from SCA1+ progenitor cells +/- G9ai. *Image adapted from Rowbotham et al., bioRxiv, 2021.*

one key aspect of aging in the lung may be the abrogation of epigenetic regulation mediated by G9a, which changes the dynamics of how different progenitor cell types are used in repair and regeneration (Rowbotham et al., bioRxiv, 2021).

Recent studies in lung biology have identified lung progenitor cells that may one day be used to treat varying pulmonary diseases (Barkauskas et al., 2013; Hong et al., 2001; Kathiriya et al., 2020; Kim et al., 2005; Rawlins et al., 2009; Louie et al., 2022). Current treatment modalities for patients suffering from pulmonary diseases largely rely on whole or partial lung transplantation from donor lungs. Due to the shortage of donor lungs, an alternative to whole and partial lung transplantations would be useful in combating these varying pulmonary diseases. A potential alternative may be to utilize lung progenitor cells as a treatment modality. For example, alveolar type I and AT2 cells may be useful in treating idiopathic pulmonary fibrosis, as these are the lung cell types impacted throughout the course of this disease. Our laboratory has tested the potential of lung organoid cells to be transplanted into the lung while retaining progenitor status, a critical aspect

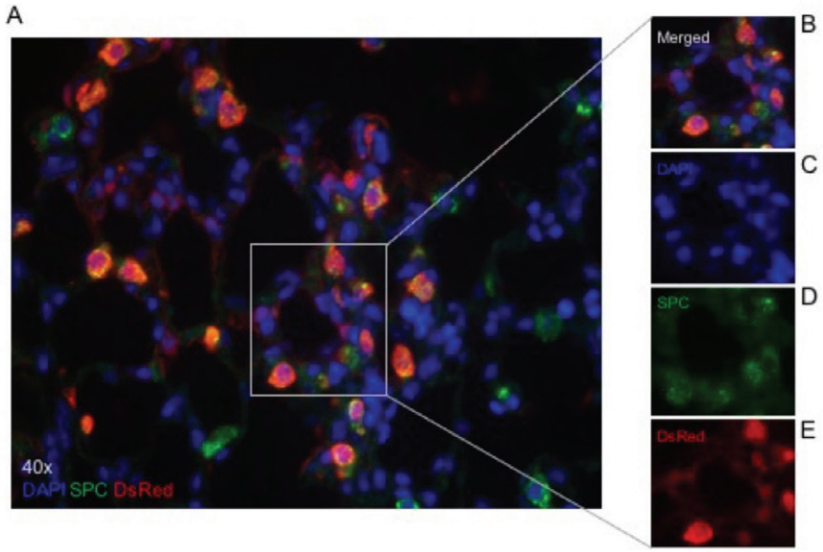

Figure 5. Lung organoid cells are retained in the lung after transplantation. (A) Representative picture of IF staining on mouse transplanted with alveolar organoid cells. Images taken at 40x magnification. (B) Representative picture of IF staining of applicable lung area showing blue, DAPI; green, SPC; and red, DsRed. (C) IF staining of DAPI. (D) IF staining of SPC. (E) IF staining of DsRed. *Modified from Louie et al., Cell Reports, 2022.*

for use in future treatment modalities (Louie et al., 2022). We conducted a multi-faceted study, analyzing the engraftment of these cells, their gene expression program via single-cell RNA sequencing, and their proliferative potential after re-injury in recipient mice. We transplanted organoids derived from Sca1- cells and organoids derived from Sca1+ cells into bleomycin injured mice. We then analyzed mice at varying time points and investigated the transplanted cells via flow analysis, immunohistochemical staining, and RNA sequencing. Transplanted AT2 cells expressed the AT2 marker surfactant protein C (SPC), suggesting engraftment of the organoid cells (Figure 5). Single cell RNA sequencing revealed that transplanted and native cells were transcriptionally similar but distinct from the organoid cluster. Furthermore, flow cytometry analysis and immunostaining showed a similar proliferation potential for transplanted cells and native AT2 cells in the lung (Louie et al., 2022). In conclusion, we were able to show that alveolar cells maintained in organoid cultures retain progenitor cell activity after transplantation, an important concept for their potential future use in therapy.

Besides the relevant uses of the organoid system as a tool for investigating lung cancer and disease using mouse models, there have been many new advances that shed new light on the identity of human lung progenitor cells and how to maintain the diversity of lung cell types in organoid cultures. For example, recent work has identified progenitor cell populations in anatomical structures that only exist in the human lung (Basil et al., 2022, Murthy et al., 2022). Such discoveries and many more are required to understand the complexity of human lung biology and how to intervene when lung homeostasis goes awry in a clinical setting. Organoids may serve as a useful and feasible treatment modality for lung diseases in the future, depending on the injury and cell populations impacted. Alveolar organoids provide treatment for surfactant protein deficiency, Hermansku-Pudlak Syndrome, pulmonary fibrosis, and bronchopulmonary dysplasia. Airway organoids may help in the treatment of chronic obstructive pulmonary disease, cystic fibrosis, and bronchiolitis. Future treatment options made possible through organoid modeling or organoid transplantations have the potential to treat a variety of chronic and acute diseases, impacting many patients worldwide.

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Modeling the Human Brain in Development and Disease

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Abstract

Inspired by the iron-clad tenacity of past scientists we set out to understand the mechanisms of life's bricks, how they divide, which genes direct them, how they differentiate into specific lineages and how this intricate orchestration goes awry.

Much of the bridging between past and contemporary neuroscience lies in maintaining a close-knit relationship between the sciences that study the mind and reductionist science. It is through health that we conceptualize the fundamental properties of its nature but through disease that we appreciate them. Neurological disorders gather our attention as they challenge and alter communication and relationships by limiting and then extending them. To study phenotypes associated with human brain development, function, and disease, it is necessary to use experimental systems that are accessible, ethically justified, and can replicate human context.

Human pluripotent stem cell (hPSC)-derived brain organoids offer such a system, which faithfully reiterates features of early human neurodevelopment *in vitro*, including the generation, proliferation, and differentiation of neural progenitors into neurons and glial cells and the complex interactions among the diverse, emergent cell types of the developing brain in three-dimensions (3D). In recent years, numerous brain organoid protocols and related techniques have been developed to recapitulate aspects of embryonic and fetal brain development in a reproducible and predictable manner. Coupling our ground-breaking cerebral organoid technology with elegantly tailored cutting-edge genetic manipulations has enabled us to efficiently screen for disease-linked mutations serving as a boon when studying human neurobiology and neurodevelopmental disorders. Altogether, these different organoid approaches provide distinct bioassays to unravel novel, disease-associated phenotypes and mechanisms.

★ ★ ★

To say that the human cerebral cortex is the organ
of civilization is to lay a very heavy burden
on so small a mass of matter. – C. Judson Herrick (1926)

Understanding the brain is probably the greatest task of human biology for it encapsulates the purpose of humankind. The brain births ideas, feelings, cultures, motion, reflex, wisdom, and identity. It is an organ that has nothing short of divided and fused, perplexed and intrigued populations throughout history. The light shines on it; no dimming in sight, not even so much as a flicker as we gather ourselves sedulously attempting to satisfy our scorching thirst from ploughing away at its many facets for the chance of grasping its veritable nature.

The Greek philosopher Aristotle believed that memory and consciousness were found in the heart, coining what we now refer to as emotional intelligence. The Egyptians, however, first described the basic anatomy of the brain and made the connection that it controls movement. The brain as an organ has interested populations and cultures amass, many reaching the same conclusions. Much of the early knowledge was based on observations by doctors who made poignant connections between human behavior, physiology and the brain.

Descarte's portrait by Frans Hals.
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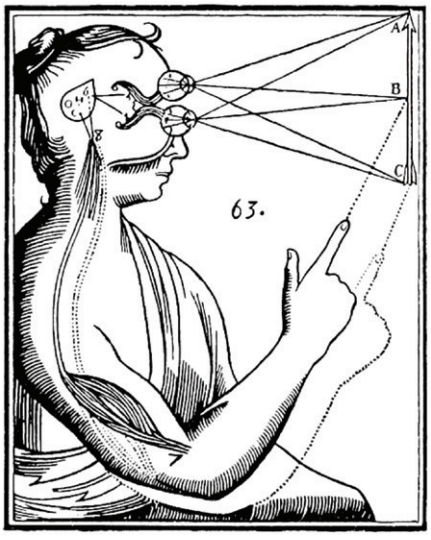

Descartes's illustration of mind-body dualism in "Treatise of Man". Public domain.

A thousand years later the French philosopher Descartes distinguished the brain from the mind, thereby introducing the pertinent notion of dualism. This planted the seed that flowered into the scientific revolution where the brain earns the leading role sprouting different fields of neuroscience research. Like all sciences, neuroscience was approached from the macroscopic to the microscopic scale, offering plenty of fascinating discoveries and contributing to deep and often controversial discussions along the way.

The fascination over the complexity and importance of the human brain ensures quick progress in teasing out its many layers of anatomy and diverse functions. Strolling along the neuroscience history aisles one notices the quick succession of discoveries with Purkinje (Purkyně) describing the neuron, Broca identifying the region responsible for speech and Pavlov examining the physiology of involuntary reflexes earning him a Nobel Prize in Medicine. Soon after, electroencephalography (EEG) is developed to measure electrical activity in the brain and Sir Charles Sherrington wins the Nobel Prize for describing the existence of synapses and how reflexes occur as a result of nerves extending into muscles. A decade later Isidor Rabi wins the Nobel Prize for discovering nuclear magnetic resonance which made the development of magnetic resonance imaging (MRI) possible. Several researchers bring evidence that solidified Julius Bernstein's hypothesis that action potential is a product of ionic conductance. These technological breakthroughs permit Joseph Erlanger and Herbert Gasser to document the existence of different action potentials across different cells which ultimately leads to their Nobel Prize winning discovery of the velocity of action potentials. Progress in physiological neuroscience is accompanied by the confirmation that acetylocholine is a neurotransmitter marking a landmark discovery for molecular neuroscience.

The variety of emerging fields quickly creates the need for more unified efforts in teasing out the complexity of our brain and it is now officially recognized as an independent discipline. The fast-paced progress in its diverse fields highlights the need for deeper understanding of our nervous system by looking down to its founding units.

At this point a set of groundbreaking discoveries are made that influence and impact biology and medicine globally in an unprecedented way. Wilhelm His and Santiago Ramón y Cajal independently notice the presence of cells from which all types of neurons arise before migrating from the place of origin to increasingly more distant locations.[1,2] Ernest McCulloch and James Till identify the existence of cells in the

adult bone marrow which can self-renew and are hematopoietic giving rise to all blood cell types, inaugurating the field of stem cell research.[3] As scientists curiously peak down the microscope into life's building blocks, cell biology and medicine have, unbeknown to them, been revolutionized. Martin Evans and Matthew Kaufman isolate and culture mouse embryonic stem cells opening up the possibility to study the function of specific genes during disease.[4] Soon after, James Thompson reports the derivation and culturing of human embryonic stem cells that retain their pluripotent state; their ability to give rise to different cell types.[5] Shinya Yamanaka astounds the scientific community by making a remarkable discovery that adult mouse fibroblasts can be reprogrammed into reacquiring a pluripotent state, much like that of mouse embryonic stem cells and calls them induced pluripotent cells (iPSCs).[6] Together with Takahashi they successfully derive iPSCs from human fibroblasts as well, alleviating the considerable and understandable ethical concerns of using human embryonic material for stem cell research.[7]

The hallmark properties of stem cells are the ability to self-renew by dividing indefinitely into daughter cells, while at the same time retaining the capacity to commit daughter cells to lineage-specific differentiation which

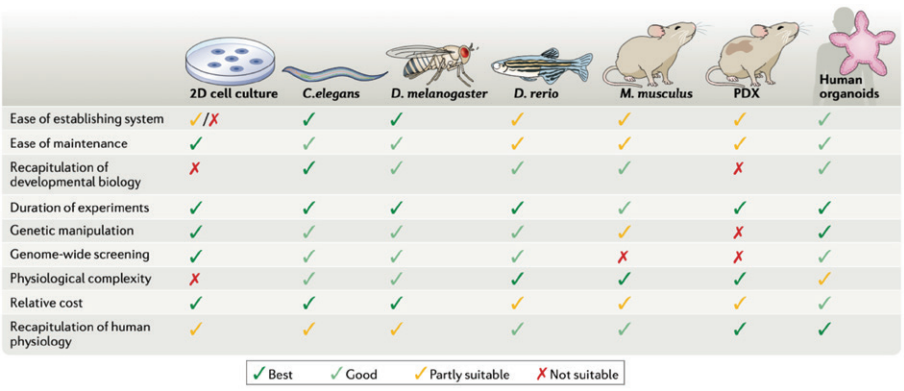

Figure 1. Comparison of organoids with other model systems. The most common model organisms that are used in biomedical research are Caenorhabditis elegans, Drosophila melanogaster, Danio rerio and Mus musculus, along with patient-derived xenografts (PDX). These models, as well as 2D cell cultures and human organoids, are assessed here for their relative benefits and limitations. Relative scores are represented as being the best (dark green tick), good (light green tick), partly suitable (yellow tick) and not suitable (red cross). Xenografts, tissues or organs transplanted between different species. (Figure from Kim, J et al., 2020).

is the more differentiated progeny that drives tissue specific development. Stem cells can be isolated from the blastocyst stage of the developing embryo, but they are also found to persist in niches of adult tissues, including the brain. Neural stem cells, like all stem cells, play important roles in tissues homeostasis, and in development. In adult organisms, they ensure continuous replacement of dying or damaged cells, while during development they generate most of the cell types in a developing organ. To fulfill this task, stem cells can maintain an undifferentiated state, but at the same time generate daughter cells that are lineage-restricted and ultimately undergo terminal differentiation. Understanding how the balance between self-renewal and differentiation is controlled within a stem cell lineage is important since defects in the control of this process can result in tissue degeneration or tumorigenesis. Neural stem cells are the focus of my lab's research, and our work has offered many insights into what is, "there".

Building on the fundamental idea that biological mechanisms are conserved throughout evolution, biomedical research focuses on animal model organisms. Animal experimentation is widely used as a proxy for understanding human embryonic development and organ function.[8] A menagerie of animal species, both vertebrate and invertebrate, are employed in an attempt to answer more direct questions. Each model offers particular strengths (Fig 1).[9] Although some extrapolations lead to valid knowledge, other speculations do not translate quite as fluently. Human physiology is profoundly different from the mouse model system: it is perhaps unsurprising that there are huge differences in metabolism between humans and laboratory models, given that humans develop far slower than the other models[10] or the fact that continuous oscillations in the hippocampus, for the purpose of spatial navigation of rodents, are found not to be true in bats or monkeys. Even further, several biological phenomena that are specific to humans are not amenable to being reproduced in animal models. The human brain, for example, is far more complex than its mouse counterpart, owing partly to human-specific developmental events and mechanisms.[11] Neurons in the human cortex, for example, arise from a cell type (outer radial glia) that is either not present – or is present only in minute numbers – in rodents.[11] Despite this and with, perhaps, a reluctant recognition that not all knowledge from the animal kingdom transcribes to the human, armies of scientists methodically reveal distinct aspects of brain development by using animal models.

Drosophila and Caenorabditis elegans models are instrumental in elucidating the principles of stem cell self-renewal and differentiation, un-

covering molecular parallels for this process in different species. Understanding that one way to generate cellular diversity during development is to segregate cell-fate determinants predominantly into one daughter cell upon division, inspires us and others to ask how this process comes to be. Work mostly done in the fruitfly, *Drosophila*, suggests two different mechanisms by which this remarkable task can be achieved.[12] Already in interphase, cells which undergo such intrinsically asymmetric divisions use apical-basal or planar polarity of the surrounding tissue to set up an axis of polarity. As they enter mitosis, this axis is used to polarize the distribution of protein determinants and to orient the mitotic spindle so that

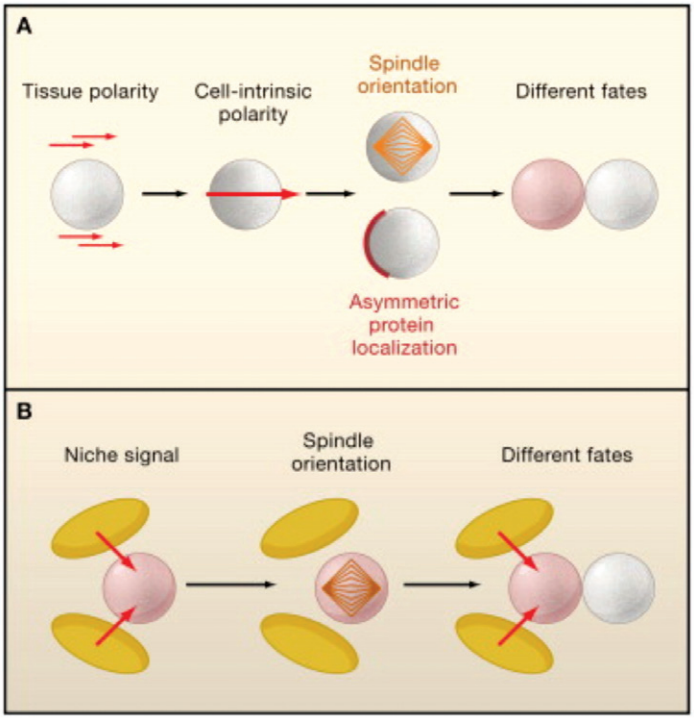

Figure 2. Extrinsic and intrinsic regulation of stem cell self-renewal by asymmetric cell division. (A) Stem cells can set up an axis of polarity during interphase and use it to localize cell fate determinants asymmetrically in mitosis. Orientation of the mitotic spindle along the same polarity axis ensures the asymmetric segregation of determinants into only one of the two daughters. (B) Stem cells may depend on a signal coming from the surrounding niche for self-renewal. By orienting their mitotic spindle perpendicularly to the niche surface, they ensure that only one of the two daughter cells continues to receive this signal and maintains the ability to self-renew. (Figure taken from Knoblich, JA, 2008).

these determinants are inherited by only one of the two daughter cells. Alternatively, they can orient their division plane so that only one of the two daughter cells maintains contact with the niche and stem cell identity (Fig. 2).[13] A stem cell, by orienting its mitotic spindle perpendicularly to the niche surface, ensures that only one daughter cell can maintain contact with the stem cell niche and retain the ability to self-renew. In contrast to intrinsically asymmetric cell divisions, which usually follow a predefined developmental program, niche-controlled stem cell divisions offer a high degree of flexibility. Occasionally, the stem cell can divide parallel to the niche, thereby generating two stem cells to increase stem cell number or to compensate for occasional stem cell loss. For this reason, niche mechanisms are more common in adult stem cells, whereas intrinsically asymmetric divisions predominate during development.

Clarifying the mechanism of asymmetric cell division in the Drosophila nervous system becomes the starting point of my contributions to stem cell biology. Building on what I learned during my post-doctoral work,[14,15] I teamed up with extraordinary and brave scientists in my lab to develop a conceptual framework for how the asymmetric cell division process occurs. We proposed, tested, and showed that an axis of polarity is established during interphase guiding both the orientation of the mitotic spindle and the asymmetric localization of protein determinants during mitosis. Over several years, we identified a near-complete set of proteins involved in the various stages of the process and achieved a mechanistic understanding of asymmetric cell division. We found that it is the asymmetric localization of the so-called Par-proteins that establishes the polarity axis to guide asymmetric cell division. In mitosis, a polarized attachment site for microtubules established by the proteins Pins, Galphai and Mud orients the mitotic spindle while the kinase aPKC detaches protein determinants (Numb, Prospero and Brat) from one side and guides their accumulation at the opposite site (Fig. 3).[16-20] This mechanism enjoyed wide acceptance in the field and became part of most developmental biology textbooks. Importantly it is conserved in mammalian stem cells highlighting the relevance of asymmetric cell division in stem cell biology, especially considering the compelling connections to tumorigenesis that begin to emerge, like, for example, the link we make between cellular metabolism and immortalization of tumor-initiating cells by performing targeted metabolomics and *in vivo* genetic screening.[21]

Matching a gene to its function is necessary in detangling developmental processes but it is also a laborious process. Genetic screens become the

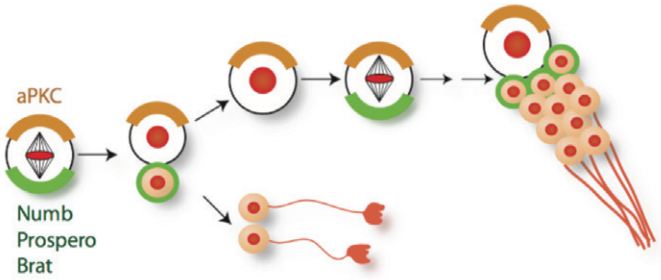

Figure 3. Axis of polarity establishment during asymmetric cell division. During interphase, Par proteins (like aPKC) localize asymmetrically creating a polarity axis which during mitosis will serve to guide asymmetric cell division. During mitosis, microtubules attach a polarized site to orient the mitotic spindle and aPKC locally phosphorylates cell fate protein determinants to guide them to the opposite site. Ultimately, the cell fate determinants Numb, Pros, and Brat segregate into the small daughter cell, the ganglion mother cell (GMC) that divides only once more to generate two differentiating neurons.

go-to method for the elucidation of developmental pathways, and work done in invertebrates is followed by an analysis of evolutionary conservation in mammalian model systems, often leading to clinical translation for humans. Pioneers Christiane Nüsslein-Volhard and Eric Wieschaus paved the way by screening through massive numbers of randomly induced mutant fly embryos for defects in developmental patterning and classified 15 genes as the key players during embryonic development in Drosophila.[22] In the wake of the discovery of stem cells and the establishment of the key tenets of stemness, another technology is appearing in an entirely unexpected way. As Andrew Fire and Craig Mello investigated gene expression regulation in C. elegans, they observed that double-stranded RNA blocks the expression of the respective gene and named this approach RNA interference (RNAi).[23] The ability to silence specific genes overcomes the main drawback of random mutagenesis approaches in that it is gene specific. RNAi allows large-scale genetic screens to reveal the functions of many genes through development. Consequently genome-wide RNAi studies are performed in mammalian stem cell cultures.[24,25]

Naturally, the wish to study stem cells *in situ* arises where the interactions with the surrounding niche and the tissue-specific characteristics of individual lineages are maintained. In *Drosophila*, this becomes possible through the establishment of a transgenic RNAi library that can be expressed in a tissue-specific manner.[26] Together with my team we became

the first to perform genetic screens in a tissue-specific manner within an entire organism. We focused on external sensory organs, where defects in asymmetric cell division or Notch signaling lead to visible phenotypes, like gain or loss of bristles. We used a library of 20,000 transgenic RNAi lines generated by Barry Dickson that resulted in informative loss-of-function phenotype data for 23% of all protein coding Drosophila genes, a data set that is still regularly queried by others in the field.[27]

Armed with all this knowledge, we now wonder how the finely tuned yet fragile homeostatic balance between stem cell self-renewal and differentiation is regulated. We perform genetic screens on neural stem cells using genome-wide transgenic RNAi and identified 620 genes that are potentially involved in controlling this balance in Drosophila neuroblasts (larval brain stem cells).[28,29] We quantified all phenotypes and derived measurements for proliferation, lineage, cell size, and cell shape. We identified a set of transcriptional regulators essential for self-renewal and integrated hierarchical clustering with interaction data to create functional networks to uncover the control of neuroblast self-renewal and differentiation. Our data revealed key roles for the chromatin remodeling Brm complex, the spliceosome, and the TRiC/CCT-complex showing that the alternatively spliced transcription factor Lola and the transcriptional elongation factors Ssrp and Barc control self-renewal in neuroblast lineages.[28,29] These efforts truly laid solid foundations for the mechanistic discoveries that ensued on stem cell immortalization and tumorigenesis.

Studies in Drosophila undoubtedly enriched our scientific acumen of neural development but the gnawing need to intimately explore the least understood organ of our body is ever-present. The complex architecture and function of the human brain enables us to perform higher cognitive functions. Abnormalities in the structure or function of the brain can lead to severe neurological and psychiatric disorders. It is becoming increasingly clear that many neurological and psychiatric disorders have their roots in neurodevelopment.[30,31] However, determining the neurodevelopmental cause and mechanisms of these brain disorders is challenging, due to the limited access to human brain tissues. Given the large evolutionary distance between mouse and human, and the immense elaboration of the primate brain in size and complexity, there are many features unique to human brain development and diseases that are not seen in rodent systems.[32]

Standing on the shoulders of giants we can now see much farther than we ever thought possible and diving into the unknowns of the human brain seems to be more within our reach. But first a bridge must be built.

A new model that does not rely on human primary material is needed. Madeline Lancaster, in my laboratory, replaces mortar and pestle with pipette and culture dish and attempts to use human pluripotent stem cells to model key developmental events of the human brain *in vitro*. By combining classical cell culture approaches with recently developed methods enabling cells to grow three-dimensionally, we developed cerebral organoids, a tissue culture method that recapitulates human brain development.[33] The gap between animal models and human beings has been bridged.

Human brain organoids, otherwise known as cerebral organoids, are hPSC-derived self-organizing human pluripotent stem cell-derived three-dimensional culture systems that develop various discrete, although

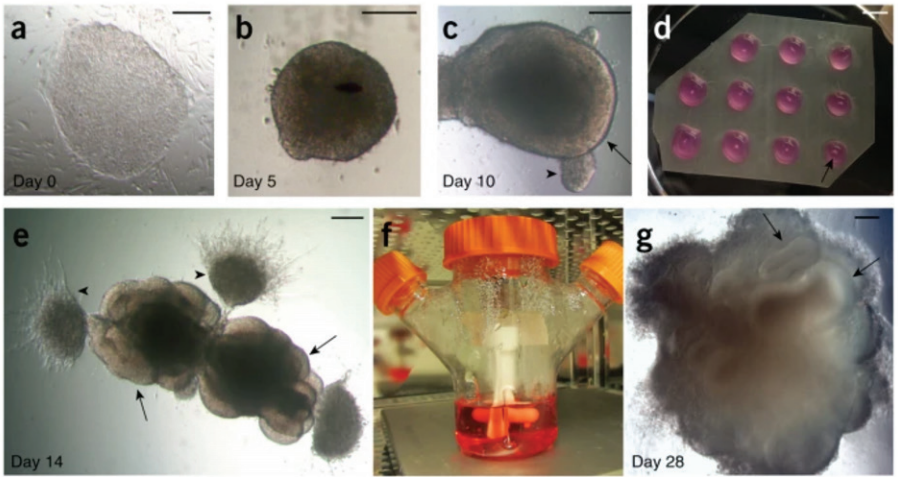

Figure 4. Progression of cerebral organoid development from human PSCs. (a) A colony of feeder-dependent human ESCs showing typical pluripotent morphology with clear boundaries and a uniform texture. (b) An EB at day 5 showing evidence of ectodermal differentiation, as indicated by the presence of brightened surface tissue, whereas the center is quite dark with dense non-ectodermal tissue. The EB also has a smooth surface, indicating healthy tissue. (c) An early organoid at day 10 showing smooth edges and bright optically translucent surface tissue consistent with neuroectoderm (arrow). This organoid also contains small buds of ectodermal tissue that is not organized radially (arrowhead). (d) Image of the neuroectodermal tissues embedded in Matrigel droplets on a sheet of dimpled Parafilm. The tissues are visible as small white specks within the droplet (arrow). (e) An organoid at day 14, after embedding in Matrigel, showing evidence of neuroepithelial bud outgrowth (arrows) that are optically clear and in several cases surround a visible lumen. Other outgrowths and migrating cells are also visible (arrowheads) that are not neuroepithelial. (f) The spinning bioreactor setup in the tissue culture incubator. Organoids are visible within the bioreactor as small white floating specks. (g) An organoid at day 28 of the protocol, revealing many large neural tissues (arrows) that have greatly expanded once embedded in the Matrigel. Scale bars, 200 μm (a–c,e,g) and 5 mm (d). (Figure from Lancaster et al., 2014).

interdependent, brain regions. Cerebral organoids recapitulate the neurodevelopmental scheme to generate 3D tissue architectures that mimic various features of the developing fetal brain pertaining to cellular composition and tissue structure.[34] hPSCs cultured in appropriate media conditions form an embryoid body[35] or a spheroid[36] and undergo neural induction to adopt the neuroectodermal fate.[33,36,37] The neuroectodermal progenitors self-organize into multiple 3D structures featuring apical lumens called neural rosettes or neural buds reminiscent of the neural tube. After 1 month in culture, organoids exhibit neuronal differentiation

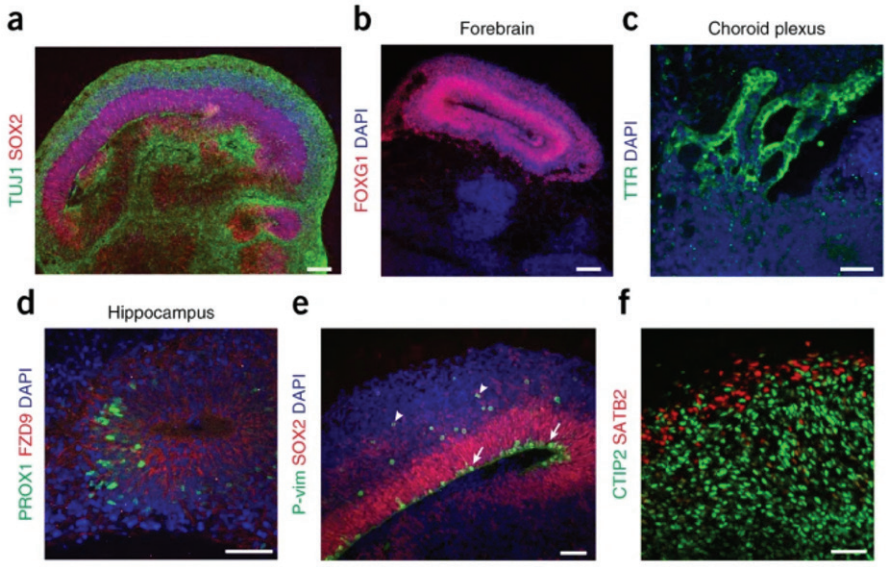

Figure 5. Staining for brain regions and neuronal cell identities. (a) Staining for neurons (TUJ1, green) and progenitors (SOX2, red) in a large continuous cortical tissue within an organoid. Note the organized apical progenitor zone surrounded by basally located neurons. (b) A forebrain region of an organoid staining positive for the marker FOXG1 (red). (c) Choroid plexus stains positive for the marker TTR (green), and it displays convoluted cuboidal epithelium. (d) Hippocampal regions stain positive for the markers PROX1 (green) and FZD9 (red), although the cells fail to spatially organize into recognizable dentate gyrus and CA regions. (e) Staining for mitotic radial glia (P-vimentin (P-vim), green) in a cortical region reveals inner radial glia undergoing mitosis at the apical membrane (arrows), whereas outer radial glia undergo mitosis outside the ventricular zone (arrowheads). All radial glia are marked by SOX2 (red). (f) Staining for cortical layer identities of advanced organoids (75 d). Later-born superficial-layer identity (SATB2, red) neurons populate more superficial regions of the organoid, whereas early-born deep-layer identity (CTIP2, green) neurons populate deeper regions of the organoid. DAPI in a–e labels nuclei (blue). Samples in a–e are 30-35 d after initiation of the protocol. Scale bars, 100 μm (a,b) and 50 μm (c–f). (Figure from Lancaster et al., 2014).

(TUJ1, fig. 4), leading to progressive expansion and thickening of cerebral tissues (Fig. 4).[38] By 2 months, different brain regions are visible, including forebrain and hippocampus (Fig. 5).[38]

The path to cerebral organoid generation is exciting but nothing short of challenging. Knowing we have made the first big leap into the systematic investigation of human brain development and disease we dedicate much time and effort to deepen our understanding and broaden our tool kit. We use brain organoids to examine the cell biological basis of a form of microcephaly, a disorder involving small brain size.[33,39,40] In fact, a variety of neurological disorders can be examined in cerebral organoids. We use RNA interference and patient-specific induced pluripotent stem cells to model microcephaly, a disorder that has been difficult to model in mice. We demonstrate premature neuronal differentiation in patient organoids, a defect that could help to explain the disease phenotype.

We then initiated the development of more organoid-based human disease models.[41] We were able to reproduce the events leading to human brain cancer formation by electroporating mutagenic DNA constructs and introducing brain cancer specific mutations. Importantly, the new methodology allowed some of the key events in human brain cancer, like the invasive nature of cancer cells, to be replicated *in vitro.*[41] We demonstrated the usefulness of our cancer models for drug treatment by inhibiting tumor growth using EGFR inhibitors and predicting drug effects in a patient and tumor type specific manner. Cerebral organoids become very useful for elucidating and characterizing the teratogenic effects of the ZIKA virus and for predicting its mechanism of infection.[42,43] In an attempt to extend those observations, we modelled the pathology of Herpes Simplex Virus in organoids. We succeeded in recreating the pathology and could identify a potential therapeutic strategy for eliminating the virus from the fetal brain.[44] We therefore convincingly showed that three-dimensional organoids can recapitulate development and disease even in this most complex human tissue.

While we agree that organoids enable disease modeling in complex and structured human tissue, *in vitro*, like most 3D models, they lack sufficient oxygen supply, leading to cellular stress. We hypothesized that drawbacks might prevent proper lineage commitment. We therefore set out to analyze brain organoid and fetal single cell RNA sequencing (scRNAseq) data using our own and other datasets totaling over 190,000 cells. We described a unique stress signature found in all organoid samples, but not in fetal samples. We demonstrated that cell stress is limited to a defined organoid

cell population, and developed Gruffi, an algorithm that uses granular functional filtering to filter out stressed cells from any organoid scRNAseq dataset in an unbiased manner.[45] In this way, we offer a robust way to bioinformatically control for the adverse effects of cell stress thereby improving developmental trajectories and strengthening resemblance to fetal data.

In parallel we developed variable organoid protocols that permitted us to study different parts of brain development. We pushed the boundaries by achieving the faithful reproduction of long-range neuron migration in the human brain by assembling dorsally and ventrally patterned organoids (Fig. 6). We recreated a polarity axis and demonstrated that this axis is maintained throughout the organoid culture, leading to correct interactions between the two brain parts. Like in the real human cortex, interneurons within these cultures correctly migrated from the ventral to the dorsal part allowing us to investigate their migration in real time and to test the effect of specific signaling pathways by using specific inhibitors.[46]

We and others share our appreciation for what seems to be a new technology with enormous potential. We now have a versatile tool that can be coupled with genetic screening permitting us distinct bioassays to unravel novel, disease-associated phenotypes and mechanisms. It comes as no surprise that we are enthusiastically combining decades of multi-disciplinary research outcomes and organoid technology to investigate what other model organisms helped frame the hypotheses on. In recent years, multiple breakthrough discoveries have been made, and groundbreaking methodologies have been developed. The most prominent of these is the development of the CRISPR-LICHT approach, which is a method for genetic screening in 3D organoid systems that can now be applied to any set of human disease genes and any organoid system (Fig. 7).[47] The development of the CRISPR-Cas9 endonuclease technology has made diverse methods of genetic engineering readily available to all researchers.[48-51] Unlike the previous technologies, the Cas9 endonuclease is guided to the genomic sequence of interest as a means to generate a DSB by a guide RNA sequence (gRNA), making the system highly versatile and easy to apply.[52] We combined CRISPR/LICHT, CRISPR/Cas9 dropout screening with lineage barcoding to overcome the intrinsic variability of organoids (Fig. 7). This genetic loss-of-function screening within the modern era of organoid technology allows us to search through sets of genes that are suspected to be involved in a specific human brain disorder.[47] We gather definite proof of gene-specific disease relevance while generating an organoid disease model that can be further exploited by the community to portray the disease mechanism

or test therapeutic targets. The CRISPR-LICHT technology permits us to establish a mathematical model for organoid growth and to perform a statistical power analysis for the screen to define its scale and interpret its result. Using the methodology, we screened through 173 candidate microcephaly genes, identifying the unfolded protein response as a new process determin-

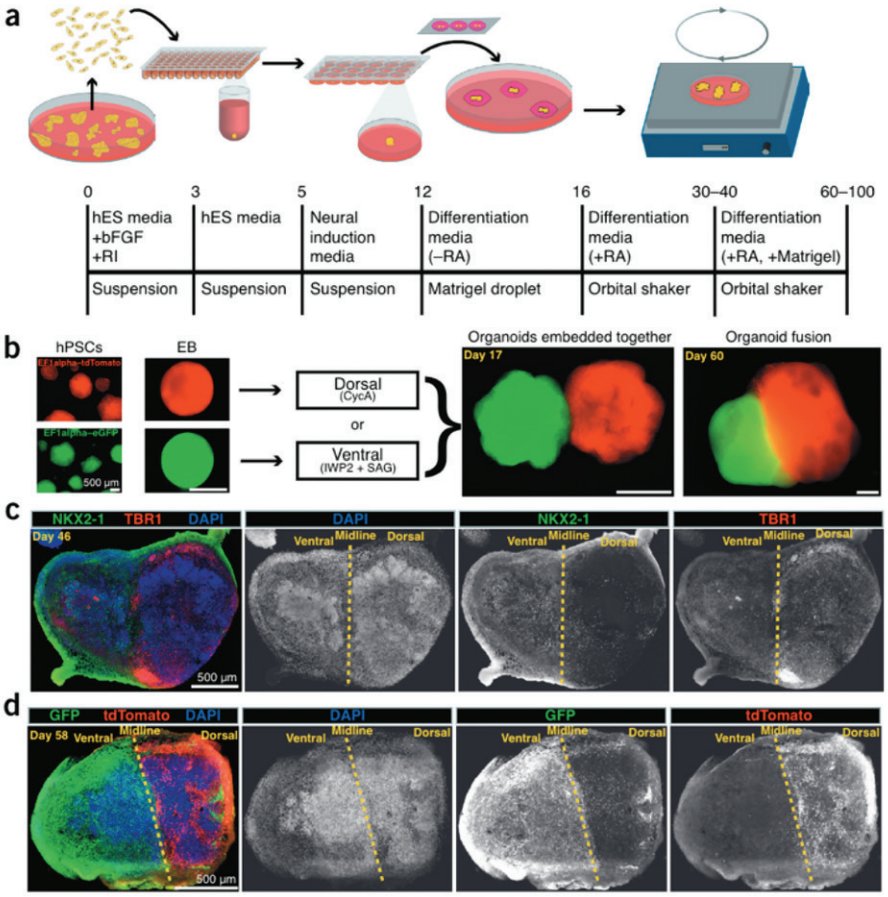

Figure 6. Fused cerebral organoids as a model for cell migration. (a) Schematic of the cerebral organoid fusion coculture method. (b) Representative widefield images at different stages of the organoid fusion procedure. Organoids are independently labeled with the indicated fluorescent reporters. (c) Tile-scan image of a of a ventral::dorsalCycA organoid fusion cryosection immunostained for a ventral (NKX2-1+) and a dorsal (TBR1+) marker. (d) Tile-scan image of a ventral/GFP+::dorsalCycA/tdTomato+ organoid fusion cryosection immunostained for GFP and tdTomato. (Figure from Bagley et al., 2017).

ing brain size in humans. Not only are we able to identify microcephaly genes with CRISPR-LICHT, but we also pinpoint a specific mechanism involved in controlling the size of the brain. The endoplasmic reticulum (ER) was identified as a main hub in controlling extracellular matrix protein secretion (Fig. 8). This mechanism affects the integrity of the tissue, and thus the brain size, and was identified as one cause of microcephaly.

The speed of discoveries is gathering momentum and we seem to be reaching one goal after the other with what appears to be effortless poise but which I can attest to being the merited success of many dedicated and charismatic scientists that lend their talent to stem cell research. It is true that the hypothesis-driven scientist fearlessly tests the *status quo* putting knowledge to practice and creating platforms for what will be the next ordinary or extraordinary step when claiming the unknown. As we approach the present times there is one more discovery we eagerly share. While using cerebral organoids to show that overproduction of mid-gestational human interneurons causes Tuberous Sclerosis Complex (TSC), a severe neuro-developmental disorder, we identified a previously uncharac-

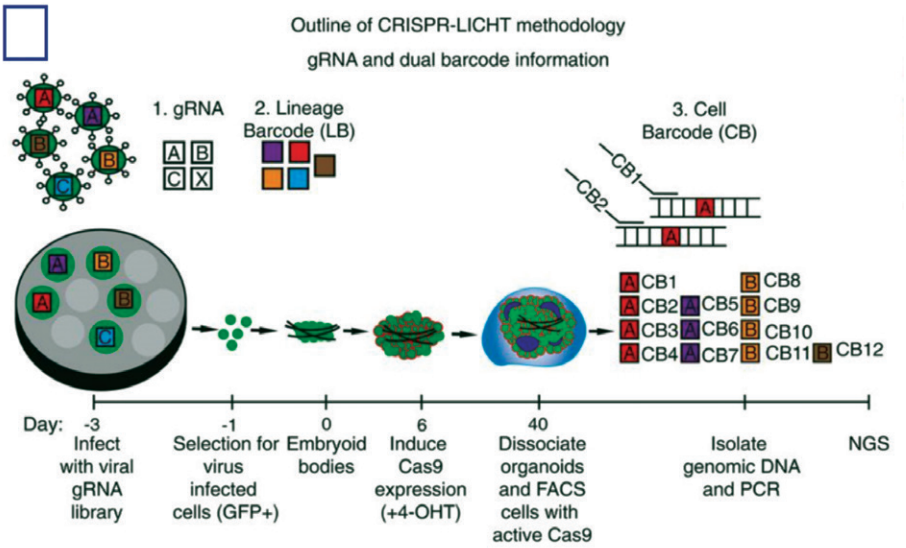

Figure 7. Screening in human cerebral organoids using CRISPR-LICHT identifies microcephaly-associated genes. Scheme describing the screening methodology. Stepwise introduction of gRNA and lineage barcode (LB) as well as cell barcode (CB) are indicated. FACS, fluorescence-activated cell sorting; NGS, next generation sequencing. (Figure from Esk et al., 2020).

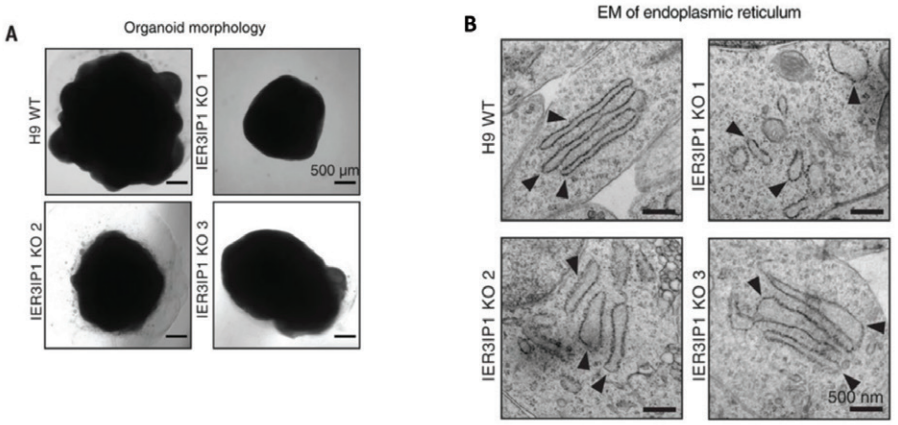

Figure 8. *IER3IP1* deletion results in a microcephalic phenotype and increased UPR and ER stress. (A) Bright-field images of WT and IER3IP1 KO lines 1 to 3 at day 42. Scale bars, 500 µm. (B) EM images of ER structures in neural rosette areas of WT and IER3IP1 KO organoids 1 to 3. Arrowheads indicate ER structures. (Figure adapted from Esk et al., 2020).

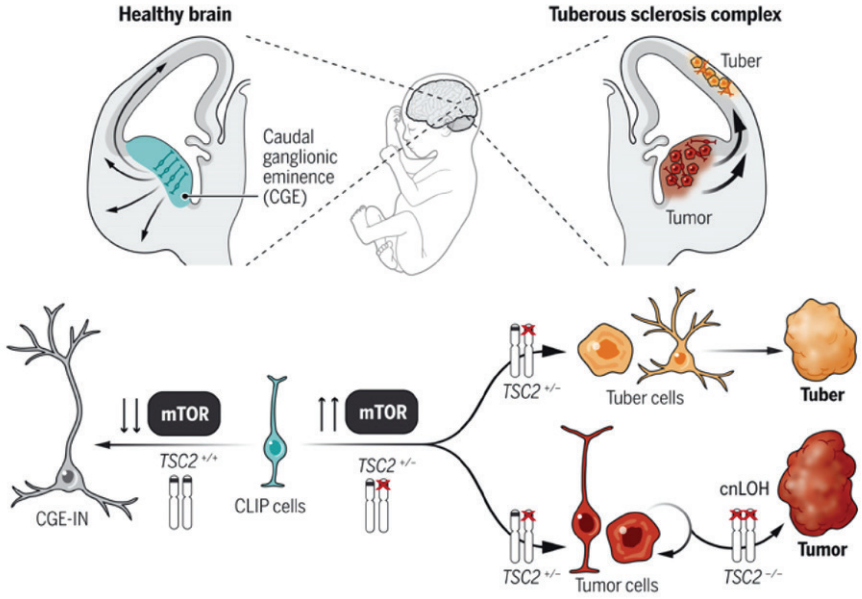

Figure 9. During mid-gestation, CLIP cells residing in the CGE generate interneurons that migrate into the cortex. (Top right) In TSC, CLIP cells generate brain tumors and cortical tubers. Heterozygous mutations in TSC2 result in excessive proliferation of CLIP cells, generating cell types of cortical tubers (orange) as well as brain tumors (red). During progression, the healthy allele is lost because of cnLOH, increasing tumor proliferation. Image: Kellie Holoski/*Science*.

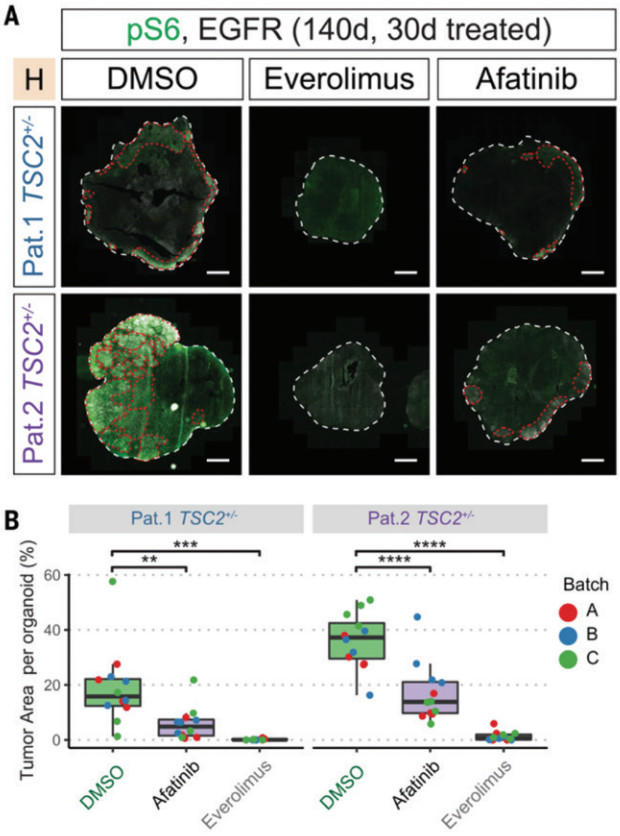

Figure 10. EGFR inhibition reduces tumor burden. (A) Immunostaining for pS6 and EGFR identified tumors (red lines) in the control group (DMSO) in both patients. Tumors were reduced through Afatinib and Everolimus treatment. (B) Quantification of tumor area per organoid. Tumors were identified as regions of overlapping pS6 and EGFR staining. Although tumors were detected in DMSO-control for both patients, Afatinib or Everolimus treatment both reduce tumor burden Scale bars, (A) 1 mm. (Figure from Eichmüller et al., 2022).

terized population of caudal late interneuron progenitors, the CLIP-cells (Fig. 9).[53] We showed that developmental processes specific to humans are responsible for malformations of cortical development (MCDs), which result in developmental delay and epilepsy in children. In TSC, CLIP cells over-proliferate, generating excessive interneurons, brain tumors, and cortical malformations (Fig. 10). Epidermal growth factor receptor inhibition reduces tumor burden, identifying potential treatment options for TSC

and related disorders. The identification of CLIP cells reveals the extended interneuron generation in the human brain as a vulnerability for disease. In addition, this work demonstrates that analyzing MCDs can reveal fundamental insights into human-specific aspects of brain development. We predict that this work will have a long-lasting fundamental impact on the entire field of brain research and will sooner or later find its place in freshly updated textbooks on the disease.

Concluding remarks

The brain, like no other organ, births all thoughts and involuntary triggers. As a consequence, it is a treasure chest storing cardinal information; hard to penetrate, retrieve and even understand. It is perhaps not random that many neuroscientists have originally studied philosophy while others moved from bench science to questioning the mind, its powers and limitations, its flexibility to expand and collapse on the path to enlightenment. Such dynamic processes hosted within a tissue that is highly organized and almost fragile.

Human brain development is, therefore, correctly described as a complex series of dynamic and adaptive processes that are genetically determined and environmentally influenced and which operate throughout development, finally resulting in an organ that is responsible for the widest array of functions we know to exist. Attempting to decipher the genetic codes and molecular pathways that govern cellular function during brain development has been a long and arduous process. The discovery and characterization of stem cells revealed the tangles within the process while instrumentally influencing our understanding by offering the opportunity to make stem cell attributes our asset. Taking advantage of their self-organizing ability, we built a model that was the missing link between knowledge acquired from animal models and the mystery of the human brain.

Born from the passion for evolving science to understand life, methodological developments have always been central to overcoming seemingly impossible obstacles turning improbable outcomes to acquired knowledge. Our lab has significantly contributed in shaping contemporary and future research on brain development in health and disease by careful, almost pedantic experimentalism, and with concentrated focus on multi-disciplinary, expansive and strategic research that pushes the boundaries daring to address the big open questions. At the heels of brain organoid technology, we intertwined quick and efficient gene editing to model diseases that were intractable in mice, including forms of microcephaly and brain cancer. The

comparable complexity between human cerebral organoids and primary tissue promises to further our grasp of human-specific complex diseases such as autism and epilepsy but also as patient-specific *in vitro* cancer models, and for predictive drug testing. Single-cell technologies enhanced our ability to analyze molecular phenotypes at cellular resolution and detect emergent phenotypes that are difficult to tease out with traditional investigation methods.[54] Further adaptation of diverse single-cell -omics technologies to brain organoids expanded the set of discernable phenotypes, otherwise hidden, such as novel cell types and/or states that are altered during disease like in our microcephaly and tuberous sclerosis models. Without a doubt the field of brain organoid research is still young, but its applicability, diversity and potential encourage expedient progress. Given the rapid technical advances in the field, we believe that human organoid systems will provide unprecedented opportunities to improve human health.

To conclude, the brain is an organ that is more understood now than ever before but still to such a narrow degree. Despite the jaw-dropping advancements across all scientific disciplines of neuroscience, we remain far away from fully understanding it, although we appreciate the circuitous complexity of its nature. Attempting to predict what the path will look like in the future would only prove us wrong but what we know for certain is that, as described in this review, neuroscience research resembles a sequel that always has you wishing for more.

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STEM CELLS AND DISEASE STATES

Targeting the Gerozyme 15-PGDH to Regenerate and Rejuvenate Aged Muscles

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Introduction

Aging is an inescapable fact of life. The young and vibrant babe grows to glowing adulthood and then progressively ages, becoming decrepit and worn, as stunningly depicted by the Viennese painter, Gustav Klimt (Fig. 1). Aging is thought to bring wisdom, but for many individuals, their later years are characterized by decline and discomfort as body systems slow and eventually fail. This is a problem that impacts billions of people around the world. Throughout history people have sought means to reverse aging, or to maximize healthspan, time spent free from ailments. From Idun and her magic apples, to the Peaches of Immortality of Chinese Mythology, to the Ambrosia of the Ancient Greeks, to Sir Galahad and the Holy Grail, to Ponce de Leon's Fountain of Youth, humanity has been on a quest for a balm to ameliorate aging.

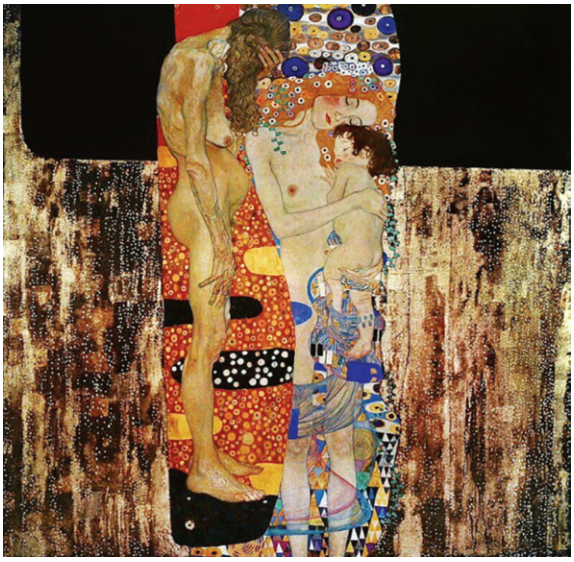

Figure 1. The Three Ages of Woman by Gustav Klimt. Completed in Austria in 1905, the painting features three women at various stages, symbolizing the cycle of life.

To halt aging, a number of strategies have been invoked such as caloric restriction, telomere extension, inducing pluripotency reprogramming in vivo, reversing the methylation clock, and senolytics. Yet to date, these approaches have yet to be proven efficacious in mammals, let alone humans. As depicted by *Nature* (2018), while our lifespan has increased, our healthspan has not (Fig. 2). Both men and women are living longer, however, the increased numbers of years are plagued by chronic disease.[1] The question of utmost importance is: How can we extend quality of life?

An organism, in all its complexity, arises from a single cell. That first cell is pluripotent, it has the potential to become every cell type the body needs. All the information required is encoded within its DNA. Cells divide and specialize during development. The process is highly regulated but can be influenced by both genetic and environmental factors throughout fetal development, infancy, and childhood. Young adulthood can be considered a period of maintenance, where cells persist in homeostasis, a

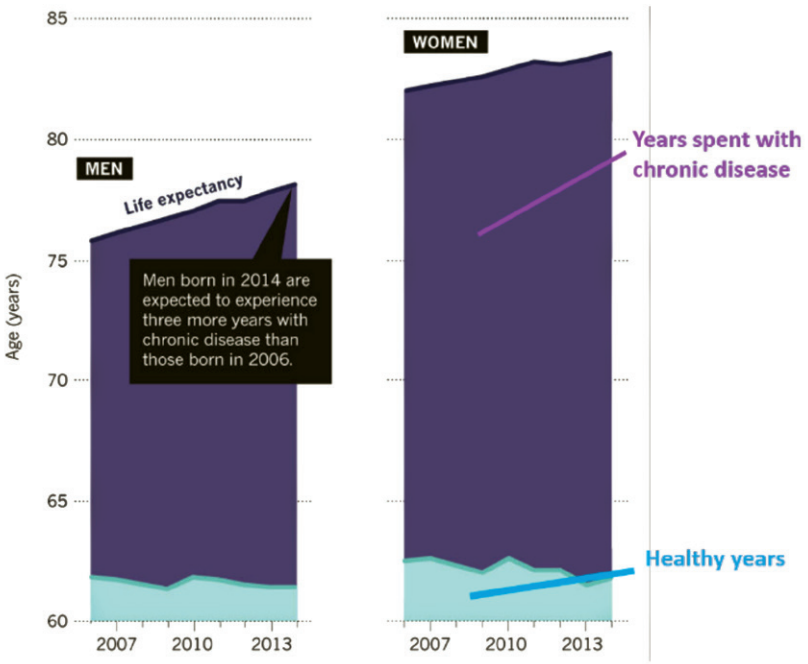

Figure 2. Life expectancy has increased, but healthspan remains the same. Advances in modern medicine have significantly extended human lifespan, however many of the years gained are spent with chronic disease. Healthspan, or the years spent in good health free of such diseases, has not increased proportionally. Figure adapted from Bellantuono et al., *Nature*, 2018.

stable state of equilibrium. However, as we age, cells and tissues change again, becoming progressively dysfunctional.

The hope of stem cell biology and regenerative medicine is that this process can be stabilized or even reversed to allow for robustly enhanced healthspan. By targeting stem cells, the building blocks of the body's specialized tissues, and elucidating mechanisms and drugs that can rejuvenate their function, we seek to enhance the quality of life. Below I chronicle work by my group and others, focused on understanding cell plasticity and stem cells, particularly as they relate to muscle aging and rejuvenation.

Plasticity of Cell Fate

In the 1980s, a long-held view in the field of cell and developmental biology was that the differentiated state of mammalian cells was fixed and irreversible. As an assistant professor I was inspired to challenge this dogma by John Gurdon's groundbreaking research showing that a highly specialized intestine cell nucleus could be reprogrammed to give rise to an entire frog if transplanted into an enucleated oocyte.[2,3] Using a non-dividing heterokaryon cell-fusion system, my lab showed that silent muscle genes could be activated in specialized human cells of all three embryonic lineages: endoderm, mesoderm, and ectoderm.[4,5] Building upon a large body of work that potently employed interspecies cell hybrids to monitor gene expression, map genes to chromosomes, and establish the existence of trans-acting repressors,[6-8] we developed a heterokaryon system in which the two fused cell types did not divide and remained stably intact. In this syncytial cell, chromosomes were not lost or reorganized, and the existence of transacting activators of gene expression could be definitively established for the first time. This system enabled us to show that previously silent muscle genes could be activated in human cells specialized as keratinocytes, fibroblasts, or hepatocytes, providing evidence that the mammalian differentiated state could be altered. The success of our approach arose from the crucial insight that gene dosage must be biased in favor of the desired cell fate outcome – in other words, an excess of muscle nuclei encoding trans-acting factors is required to activate expression of muscle genes in the nonmuscle cell type. Importantly, our experiments showed that the mammalian differentiated state is not terminal, but instead requires constant management to be maintained.

This body of work changed how the field viewed cell fate and was featured on the front cover of the "Frontiers in Biology" issue of *Science* in 1985.[4,5,6,7] The broad significance of our work across phyla is

highlighted in a review I wrote with David Baltimore, "Differentiation requires continuous regulation".[9] Our findings established that the stable differentiated cell state is controlled by mechanisms that are dynamic and reversible.

This concept and the demonstration of the plasticity of the mammalian differentiated state has since been brilliantly highlighted by the elegant experiments of Shinya Yamanaka showing that upon overexpression of four transcription factors, differentiated mammalian cells give rise to pluripotent stem cells.[12] This advance revolutionized the field of stem cell biology and regenerative medicine. It provided the ultimate demonstration that cell fate could be changed and allowed the modeling of human development and disease in culture in a manner not previously possible.

The demonstration of cell fate plasticity has prompted new thinking about the process of aging. Is it possible that aged tissues and stem cells can be reprogrammed to a "youthful" state? Could this state be propagated via a heritable molecular memory? Would such an approach rejuvenate the function of a tissue? Is this a way to increase healthspan?

Overcoming Blocks to Cell Plasticity

While robust regeneration of amputated limbs or injured hearts occurs via a process of dedifferentiation in several orders of lower vertebrates including urodele amphibians and zebrafish, mammalian differentiated cells in an intact organism only exhibit limited regeneration under normal circumstances. Pioneering work with these species has provided many seminal insights.[13,14] A critical step in the process of urodele limb and heart regeneration is the acquisition of proliferative potency. This is achieved by reentry into the cell cycle of postmitotic cells that retain their specified identity. Jason Pomerantz, Kosta Pajcini and I postulated that mammals gained tumor suppressors at the expense of regeneration. We used an evolutionary comparison to identify differences between humans and newts that could underlie their disparate regenerative capacity.[15-17] Newts lack the cell cycle regulatory protein p19/Arf (encoded by the Ink4a locus, a tumor suppressor which first arose in chickens; designated as p14 in humans). Additionally, newt limb regeneration involves inactivation of another tumor suppressor, the cell cycle regulator Rb, by phosphorylation. We wondered if Rb and Arf could also be the major cell-cycle regulators that restrict dedifferentiation in mammals? We addressed this question by showing that following transient inactivation of both Rb and Arf, post-mitotic murine skeletal muscle cells were induced to dedifferentiate,

proliferate, and contribute to muscle repair in vivo. The use of photoactivated laser microdissection and laser pressure catapulting to isolate single, morphologically intact, adherent, primary differentiated cells (myocytes) allowed the unambiguous identification of clones from individual post-mitotic cells. Our results show that differentiation of mammalian cells is reversed by inactivation of Arf and Rb, and support the hypothesis that Arf evolved at the expense of regeneration. The induction of dedifferentiation demonstrated here catalyzed alternative approaches to stem cells for the regeneration of mammalian tissues such as the heart.

Characterization of Muscle Stem Cells

Despite these advances, regenerative medicine stalled for years, as we lacked a method to isolate and purify muscle stem cells in order to probe their properties and discern how to augment their function in culture. The muscle stem cell (MuSC) was first identified by Alexander Mauro and depicted in a single electron micrograph as a satellite cell adjacent to a my-

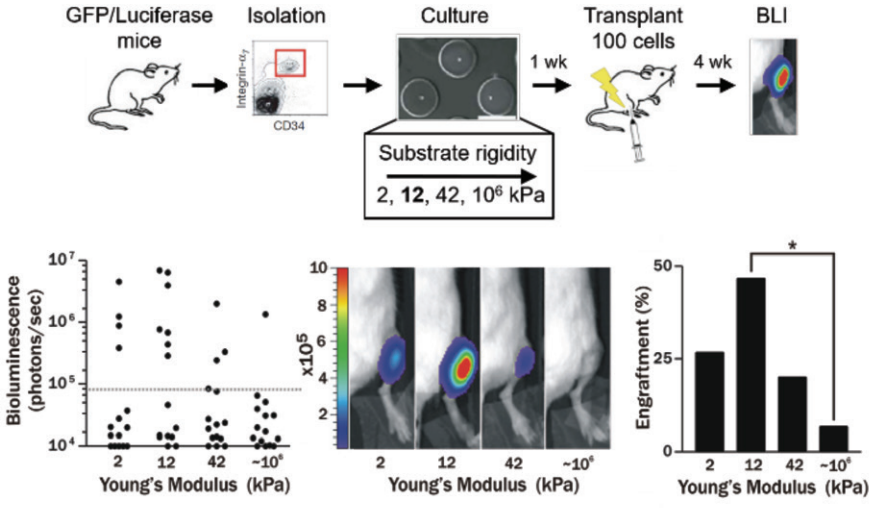

Figure 3. Substrate rigidity is key to maintaining 'stemness' in culture. MuSCs have robust regenerative capacity in vivo that is rapidly lost in culture. We combined a novel bioengineered substrate that recapitulates key biophysical and biochemical niche features and a highly automated single-cell tracking algorithm to show that substrate elasticity is a potent regulator of MuSC fate in culture. MuSCs cultured on soft hydrogel substrates that mimic the elasticity of muscle proliferate in vitro and promote regeneration when transplanted into mice as shown by BLI. Figure adapted from Gilbert et al., *Science*, 2010.

ofiber.[18] However, not until a half a century later did scientists acquire a means to prove Mauro right. Identification of the hallmark MuSC transcription factors by Michael Rudnicki[19] and Margaret Buckingham[20] were major breakthroughs. I recruited engineers, chemists, and artificial intelligence experts to address the problem of muscle stem cell isolation and maintenance in culture. Building on the foundational work of prior investigators, we established parameters for prospectively isolating skeletal muscle stem cells from adult tissues by fluorescence activated cell sorting.[21] We developed a second technique, bioluminescence imaging (BLI), to validate the 'stemness' of the isolated cells by single cell transplantation.[21] BLI enabled assessment of the dynamics and magnitude of muscle stem cell engraftment over time. Further, single stem cell transplants assessed by BLI showed that MuSCs met the quintessential definition of a stem cell established for hematopoiesis: self-renewal and differentiation. Using bioengineered 'niches', we overcame a major hurdle: the stem cell properties of MuSCs were rapidly lost in culture. We showed that MuSC function is exquisitely sensitive to substrate elasticity (Fig. 3, 4). If plated on stiff tissue culture plastic, MuSC function is lost, whereas on hydrogels with a stiffness matching healthy young muscle, stemness is preserved.[22] This work provided a functional link between substrate elasticity and maintenance

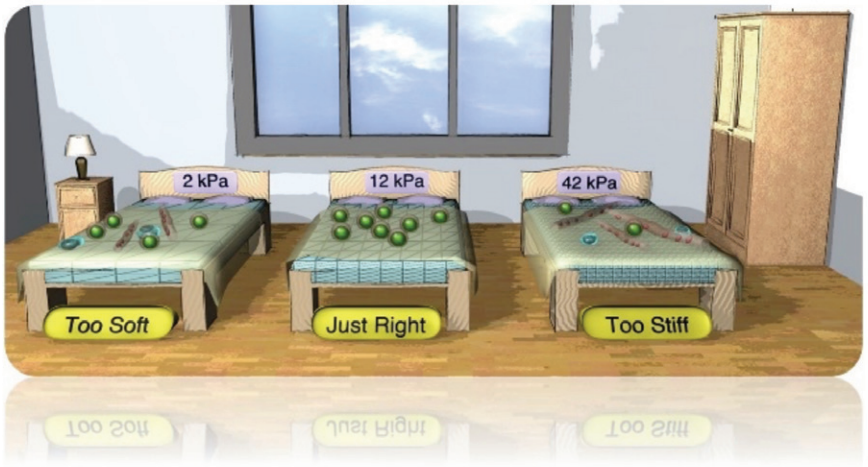

Figure 4. Cells are exquisitely sensitive to substrate stiffness. Some surfaces can be too soft (2 KPa), and others are too stiff (42 KPa). The natural stiffness of muscle tissue found in our bodies (12kPa) is just right; hydrogels mimicking that elasticity maintain the stem cell state *in vitro*. Image by Stephane Corbel/Blau lab.

of muscle stem cell self-renewal following transplantation, establishing a niche paradigm with broad utility for enhancing the regenerative behavior of stem cell types in diverse specialized tissues.

Aged Muscle Stem Cells Have Diminished Regenerative Capacity

Using these techniques, we encountered another roadblock. We found that muscle stem cell function declines markedly during aging due to the acquisition of cell intrinsic defects, which in conjunction with cell-extrinsic systemic factors, are largely responsible for the decreased regeneration observed with aging (Fig. 5A). A small molecule screen identified an inhibitor (SB202) of the aging-associated p38 MAP kinase that enabled stem cell expansion in vitro, but the cells remained dysfunctional. We surmounted this problem by cultivating the stem cells on elastic hydrogels together with SB202 (Fig. 5B-D). This work provided fresh insights that a synergy of biophysical and biochemical signals could expand and rejuvenate the function of the aged stem cell population. Indeed, not only was stem cell engraftment robustly enhanced post injury, but also strength, as aged mice transplanted with the treated stem cells exhibited twitch and tetanic forces on par with young mice (Fig. 5D).[23,24] In parallel, other groups targeted this and other signaling pathways to restore function to aged MuSCs.[23-31] This body of work suggests a therapeutic strategy for the development of an autologous cell therapy to treat localized muscle wasting.

Capitalizing on Immune Signaling to Augment and Rejuvenate Muscle Regeneration

All forms of muscle injury, from exercise to severe trauma, generate an inflammatory response. We sought to capitalize on mechanisms associated with this natural wound healing mechanism. In particular, we wished to increase the regenerative function of the endogenous MuSCs resident in muscle tissues. We hypothesized that a transiently induced inflammatory mediator could regulate MuSC function and play a crucial role in regeneration. Mining the transcriptomic database from our lab and others for genes expressed by activated MuSCs, we found that the G-protein coupled receptor, EP4, a prostaglandin E2 (PGE2) receptor, was among the top hits.

Further research in my lab uncovered Prostaglandin E2 as an essential component of the immune response and repair process that is orchestrated by MuSCs after injury,[32] in accordance with findings by Pavlath showing that regeneration is impeded in mice in which the PGE2 synthetic enzyme, COX-2, is inhibited.[33] My lab found that genetic ablation of the PGE2

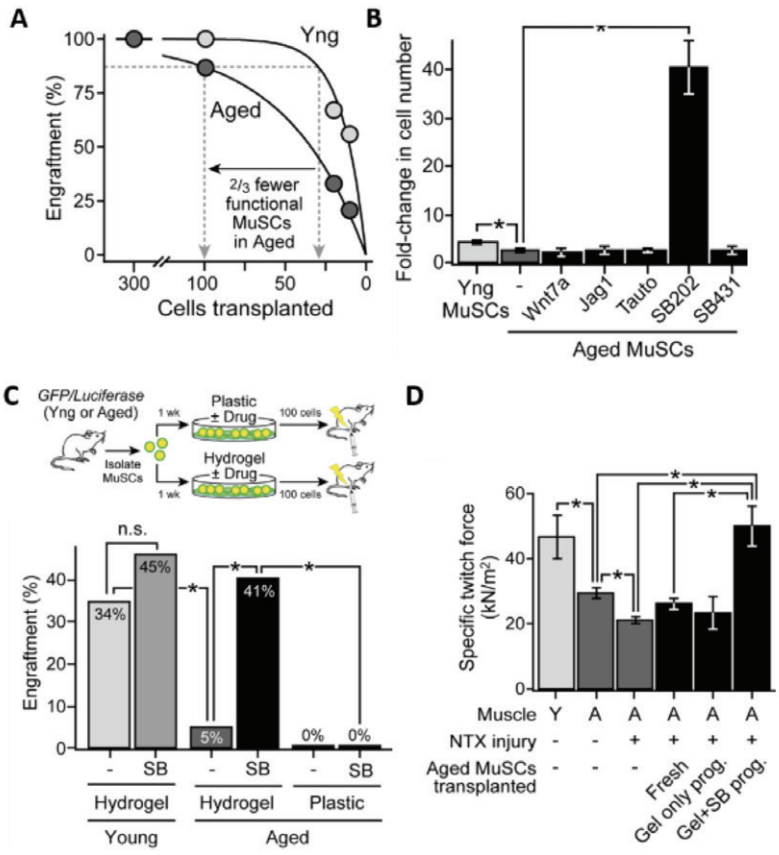

Figure 5. SB rejuvenates function of Aged MuSC populations on soft hydrogels. A. Aged MuSCs engraft less efficaciously than young. B. Screen identified p38MAPK inhibitor SB202 as a factor that improves aged MuSC proliferation. C. SB202, combined with culture on soft hydrogels renders engraftment of aged MuSCs on par with young. D. increase in engraftment leads to a rescue of function. Mice transplanted with SB202 treated MuSCs grown on hydrogel exhibit increased regenerative potential and restoration of strength on par with young. Figures adapted from Cosgrove et al., *Nature Med* 2014 and Blau et al., *Nature Med* 2015.

receptor EP4 on MuSCs leads to a loss of strength after muscle damage (Fig. 6). Similarly, non-steroidal anti-inflammatory drugs, commonly used to treat pain after exercise (a form of muscle injury), inhibit endogenous PGE2 synthesis and hinder regeneration leading to decreased strength. These loss of function experiments highlight the crucial role of PGE2 in muscle repair. PGE2 is essential to the proliferation and survival of MuSCs,

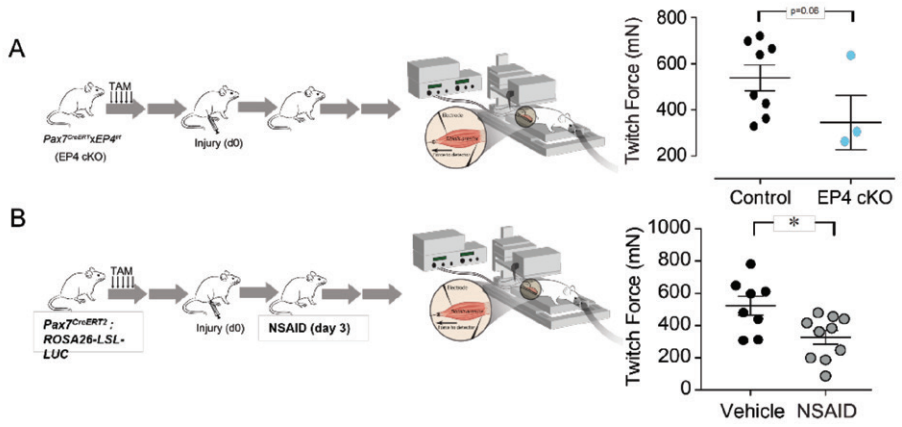

Figure 6. Loss of PGE2 signaling in MuSCs impairs regeneration and recovery of strength post-injury. Genetic ablation of the EP4 receptor blocks PGE2 signaling and impairs muscle regeneration. Consequently, EP4KO injured mice recover less well than controls. Their muscles have lower Twitch force (strength). (A). The use of NSAIDS, such as ibuprofen or Tylenol, inhibits PGE2 synthesis, part of the natural healing response, and show a similar loss of force (B). Figure adapted from Ho et al., *PNAS* 2017.

evident from single cell lineage tracking experiments by timelapse microscopy and from transcriptome analysis of PGE2 treated cells. Importantly, an acute treatment entailing co-injection of PGE2 together with FACS-purified MuSCs augments their engraftment and regeneration of damaged muscles. Moreover, injection of PGE2 alone into injured muscle suffices to induce endogenous stem cells resident in the tissue to increase in numbers and enhance muscle repair. These findings suggest PGE2 as a therapeutic to speed recovery from muscle damage due to trauma or disease.

A Strategy for the Treatment of Sarcopenia, Age-associated Muscle Wasting

Sarcopenia, or the age-associated loss of muscle mass and function, has a profound effect on survival. Sarcopenia is associated with prolonged recovery from falls and surgical interventions, which in turn is associated with loss of independence, increase in hospitalization, and ~$20 billion in annual healthcare expenditures in the United States alone. An estimated 5% of people aged >65 have sarcopenia, rising to ~30% by age 80. Owing to its multifactorial etiology, untangling the causal molecular pathways underlying sarcopenia has proven challenging and there are currently no treatments to counter it.

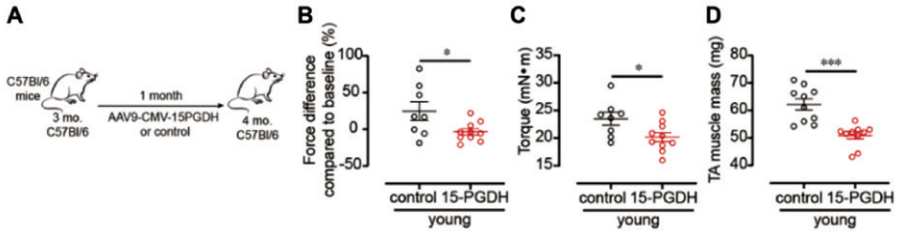

Figure 7. 15-PGDH overexpression in young mice leads to muscle atrophy and loss of strength. Young mice treated with SW, a small molecule that inhibits degradation of PGE2 by 15-PGDH, effectively boosting PGE2 levels (A). Compared to vehicle-treated controls, the muscles of young SW-treated mice are less strong (B, C) and are smaller (D), phenotypes typically seen in aged mice. Figure adapted from Palla et al., *Science* 2021.

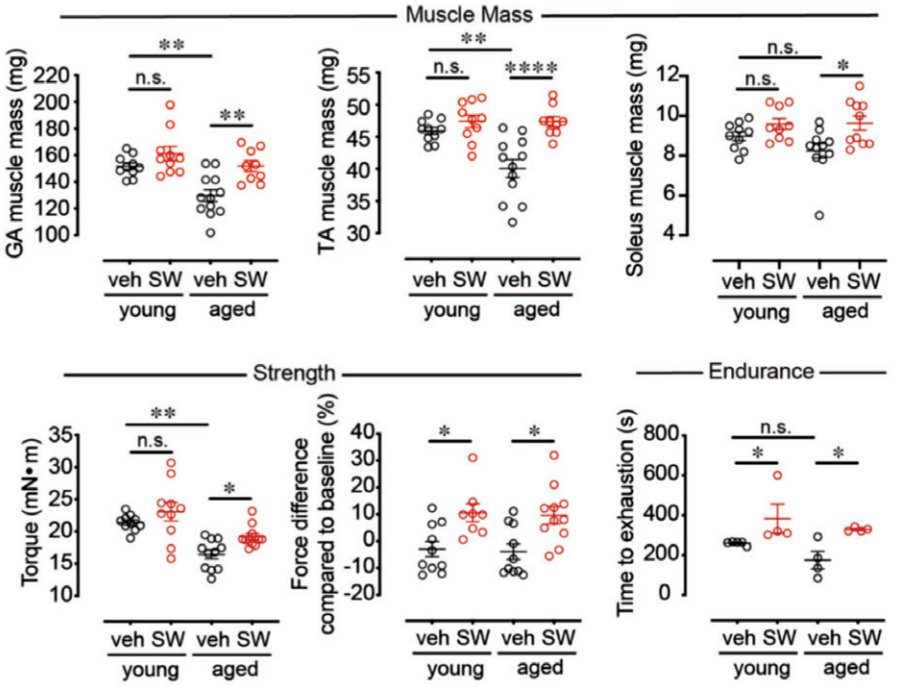

Figure 8. 15-PGDH pharmacological inhibition augments aged muscle mass, strength, and endurance. Aged mice were treated with SW to boost PGE2 levels by inhibiting 15-PGDH. Compared to vehicle-treated controls, the muscles of aged SW-treated mice are larger (top) and stronger (bottom left). In addition, SW treated mice have improved endurance as demonstrated by time to exhaustion running on a treadmill (bottom right). Figure adapted from Palla et al., *Science* 2021.

We hypothesized that a systemic increase in PGE2 might have a widespread beneficial effect on muscles of aged individuals. This led us to the discovery that the enzyme which degrades PGE2, 15-Hydroxprostaglandin Dehydrogenase (15-PGDH), is a pivotal molecular determinant of aging,[34] which we term a "gerozyme". We found that dysregulation of PGE2 homeostasis is a major driver of skeletal muscle wasting in aging. Increasing 15-PGDH levels in young mice shrinks and weakens their muscles, mimicking the effect of years of aging in one month (Fig. 7). Conversely, reducing the activity of 15-PGDH by genetic knockdown or using a small molecule inhibitor in old mice increases PGE2 and leads to improvement in their overall health. After one month of treatment, old mice can run longer distances on a treadmill, their muscles are larger, and 10-15% stronger (Fig. 8).

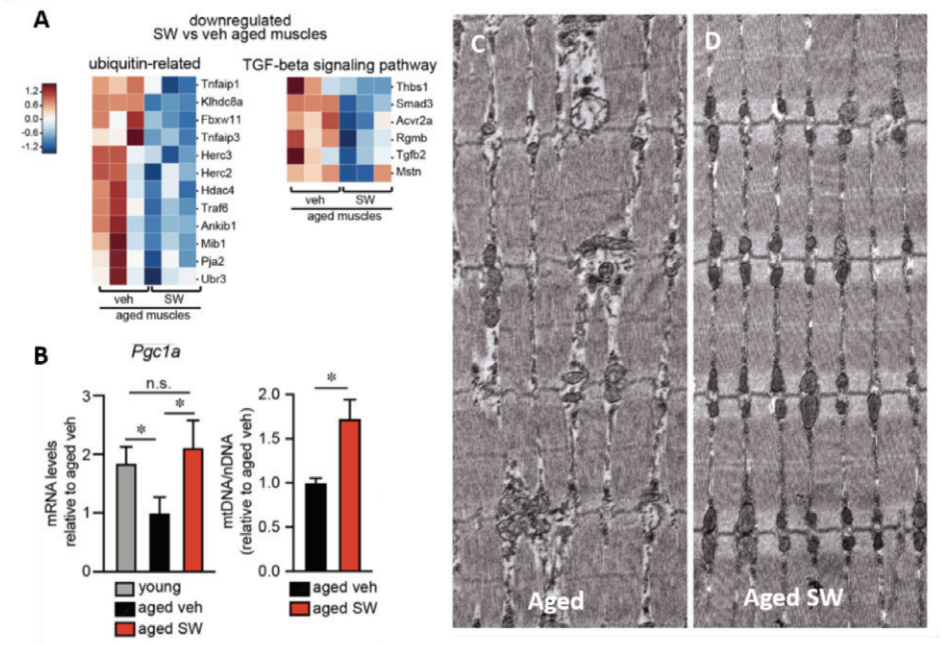

Figure 9. 15-PGDH inhibition impacts multiple pathways to improve aged muscle function. A. Treatment of mouse muscle with SW downregulates the expression of ubiquitin-related genes and atrogenes to levels more typical to those seen in the muscles of young mice. B. We also observed rescue of the mitochondrial master regulator PGC1a to levels typical of young mice in old mice treated with SW. C, D. This led to a restoration of mitochondrial morphology to a youthful state. Adapted from Palla et al., *Science* 2021.

Sarcopenia is a multifactorial disease that results from a compendium of dysregulated signaling pathways that culminate in chronic inflammation, muscle denervation, and defective mitochondria. The pleiotropic beneficial effect that modulating 15-PGDH activity has on sarcopenic muscle suggests that PGE2 is upstream of the molecular changes characteristic of sarcopenic muscle. Restoration of PGE2 levels in aged mice to those found in young reverses the mitochondrial damage and loss of function in sarcopenic muscle, in accordance with other cAMP-inducing agents. PGE2 reduces deleterious TGF-beta signaling components, including myostatin expression, a common target of muscle anti-aging strategies (Fig. 9A). Expression of atrogenes, the ubiquitin ligases that lead to protein degradation, is diminished by PGE2 (Fig. 9A). Autophagy, which is increased when PGE2 is elevated, is likely responsible for the dramatic tissue remodeling seen after SW treatment of aged mice. Strikingly, myofibril alignment is markedly improved, and the distended vacuous mitochondria characteristic of aged muscles are replaced by organelles with the compact morphology and augmented metabolic function characteristic of young muscles (Fig. 9B-D). These synergistic interactions induced by PGE2 following 15-PGDH inhibition drive the dramatic remodeling and rejuvenation of aged muscle tissue structure, mass and strength.[34]

PGE2 signaling is critical for muscle maintenance, regeneration, & rejuvenation

PGE2 rejuvenates aged MuSC function in regeneration:

- PGE2 is an essential inflammatory metabolite for muscle regeneration – body's natural healing mechanism
- PGE2 is required and sufficient for muscle stem cell (MuSC) proliferation, survival, expansion and engraftment
- PGE2 heritably alters MuSC function – epigenetic modifications

PGE2 rejuvenates aged muscle fiber function:

- 15-PGDH degrades PGE2 and is a novel hallmark of aged muscles and other aged tissues
- Benefit derives from physiologic modulation of "pro-inflammatory" metabolite to youthful level
- Mouse muscle strength is increased 15% in one month – humans >50 yr lose 10% muscle mass/decade
- Targeting pivotal regulator of muscle aging, 15-PGDH, may be a therapeutic strategy to counter sarcopenia

Conclusion

The potency of PGE2 arises from its dual targets: muscle stem cells and myofibers. Because the PGE2 degrading enzyme,15-PGDH, can be inhibited using a small molecule drug, there is potential to translate this discovery to the clinic for the treatment of sarcopenia or other muscle wasting disorders. Our finding that endurance, evident as time to exhaus-

tion on a treadmill is increased, suggests that in addition to muscle, the function of other tissues may be augmented. Indeed, our findings likely have relevance of broad scope, as elevated 15-PGDH expression is detected in numerous aged tissues. We suggest that the 15-PGDH "gerozyme" is a master regulator of muscle aging.

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Cellular Reprogramming Approaches to Cardiovascular Disease

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Introduction

Heart disease remains the number one non-infectious cause of death worldwide in adults and newborns. Survivors with heart disease are often left with damaged hearts due to the inability of the mammalian heart to regenerate itself. As a result, globally over 25 million people suffer from heart failure where the cardiovascular system is unable to support the body's normal activities. Genetic causes of valve disease and congenital malformations, present in one percent of all live births, have been discovered, but the cause for a majority is still unknown and no disease-modifying therapies are available clinically.

A deep understanding of gene networks involved in cardiac cell fate and morphogenesis during development has enabled relatively precise control of cell fate decisions in vitro. The ability to control programming and reprogramming of human cells is now possible and is used to not only generate human induced pluripotent stem (iPS) cells, but also to differentiate pluripotent cells into desired cell types for transplantation, to understand disease mechanisms, and to discover new therapeutics. Here we will provide some examples of how control of fate decisions involving cellular reprogramming is providing hope for cardiac regeneration and new therapeutic development.

Cellular Reprogramming for Regenerative Medicine

In recent years, redeployment of key networks by discrete combinations of transcription factors (TFs) and translational regulators has enabled reprogramming of somatic cells to induced pluripotent stem (iPS) cells, demonstrating the power of a limited number of factors to dictate cell-fate decisions [1,2]. More recently, somatic cells have been reprogrammed directly toward multiple cell types *in vitro* using combinations of transcription factors and/or microRNAs with varying degrees of efficiency, including

the cardiomyocyte, neuronal, and hepatocyte fates, among other cell types [3]. Because the heart has limited regenerative potential and has a vast pool of fibroblasts, the ability to reprogram endogenous cardiac fibroblasts into cardiomyocyte-like cells has emerged as a promising approach to restore function to damaged hearts.

Our lab initially pioneered the conversion of cardiac fibroblasts toward a cardiomyocyte-like cell with a combination of Gata4, Mef2c and Tbx5 (GMT) [4,5]. Many groups have reproduced and advanced the technology with various combinations of genes and microRNAs and chemicals [6]. We termed these cells "induced cardiomyocytes" (iCMs), as they developed sarcomeric structures and calcium transients typical of cardiomyocytes. Although there was significant heterogeneity to the iCMs, the more fully reprogrammed iCMs had action potentials that were most similar to adult ventricular myocytes, consistent with lineage tracing evidence that reprogrammed cells did not go through a cardiac progenitor stage during their transition [4]. However, most of the cells transdifferentiated in vitro were only partially reprogrammed.

Because the objective with this strategy was to harness the endogenous cardiac fibroblasts for regeneration without needing to use cell-based therapy, we delivered GMT in vivo retrovirally after ischemic injury in mice and successfully converted resident non-myocytes to cardiomyocyte-like cells. Genetic lineage-tracing studies in mice were performed to demonstrate that dividing non-myocytes infected by retroviruses could be converted into iCMs [5] progressively over a period of 4 weeks. iCMs in this setting developed sarcomeres, with ~50% of reprogrammed cells develop-

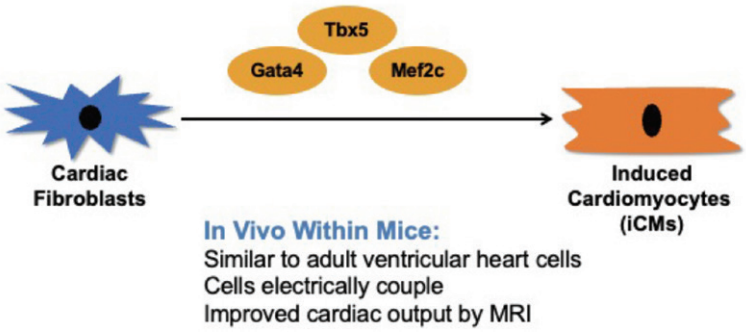

Figure 1. Direct conversion of fibroblasts to induced cardiomyocyte-like cells (iCMs).

ing contractile activity when isolated in single-cell suspension, compared to less than 0.1% when reprogrammed *in vitro*. Importantly, we found evidence for electrical coupling of the *in vivo* reprogrammed iCMs with endogenous cardiomyocytes and other iCMs, and reprogrammed cells were most similar to ventricular cardiomyocytes electrically and transcriptomically. *In vivo* delivery of GMT intramyocardially decreased scar size and attenuated cardiac dysfunction after coronary ligation, as assessed by MRI and echocardiography. As expected, cardiomyocytes within the scar area of GMT-treated mice represented newly born iCMs as determined by lineage tracing experiments (Fig. 1).

To advance cardiac reprogramming technology, we and others described overlapping but distinct combinations of factors that could reprogram human fibroblasts into a more cardiomyocyte-like state. This included a combination of MEF2C, its co-activator, MYOCARDIN, and TBX5 [7,8]. This technology is now being developed toward a clinical trial by Tenaya Therapeutics, where reprogramming factors have been packaged into a single AAV vector with initial efficacy observed in the pig model. Future clinical trials in patients with heart failure will determine if this regenerative approach can result in improved cardiac function and avoidance of heart transplant or death.

Cellular Reprogramming for Disease Modeling and Drug Discovery

Human mutations with large effect size, as observed for monogenic diseases, hold the greatest promise for successful disease modeling using human iPS-derived cells, with the hope that underlying mechanisms will be relevant to more common forms of disease. However, use of iPS-derived models for understanding more complex disease will also be important, albeit more challenging. Here, we will consider examples of both cases as they relate to heart disease.

iPS-Derived Therapeutic Development for Calcific Aortic Valve Disease

Calcific aortic valve disease (CAVD) is the third leading cause of adult heart disease and is responsible for over 100,000 valve replacements annually in the United States alone. The disease progresses in an age-dependent fashion. Bicuspid aortic valve (BAV), a congenital malformation which occurs in 1-2% of the population and involves the formation of two rather than the normal three valve leaflets, is a major risk factor for early valve calcification, although the mechanism for the calcification had been unknown. Our group previously reported two families with heterozy-

gous nonsense mutations in the membrane-bound transcription factor, NOTCH1 (N1), which led to BAV and severe aortic valve calcification in adults [9]. Valve thickening also occurred, and pathology ranged from neonatal to adult onset. Further studies have identified *N1* mutations in additional familial cases of BAV and CAVD, as well as in approximately 4% of sporadic CAVD cases, underscoring the importance of *N1* in this disease [10,11]. In mice, endothelial cell (EC)-specific deletion of the N1 ligand JAGGED1 results in aortic valve calcification and malformed valves [12], consistent with a critical role for the ECs lining the valve in the malformation. Despite the recognition of *N1* mutations as a cause of CAVD, as well as SMAD6 [13], there are currently no medical treatments available for CAVD patients.

Recent studies from our group suggest the pathology involves reprogramming of valve endothelial cells into osteoblast-like cells with activation of gene networks that promote calcification. Using human iPSC-derived ECs, we showed that heterozygous nonsense mutations in *N1* disrupt the epigenetic architecture, resulting in de-repression of latent pro-osteogenic and -inflammatory gene networks (Fig. 2) [14]. Hemodynamic shear stress activated anti-osteogenic and anti-inflammatory networks in $N1^{+/+}$, but not $N1^{+/-}$ iPSC-derived ECs [14]. *N1* haploinsufficiency altered H3K27ac at N1-bound enhancers determined by chromatin immunoprecipitation followed by sequencing (ChIP-seq), dysregulating downstream transcription of over 1000 genes. The gene pathways that were perturbed were implicated in osteogenesis and inflammation. Computational analyses of the disrupted N1-dependent gene network by integrating datasets revealed regulatory nodes, particularly the transcription factors, SOX7, TCF4 (mediating Wnt signaling) and SMAD1 (mediating Bmp signaling), that were upregulated in the mutant setting (Fig. 2). Remarkably, knockdown of just SOX7 and TCF4 restored the gene network dysregulated by *N1* haploinsufficiency toward the wild-type (WT) state (14). Importantly, primary ECs grown from explanted valves from patients with CAVD show a similar dysregulation in gene expression [15], supporting the clinical relevance of these findings.

We introduced the mouse $N1^{-}$ allele into mice lacking the telomerase RNA component (TERC), resulting in shortened telomeres over successive generations of breeding. $TERC^{-/-}$ mice are relatively normal for up to 5 generations of breeding, each with progressively shorter telomeres. Although $N1^{+/-}$ mice have normal echocardiographic findings at baseline, crossing this strain to mice lacking TERC over successive generations ($mTR^{-/-}$generation 1-3, referred to as mTR^{G1}-mTR^{3}) demonstrated that in

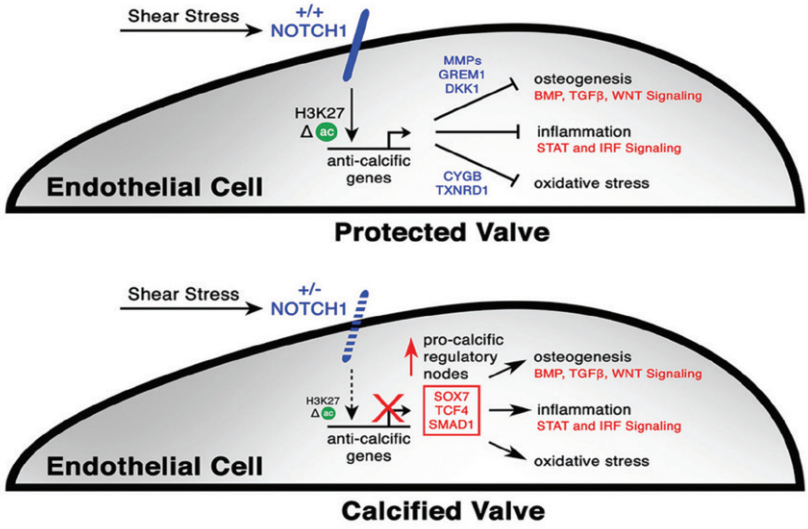

Figure 2. Disruption of Notch 1 signaling derepresses osteogenic and inflammatory gene networks in endothelial cells, resulting in valve calcification. (Theodoris et al., *Cell*, 2015).

the setting of shortened telomeres, $N1^{+/-}$ mice develop age-dependent AV thickening, calcification, and stenosis as well as pulmonary valve (PV) stenosis, mimicking the range of human disease caused by *N1* haploinsufficiency [16]. 40–50% of $N1^{+/-}$ mTR^{G2} mice developed significant histologic evidence of aortic valve calcification within 2 months of age, and ~30% had severe enough aortic or pulmonary valve stenosis to detect echocardiographically, represented by acceleration of blood flow across the valve. Most strikingly, immunohistochemistry of aortic valve sections revealed the presence of Runx2-positive cells in the valve. Runx2 is a "master transcriptional regulator" of the osteoblast fate, consistent with the conclusion from the human iPSC study that the underlying pathology in CAVD is a cellular reprogramming event of a valve cell into an osteoblast-like state. Thus, this mouse model both effectively recapitulated many aspects of the human disease state and supported conclusions of the human iPSC model. It also provided an in vivo model in which to test potential therapies.

Given the mechanistic insights we developed through our gene network analyses, we sought to identify small molecules that could correct the gene expression dysregulated by *N1* haploinsufficiency. Small molecules are traditionally screened for their effects on one to several outputs

at most, from which their predicted efficacy on the disease as a whole is extrapolated. However, determining the gene regulatory networks driving human disease allows the design of therapies targeting the underlying disease mechanism rather than primarily symptomatic management. In principle, mapping the architecture of the dysregulated network could enable screening for molecules that correct a gene network's core regulatory elements rather than peripheral downstream effectors that will likely have only limited influence on the disease process.

Accordingly, we designed a targeted RNA-seq strategy assaying expression of over 100 genes that were either predicted central regulatory nodes or peripheral genes positioned within varied regions of the N1-dependent network in human iPSC-derived ECs determined by whole transcriptome RNA-seq [14,15]. We evaluated these genes in isogenic wild-type (*WT*) or $N1^{+/-}$ iPS-derived ECs exposed to either DMSO or each of 1595 small molecules. To screen small molecules for this effect, we used machine learning approaches to classify the network gene expression by targeted RNA-seq as *WT* or $N1^{+/-}$ based on isogenic ECs of each genotype exposed to vehicle. This strategy resulted in a total of 7 hits that were ultimately validated. The molecule, XCT790, had the largest corrective impacts on the network.

We performed a pre-clinical trial with the drug candidates by treating 4-week-old $N1^{+/-}$ mTR^{G2} mice with daily intraperitoneal injection

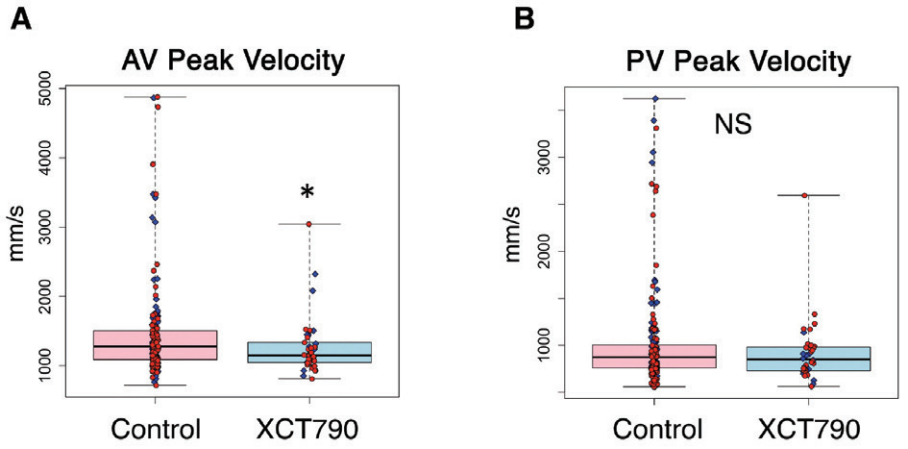

Figure 3. XCT790 inhibits valve stenosis in Notch1$^{+/-}$Terc$^{-/-}$ mice. (A) Aortic valve (AV) or (B) Pulmonary valve (PV) peak velocity by echo-cardiography in N1$^{+/-}$/mTRG2 mice treated with XCT790 or control solvent (AV *P = 0.017). (Theodoris et al., *Science*, 2021).

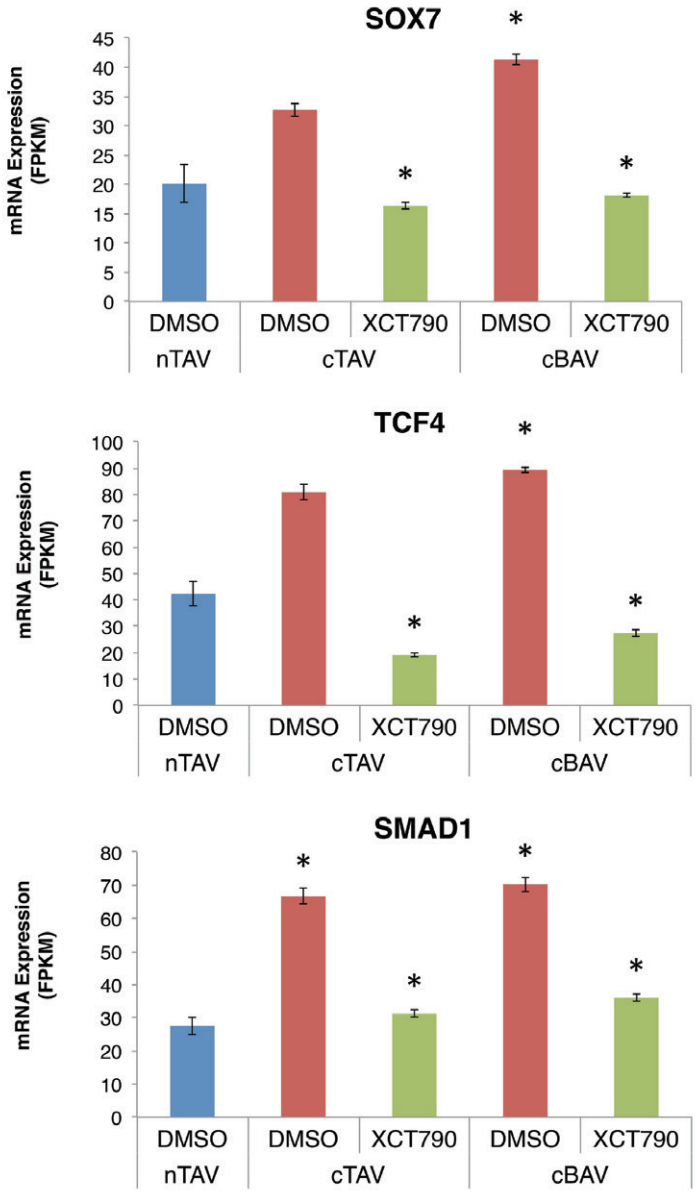

Figure 4. XCT790 reverses gene dysregulation in primary aortic valve endothelial cells from explanted valves of CAVD patients. Expression of the key regulatory nodes SOX7, TCF4, and SMAD1 in normal tricuspid aortic valves (nTAV), calcified TAV (cTAV), or calcified bicuspid aortic valves (cBAV) ECs by RNA-seq (*P ‹ 0.05). nTAV, n = 5; cTAV, n = 9; cBAV, n = 12. (Theodoris et al., *Science*, 2021).

of each compound for 30 days. Consistent with the gene network shift in human iPSCs, XCT790 treatment was the most effective in vivo and was sufficient to prevent aortic valve stenosis in vivo by echocardiography and showed a trend of reducing pulmonary valve stenosis by echocardiography (Fig. 3). Compared to control solvent, XCT790 also significantly reduced the thickness of treated aortic and pulmonary valves, and calcification of aortic valves.

We tested whether the effect of XCT790 could generalize to primary AV ECs from multiple patients with sporadic CAVD. We performed RNA-seq in primary human AV ECs cultured from explanted normal tricuspid AVs (nTAVs, n=5), calcified tricuspid AVs (cTAVs, n=9), and calcified bicuspid AVs (cBAVs, n=12) treated with XCT790 or DMSO. Overall, there was a significant overlap in genes dysregulated in N1-haploinsufficient ECs with those dysregulated in the same direction in cTAV ECs and cBAV ECs. XCT790 was effective in broadly correcting the dysregulated genes back to the normal state in both primary cTAV and cBAV ECs, including the key nodes regulators, SOX7, TCF4 and SMAD1 (15) (Fig. 4).

XCT790 is annotated to be a highly specific compound that targets the orphan nuclear receptor ERRα (estrogen-related receptor α), with little cross-reactivity with the estrogen receptor (ER). Thus, by inhibiting ERRα, XCT790 may function to block the aberrantly activated pro-osteogenic signaling in *N1*-haploinsufficient cells to prevent valve stenosis and calcification. Further development of XCT790 or a chemical derivative is underway for clinical advancement of this potential medical therapy which was based on human genetic studies, iPS-based interrogation of mechanism, gene network correcting drug discovery, and in vivo drug efficacy.

Discovery of novel gene candidates for congenital heart disease by intersecting iPS-based proteomics and genetics

While monogenic causes of heart disease have been informative, these are relatively rare. Genetic analyses of over 3,000 proband-parent trios in the Pediatric Cardiac Genomics Consortium (PCGC) revealed that *de novo* monogenic aberrations were found to collectively contribute to ~10% of congenital heart disease (CHD) cases, while rare inherited and copy number variants have been identified in ~1% and 25% of cases, respectively [17]. Additionally, polygenic and oligogenic inheritance models, where multiple genetic variants with epistatic relationships are implicated, have been proposed as mechanistic explanations for certain complex phenotypes. A recent study from our group highlighted the involvement of genetic modifiers in

human cardiac disease [18], but the net contribution of oligogenic inheritance remains to be determined. A barrier to a complete understanding of CHD's etiology is its immense genetic heterogeneity. Estimates based on *de novo* mutations alone indicate that more than 390 genes may contribute to CHD pathogenesis [19]. Despite the growing catalogue of human genome variants, the cause of over 50% of CHD cases remains unknown [17].

Cardiac malformations have been linked to variants in tissue-enriched cardiac transcription factors that are expressed more widely. Such transcription factors typically form complexes with other tissue-enriched and ubiquitous proteins to orchestrate specific developmental gene programs. Missense variants in transcription factors can disrupt specific interactions with other proteins, affecting their transcriptional cooperativity and causing disease [20]. In CHD specifically, an excess of protein-altering *de novo* variants from the Pediatric Cardiac Genomic Consortium's cohort were found in ubiquitously expressed chromatin regulators that partner with cardiac transcription factors to regulate the expression of key developmental genes [21]. This led us to hypothesize that protein-protein interactors of transcription factors associated with CHD may be enriched in disease-associated proteins, even if these proteins are not tissue-specific.

GATA4 and TBX5 are two essential transcription factors and among the first identified monogenic etiologies of familial CHD. Subsequent studies demonstrated that TBX5 and GATA4 cooperatively interact on DNA throughout the genome to regulate heart development [20,22]. Disruption of the physical interaction between these proteins or with other specific co-factors by missense variants can impair transcriptional cooperativity and lineage specification, and ultimately cause cardiac malformations [20,23]. Therefore, the unbiased identification of human GATA4 and TBX5 (GT) protein interactors during cardiogenesis could highlight disease mechanisms and aid in predicting the impact of protein-coding variants in CHD.

To identify the GATA4 and TBX5 protein interactome (GT-PPI) in the relevant human cardiac cells, we used human induced pluripotent stem cell-derived cardiac progenitors (CPs) and identified antibodies against each endogenous factor that were effective for affinity purification and mass spectrometry (AP-MS). Using CRISPR Cas9-gRNA ribonucleoproteins, we generated clonal *TBX5* or *GATA4* homozygous knockout hiPSC lines as negative controls. This approach yielded 272 proteins in total, which comprised several of the previously reported GATA4 and TBX5 interactors as well as novel interactors (24). Mutations in several of these

interactors have been previously associated with human or mouse cardiac malformations, highlighting the potential of this approach for disease-gene discovery. Use of human iPS-derived cardiac progenitors was essential for this approach, as the same type of AP-MS resulted in a set of interactors that were not enriched for variants in CHD probands.

To determine whether the GT-interactors identified in human CPs might help predict genetic risk factors for CHD, we assessed their intersection with *de novo* variants (DNVs) and very rare (minor allele frequency (MAF) $<10^{-5}$) inherited loss-of-function variants found in over 3,000 CHD probands from the PCGC. We used a permutation-based statistical test to analyze the frequency of variants in GT-interacting proteins among the CHD probands compared to the control group. The analysis indicated that protein-altering DNVs were significantly more likely to be found within GT interactors in the CHD cohort relative to the control cohort by nearly 6-fold for GATA4 interactors, and 4-fold for TBX5 interacting proteins (Fig. 5). By contrast, very rare inherited loss-of-function variants occurred in GT-PPI proteins with the same frequency in control and CHD groups.

We developed a prioritization score for the DNVs that incorporated gene, residue and proband information that appeared to effectively rank the potential impact of each missense DNV. To experimentally test the importance of missense mutations predicted to play a role in CHD, we focused on a high-scoring GATA4-interacting protein, GLYR1, which is a chromatin reader involved in chromatin modification and regulation of gene expression through nucleosome demethylation [25]. The *GLYR1*

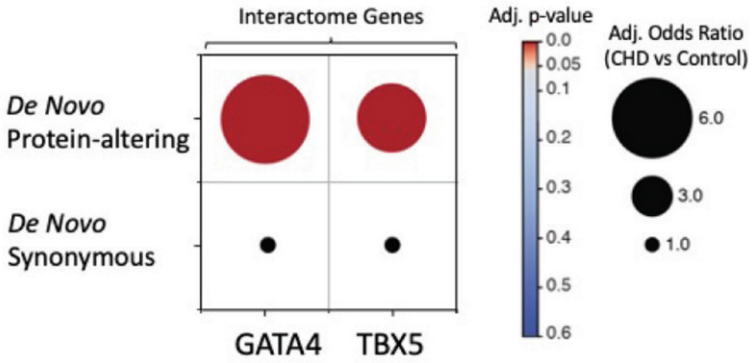

Figure 5. GATA4 and TBX5 interactome reveals enrichment in de novo variants associated with CHD. Results of permutation-based test for genomic variation indicated from PCGC CHD and control cohorts within the GATA4 or TBX5 inter-actomes in cardiac progenitors.

missense CHD DNV we detected involved the substitution of a highly conserved proline with a leucine at amino acid (aa) 496. This amino acid change disrupted GLYR1 interaction with GATA4 [24].

Analyses of DNA occupancy in iPS-CPs found a statistically significant overlap between GLYR1 and GATA4-bound gene bodies, identifying a defined subset of GATA4 and GLYR1-bound genes, mostly upregulated in CPs vs hiPS cells and with greater enrichment in heart development GO terms compared to GLYR1-only and GATA4-only occupied gene bodies.

Evaluation of a human iPS cell line containing the GLYR1 proline to leucine (P496L) missense mutation found in the patient suggested that the P496L variant affects GLYR1 DNA occupancy and transcriptional regulation of a discrete set of target genes co-bound by GATA4, several of which have been involved in human cardiac malformations and cardiomyopathies. Overall, these data demonstrated a detrimental impact of the GLYR1 P496L variant in CM differentiation, associated with altered GLYR1 genomic occupancy and gene regulation at a discrete set of loci co-bound by GATA4.

In order to assess the biological importance of the GLYR1 P496L variant *in vivo*, we generated a mouse line harboring a P495L single nucleotide variant in *GLYR1* (*Glyr1*$^{P495L/+}$), homologous to human P496L, using CRISPR-Cas mediated genome editing. Over half of the mutant mice died at birth, many with ventricular septal defects (VSDs). Thus, this model provided evidence for the biological importance of GLYR1 in cardiac development, and demonstrates a deleterious effect of the P495L variant *in vivo.*

To assess whether there was a GATA4-GLYR1 genetic interaction in mice, we crossed *Glyr1*$^{P495L/+}$ mice to GATA4-mutant mice. Whole mount, histology and echocardiography analysis showed that compound *Glyr1*$^{P495L/+}$*:Gata4*$^{+/-}$ hearts had complete penetrance of cardiac septal defects, including about 80% represented by atrio-ventricular septal defects (AVSDs). These data provide *in vivo* evidence for the biological relevance of the *GLYR1* P496L variant and its interaction with GATA4 in human disease. Furthermore, the results experimentally validate the approach of identifying the interactomes of disease-causing proteins and evaluating genetic variants in the interactors.

Conclusions

In summary, the use of cellular reprogramming technologies has facilitated novel methods of cardiac regeneration by directly reprogramming resident cardiac fibroblasts into new cardiomyocyte-like cells, led to the

recognition of aberrant cellular reprogramming as the basis for aortic valve disease, and revealed novel genes contributing to CHD. Each of these findings establish the foundation for novel therapeutic approaches to human heart disease.

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Towards Combined *Ex Vivo* Cell and Gene Therapy for Epidermolysis Bullosa

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Over three decades of research on both adult stem cells and somatic derivatives of pluripotent stem cells are culminating in remarkable clinical results. The combined disciplines of cell therapy, gene therapy and tissue engineering, broadly known as regenerative medicine, have the potential to revolutionize the treatment of previously incurable injuries and rare, orphan diseases.[1]

In 1975, the first human epidermal keratinocyte culture was established by Prof. Howard Green. Less than 10 years later, the lives of two children suffering from full-thickness burns on over 95% of their body surface were saved using autologous keratinocyte cultures.[2] Since then, such cultures have been used worldwide to treat thousands of patients with massive third-degree burns.[3]

This stunning achievement heralded the age of modern regenerative medicine and paved the way toward the development of *ex vivo* cell therapies of other epithelial injuries, as, for instance, severe ocular burns.[4] Corneal transparency is critical for visual acuity and relies on stromal avascularity, proper organization of collagen fibers and epithelial integrity. Repair and renewal of the corneal epithelium are sustained by stem cells located in the basal layer of the limbus, the narrow zone between the cornea and the bulbar conjunctiva.[5] Ocular burns affecting the central part of the cornea can be treated by a keratoplasty, but extensive chemical burns affecting also the limbus lead to limbal stem cell deficiency (LSCD), making keratoplasty ineffective.[5] LSCD is characterized by neovascularization, chronic inflammation, stromal scarring, and invasion of the cornea by bulbar conjunctival cells, with consequent corneal opacification and loss of vision. In unilateral LSCD, an option to prevent the conjunctival over-growth is to restore the limbus by grafting limbal fragments taken from the uninjured eye. The finding that limbal cultures include corneal stem cells fostered the therapeutic application of autologous limbal cultures, which led to regeneration of a fully functional corneal epithelium and restoration of visual acuity not only in unilateral LSCD but also in

severe bilateral corneal damage.[4,5] Indeed, 1-2 mm2 of healthy limbus in one eye suffices to generate limbal cultures able to restore the corneal epithelium of both eyes[4,6] (Figure 1A).

The knowledge acquired during the implementation of such advanced therapies fostered the development of combined cell and gene therapy for genetic skin diseases, such as Epidermolysis Bullosa (EB).

Epidermolysis Bullosa

EB is a heterogeneous group of rare, dominantly or recessively inherited, genetic disorders characterized by recurrent blistering of the integument. Blisters arise, spontaneously or upon minimal mechanical stress or trauma, because of the extreme skin fragility caused by mutations in genes encoding various structural proteins of the epidermal-dermal junction.[7] Common features of many EB forms include damage of ocular surface, upper airways, oral mucosa, and gastrointestinal and renal systems, as well

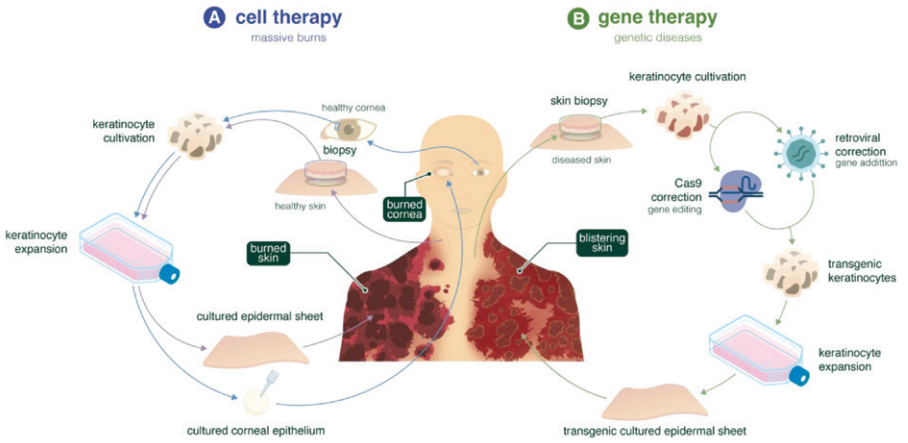

Figure 1. Advance therapies for squamous epithelia. (A) A small (1-2 mm^2) biopsy is taken from the limbus of the healthy eye of patients with unilateral chemical burn-dependent total limbal stem cell deficiency (LSCD), severe bilateral ocular burns or of unburned skin areas (4-10 cm^2). Clonogenic keratinocytes including holoclones are cultivated and used to prepare autologous fibrin-cultured grafts. Grafts are then applied on the corneal surface of injured eyes after removal of the conjunctival or on the skin burn after wound bed preparation. Within one week after grafting, the surface is covered with a fully functional epithelium. (B) *Ex vivo* combined cell and gene therapy. Clonogenic keratinocytes are cultivated from a small skin biopsy (2-9 cm^2), transduced with γRVs vector carrying the appropriate transgene or genetically modified with gene editing strategies, and used to prepare transgenic epidermal grafts, which are transplanted on surgically prepared body sites. The presence of corrected long-lived epidermal stem cells in the graft ensures a permanent skin restoration.

as hair, nail and enamel defects. More than 1,000 mutations on at least 16 structural genes cause distinct clinical manifestations, ranging from mild to severe, with local or generalized involvement and significant morbidity and mortality. This variety depends on several molecular (targeted protein, type of mutation and degree of function loss, mode of inheritance and genetic background) and phenotypic (distribution and severity of the lesions, involvement of mucosae) factors. Severe EB forms can be early lethal and generalized EB frequently leads to aggressive squamous cell carcinoma (SCC).[7]

EB encompasses 4 major forms, primarily based on the level of skin cleavage, that is intraepidermal in EB simplex (EBS), within the lamina lucida in JEB, beneath the lamina densa in DEB and at multiple levels within and/or beneath the basement membrane in Kindler syndrome (KEB).[7]

EBS is the most common form of EB and can arise from mutations in 7 different genes. Over 75% of EBS is due to dominantly inherited genetic changes affecting *KRT5* and *KRT14*, the genes encoding keratin 5 (K5) and keratin 14 (K14), which form the intermediate filament network of basal keratinocytes. Mutations in highly conserved amino acids within the helix initiation or termination motifs lead to severe EBS, which is characterized by blisters, often leading to chronic erosions, covering the entire skin surface and affecting several mucous membranes. Aminoacidic substitutions in other K5/K14 regions lead to localized EBS, marked by milder clinical manifestations usually restricted to the extremities. Very rare, severe (in some cases lethal) forms of EBS are caused by recessively inherited nonsense or missense *KRT5* and *KRT14* pathogenic variants. EBS can also be caused by mutations in plectin (encoded by *PLEC*) and dystonin (encoded by *DST*), which are hemidesmosome proteins that anchor keratin filaments to the plasma membrane.[7]

JEB is one of the most devastating forms of EB. It is due to recessively inherited mutations in genes encoding the heterotrimeric protein laminin 332 *(LAMA3, LAMB3, LAMC2)*, collagen XVII *(COL17A1)*, integrins α6β4 (*ITGA6*, *ITGB4*) and integrin α3 (*ITGA3*). The most severe forms of JEB are caused by mutations affecting laminin 332 and integrins α6β4, whilst mutations in *COL17A1* and *ITGA3* usually have a milder phenotype. Patients carrying biallelic premature termination codons leading to absence of laminin 332 or α6β4 (severe JEB) usually die within 2 years after birth. Approximately 40% of patients with intermediate JEB die before adolescence, whilst adults have a high risk of developing SCC. Missense or splicing mutations that allow residual expression of the protein, even if

truncated and only partially functional, can significantly reduce the severity of the phenotype, suggesting that low expression of one component can still sustain its interactions with the binding partners.[7]

Dystrophic EB (DEB) can be dominantly or recessively inherited and is due to over 200 mutations in *COL7A1,* the gene encoding collagen VII (C7), the main component of anchoring fibrils. Dominant DEB has a mild phenotype with blisters primarily involving the extremities. In contrast, Recessive DEB (RDEB) can be ravaging, being characterized by massive blistering and scarring, disabling joint contractures and pseudosyndactyly, all of which highly reduce the patients' quality of life. Severe RDEB usually results from biallelic *COL7A1* premature termination codons, but variants include nonsense or splice site mutations, deletions or insertions, 'silent' glycine substitutions or non-glycine missense mutations within triple helix or non-collagenous NC-2 domains. The nature and the positions of these mutations correlate with the severity of the phenotype. Patients with generalized RDEB almost invariably develop aggressive, highly metastatic SCC.[7]

KEB is caused by mutations in *FERMT1*, the gene encoding fermitin family homolog 1 (kindlin-1), an intracellular protein of focal adhesions. Blisters can occur within basal keratinocytes, along the lamina lucida and below the lamina densa of the basement membrane. Features include skin fragility and mild photosensitivity, poikiloderma, palmoplantar hyperkeratosis and high risk of developing SCC in adulthood. As with other EB forms, several mucous membranes can be involved.[7]

Combined Cell and Gene Therapy for Junctional Epidermolysis Bullosa

LAMB3-dependent generalized intermediate JEB was the first genetic skin disease successfully tackled by *ex vivo* combined cell and gene therapy.[8] Autologous epidermal cultures transduced with a gamma-retroviral vector (γRV) carrying a *LAMB3* cDNA were grafted on patients' selected skin areas, upon surgical removal of blistering epidermis and proper preparation of the wound bed.

Transgenic grafts restored large non-healing epidermal lesions in two adult JEB patients[8,9] (Figure 1B). More recently, transgenic epidermal cultures proved to be life-saving, as they restored virtually the entire epidermis of a seven-year-old boy suffering from a devastating form of JEB with very poor prognosis.[10] Through the entire 6-year follow-up, his newly formed epidermis remained robust and resistant to mechanical stress, freed from blisters or erosions. It expressed normal levels of laminin-332, had

normal thickness and continuity of the basement membrane and, notably, unveiled normal would healing upon injuries. The regenerated epidermis was entirely transgenic, as *LAMB3* mRNA and laminin 332 were both uniformly and seamlessly detected in all the analyzed skin sections. No immune response or inflammation were observed.[11]

In summary, all three patients presented a stable, fully functional, blister-free epidermis with normal expression of laminin 332 at the epidermal-dermal junction and a normal number of mature hemidesmosomes. [8-10] Despite the very high number (between 1x107 to 4x108) of transgenic clonogenic keratinocytes transplanted per patient, no adverse events have been observed (up to 16 years of follow-up).[12] In particular, neither cellular transformation nor aberrant clonal expansion have been so far detected in the regenerated transgenic skin.[11,13] An oncoming multicenter European Phase II/III clinical trial (referred to as Hologene 5) aims to confirm safety and efficacy of transgenic epidermal cultures on a larger number of *LAMB3*-JEB patients (NCT05111600).[14]

Combined Cell and Gene Therapy for Dystrophic Epidermolysis Bullosa

Autologous cultured keratinocytes transduced with a γRV carrying a *COL7A1* cDNA (earlier referred to as LEAES and today as EB-101), have been used to restore the expression of C7 on 42 skin wounds on 7 RDEB patients (NCT01263379)[15] (Figure 1B). At 2-year follow-up, more than 70% of the treated wounds healed and expressed C7 assembled in functional anchoring fibrils on at least 50% of their surface, which significantly improved the clinical picture. No adverse events related to the use of γRV-corrected cells were reported.[16] An ongoing Phase III clinical trial aims to confirm safety and efficacy on a larger number of RDEB patients (NCT04227106).

In collaboration with Johann Bauer and colleagues, we obtained similar results in a similar Phase I/II trial (NCT02984085), using autologous epidermal cultures transduced with the same type of γRV used for gene therapy of JEB. However, while the *LAMB3*-transgenic epidermis exhibits a fully functional, seamless basement membrane and a normal number of mature hemidesmosomes, *COL7A1*-transduced keratinocytes were able to partially restore the expression of C7, hence they regenerated a sort of 'mosaic' patterned epidermis (our unpublished data). This difference could be, at least in part, ascribed to a lower transduction efficiency of γRV-*COL7A1*, as compared to γRV-*LAMB3*, and to competition between untransduced and transgenic RDEB keratinocytes, which is un-

likely to occur in the JEB scenario. In fact, signals emanating from the interaction of laminin 332 with integrins α6β4 induce nuclear localization YAP, a transcriptional co-activator sustaining human epidermal stem cells.[17] *LAMB3*-JEB triggers YAP inactivation and leads to epidermal stem cell depletion, supporting the notion that JEB is an adhesion and a stem cell disease. It follows that genetic correction of *LAMB3*-JEB rescues not only cell adhesion but also epidermal stemness, thus conferring to transgenic JEB keratinocytes a selective advantage over the untransduced counterpart, both *in vitro* and *in vivo*.[17] Such a selective advantage does not hold true for RDEB clonogenic keratinocytes. This hurdle might be exceeded by a substantial improvement of the efficiency of RDEB keratinocyte transduction.

Tackling dominantly inherited EB

Gene addition strategies can successfully tackle a significant number of severe, recessively inherited genetic diseases, but are unsuitable to correct dominant mutations. The discovery of the CRISPR/Cas9 gene editing system now allows to precisely target genomic loci, hence discriminate wild-type and mutant alleles. The CRISPR/Cas9 technology is under investigation in keratinocytes, fibroblasts or induced pluripotent stem cells (iPSCs) for gene editing of many forms of EB.[18] Base editing is emerging as potentially suitable for correcting EB point mutations. The refinement of base editing in the form of 'prime editing' represents a further progress, potentially able to edit the vast majority of all pathogenic EB mutations.[19,20] However, gene editing approaches are largely at the preclinical stage. Once fully developed, they could also be applied to recessively inherited EB, provided that their efficiency in targeting epidermal stem cells (see below) would be comparable to that of gene addition strategies.

Long-term epidermal regeneration relies on transgenic epidermal stem cells, detected as holoclone-forming keratinocytes

Squamous epithelia are constantly renewed. Being the first protective barrier against the external environment, these epithelia receive daily assaults, such as wounds, that need timely repair. Long-lived keratinocyte stem cells, residing both in the epidermal basal layer and in the bulge of the hair follicle, are responsible for such regeneration and repair processes.[21] They have the unique capacity to self-renew and to generate committed progenitors – often referred to as transient amplifying (TA) cells – that generate terminally differentiated keratinocytes after a limited number of

cell divisions.[10]

Even though they were not called stem cells at the time, keratinocytes cultured in 1975 in Green's laboratory matched the definition of stem cells as we know them today. In fact, human clonogenic keratinocytes are endowed with an impressive proliferative potential and consist of stem cells and TA progenitors. A crucial step towards their identification and isolation was taken in 1987, when Barrandon and Green succeeded in cultivating human keratinocytes at a clonal level, hence identified three types of clonogenic keratinocytes giving rise to clones referred to as holoclones, meroclones and paraclones.[22] They can be isolated both from a tissue biopsy and a keratinocyte primary culture.[12]

Initially described in the skin, they were found also in other stratified epithelia, such as cornea, urethra and oral mucosa.[23] All clonal types are endowed with proliferative capacity.[23] But while paraclones can undergo only few population doublings, holoclones and meroclones can produce dozens of cell doublings. The onset of replicative senescence is determined by clonal conversion, namely progressive decline in the proportion of holoclones and meroclones and progressive increase of paraclones, the latter generating only aborted colonies.

Cultured epithelial grafts contain all clonal types. Thorough analysis of data accumulated during over 30 years of clinical application of such cultures have provided compelling, yet indirect, evidence that holoclones and meroclones/paraclones are generated by stem cells and TA progenitors, respectively. For instance, permanent restoration of a transparent, renewing corneal epithelium, as well as a renewing epidermis, strictly requires a defined number of holoclone-forming cells in the culture.4 But formal evidence of holoclone-forming cells being authentic, long-lived, self-renewing stem cells was gained only through the in-depth analysis of the transgenic epidermis that restored the skin of JEB patients. Using proviruses as clonal genetic marks, clonal tracing of the newly formed transgenic epidermis has unambiguously shown that holoclone-forming cells are long-lived, self-renewing stem cells, necessary and sufficient to sustain the human epidermis. They continuously generate meroclones and paraclones that, as expected from TA progenitors, are short-lived and, although instrumental for proper tissue regeneration and wound healing, are progressively lost during epidermal renewal. In a nutshell, clonal tracing has shown that the main feature distinguishing the holoclone-forming cell from the other keratinocyte clonal types, is its self-renewal and long-term regenerative capacity.[10]

It follows that cultured epidermal grafts must contain an adequate number of holoclone-forming cells to permanently sustain the regenerated epidermis.[24] While paraclones could be identified based on their morphology (small irregular colonies containing large and flattened cells), holoclones and meroclones cannot be distinguished based on their growth rate and behavior and/or their shape and size. Thus, by no means a colony forming efficiency assay, which measures the number (and shape) of colonies, would suffice to establish the number of holoclone-forming cells harboring a cultured epidermal graft. Such number can be attained by a formal clonal analysis.[10]

Fundamental insights into stem cells of interfollicular epidermis and hair follicle have been gathered from murine studies, but not always murine findings apply to humans. For instance, the murine epidermis does not contain the same types of clonogenic keratinocytes found in the human skin. Nevertheless, an important step toward molecular definition of human holoclones came from the discovery of p63 as a key transcription factor sustaining murine squamous epithelia.[25,26] In fact, p63-null mice lack all stratified epithelia and have major defects in their limb and craniofacial development.[25,26] This phenotype could be explained by either inability of the p63-null ectoderm to develop into epithelial lineages and/or lack of stem cell character necessary to sustain epithelial morphogenesis and renewal. Subsequently, it has been shown that ΔNp63α, a specific p63 isoform, underpins the proliferative, regenerative capacity of mammalian epithelial stem cells.[27] In humans, ΔNp63α is highly expressed by epidermal and limbal holoclones and it progressively declines during keratinocyte clonal conversion.[28] Quantification of ΔNp63αbright cells has been used as a pre-transplantation assay to evaluate the number of holoclones contained in a limbal/corneal culture.4

Strikingly, permanent restoration of a functional corneal epithelium in patients receiving limbal cultures for the treatment of severe chemical burns requires a defined number of ΔNp63αbright holoclone-forming cells in the culture.[4] This assay, however, has not yet been validated for epidermal cultures.

YAP is a transcriptional co-activator driving cell proliferation in many types of stem and progenitor cells and a key regulator of mechanotransduction. Unphosphorylated YAP translocates to the nucleus, where it induces target genes through interaction with TEAD transcription factors.

Phosphorylation of YAP in defined serine residues results in its sequestration, hence functional inactivation, into the cytoplasm by 14-3-3

proteins.[29] YAP interacts with ΔNp63α in sustaining self-renewal and proliferative/regenerative capacity of holoclone-forming cells.17 The transcriptomic profile of single human keratinocytes unveiled that FOXM1, a transcription factor member of the forkhead box family, acts downstream of YAP.30 Nuclear YAP and FOXM1 are highly expressed in epidermal holoclones but virtually undetectable in meroclones and paraclones. In contrast, phosphorylated YAP and 14-3-3σ are barely detectable in holoclones and progressively increase during clonal conversion. Accordingly, the ablation of either YAP or FOXM1 induces the selective disappearance of holoclones, whilst enforced YAP or FOXM1 (or ablation of 14-3-3σ) halt clonal conversion and sustain holoclone-forming cells indefinitely.[30]

Both microarray and single cell RNA-seq data have also shown that holoclone-forming cells display other common stem cell features, such as genes regulating DNA repair, chromosome segregation, spindle organization and telomerase activity, and are enriched in genes regulating microtubules and actin polymerization.[30]

Although a human holoclone molecular signature is thus emerging, further development of single cell genetic and epigenetic analyses is required and should give more insights that could allow to prospectively distinguish epidermal holoclones from the other clonal types.

Conclusion

There is no cure for EB. Available therapies are palliative, only partially alleviating the devastating clinical manifestations, hence they are not sufficient to provide decisive relief from pain, symptoms and mental stress achieve satisfactory living standards for these patients. Long-lasting, curative therapies are urgently needed, and several attempts have been made in this respect. As of today, gene correction in combination with cell-based approaches focus on individually designed treatments, holding promises for more effective results. The increasing number of clinical trials assessing such innovative, advanced molecular therapies resurges new hopes to definitively tackle this devastating disease. But none of these advanced approaches have yet made it to a routine therapy. The genetic and phenotypic EB heterogeneity (and the ambitiousness of a regenerative medicine approach) would require the convergence of multiple expertise and disciplines, including stem cell biology, developmental and molecular biology, genetics, tissue engineering and, not to say, a deep knowledge of all the clinical and surgical features of the different forms of the disease. Hence, a multidisciplinary collaborative effort is critical.

Acknowledgements

This work was supported by the European Research Council (ERC) Advanced Grant HOLO-GT (No. 101019289) to MDL. We thank Michele Palamenghi for Figure 1.

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Treatments for Leukemia through Understanding Hematopoietic Stem Cells: Emergence from the Cave

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Background

> *"Slowly, his eyes adjust to the light of the sun. First he can see only shadows. Gradually he can see the reflections of people and things in water and then later see the people and things themselves. Eventually, he is able to look at the stars and moon at night until finally he can look upon the sun itself"*
> Allegory of the Cave, Republic, Plato (516a)

In the Allegory of the Cave, Plato describes how prisoners in a cave see only the shadows on the wall compared with the freed prisoner who in the light of day can see things themselves (Fig. 1).[1]

During a turbulent time in history when the threat of nuclear war was looming, Till and McCulloch discovered a functional rather than a phenotypic shadow of hematopoiesis. By performing quantitative transplantation assays in irradiated mice, they identified a bone marrow-resident dormant cell with multi-lineage differentiation, homing and self-renewal potential that could resist radiation exposure – the hematopoietic stem cell (HSC).[2,3]

Subsequently, Spangrude, Heimfeld and Weissman used fluorescence activated cell sorting (FACS) analysis to prospectively purify and characterize the cell surface markers that defined mouse HSCs whereby 30 of the cells could rescue 50 percent of irradiated mice.[4] These studies were followed in close succession by the identification of human hematopoietic stem cell populations.[5] The ontogeny of both mouse and human HSCs was further defined by Mikkola and Orkin and refined by Coller, Frenette, Morrison, Suda and Trumpp who underscored the importance of quiescence for HSC maintenance in bone marrow niches.[6,7]

As a counterbalance to normal hematopoiesis, seminal research performed by Nowell, Hungerford, Rowley, Witte, Daley, Van Etten and Bal-

Figure 1. Depiction of Plato's Allegory of the Cave, Cornelis van Haarlem, 1604.

timore demonstrated that a single fusion gene, BCR-ABL, was necessary and sufficient to initiate chronic phase chronic myeloid leukemia (CML), thereby setting the stage for molecularly targeted therapeutic strategies that enabled operational cures albeit with a high risk of relapse following discontinuation of therapy.[3,8] This could be explained at least in part by the discovery by Holyoake and Eaves in mice and Jamieson and Weissman and colleagues in humans that the BCR-ABL gene occurred in a dormant HSC population but was not sufficient to drive blast crisis transformation.[3,8] Rather, malignant reprograming of granulocyte-macrophage progenitors (GMP) into leukemia stem cells (LSCs) by unregulated beta-catenin and CD47 upregulation fueled blast crisis transformation of CML.[9] Discovery of the leukemia stem cells (LSCs) in AML by Dick and colleagues demonstrated that there were clonal hierarchies that dictated therapeutic resistance as defining feature of human leukemias.[10] Moreover, the indelible yet seemingly capricious effects of environmental stress and aging on human benign, pre-malignant and malignant HSC biology continue to be an enigma.

Over the course of six decades, functional hematopoietic stem cell research has provided a strong rationale for identifying human-specific cell autonomous and non-cell autonomous drivers of accelerated aging, pre-malignant and malignant hematopoiesis and thereby enable the rapid development and implementation of interception strategies that are predicated on preventing pre-leukemia stem cell (pre-LSC) transformation into leukemia stem cells (LSCs).[2-39]

Inflammaging and Clonal Hematopoiesis of Indeterminate Potential

An increasingly thorough understanding of the molecular, phenotypic and functional underpinnings of normal HSC homeostasis has provided a framework for elucidating drivers of pre-malignant hematopoiesis in stressful microenvironments.[12,13]

As a result of momentous advances in stem cell whole genome and RNA sequencing, combined with single cell spatial genomics, transcriptomics and proteomics and perhaps more importantly transformative stem cell functional analyses that quantify tissue-specific stem cell responses to different environmental exposures, essential insights can be made into intrinsic and extrinsic drivers of HSC aging and pre-leukemic development.

While host innate and adaptive immune responses evolved to protect stem cells and other cells involved in tissue homeostasis from viral and bacterial pathogens, chronic immune activation is associated with systemic signaling driven by pro-inflammatory cytokines, such as tumor necrosis factor (TNFalpha), interferon (IFN alpha, beta, gamma) and interleukins (IL-1, IL-6), by activated T cells and tissue resident macrophages. Both mouse model studies and humanized model systems show that aging is associated with a decrease in neutrophil respiratory burst; a functional decline in macrophage production of Toll-like receptors as well as chemokine and cytokine production resulting in decreased T cell proliferative potential and reduced NK cell activity that leads to diminished immune surveillance against pre-leukemic cells. However, other aspects of immunity increase with aging as evidenced by increased production of pro-inflammatory cytokines by peripheral blood mononuclear cells from elderly compared with young individuals in response to mitogens in vitro (Fagiolo). Moreover, IL-6 levels have been shown to be higher in centenarians. Indeed, chronic inflammation has long been linked with accelerated tissue aging, particularly in the hematopoietic system, and is now termed, *inflammaging*. However, the role of stem cell inflammaging in HSC homeostasis and pre-leukemic development has not been clearly elucidated.[13]

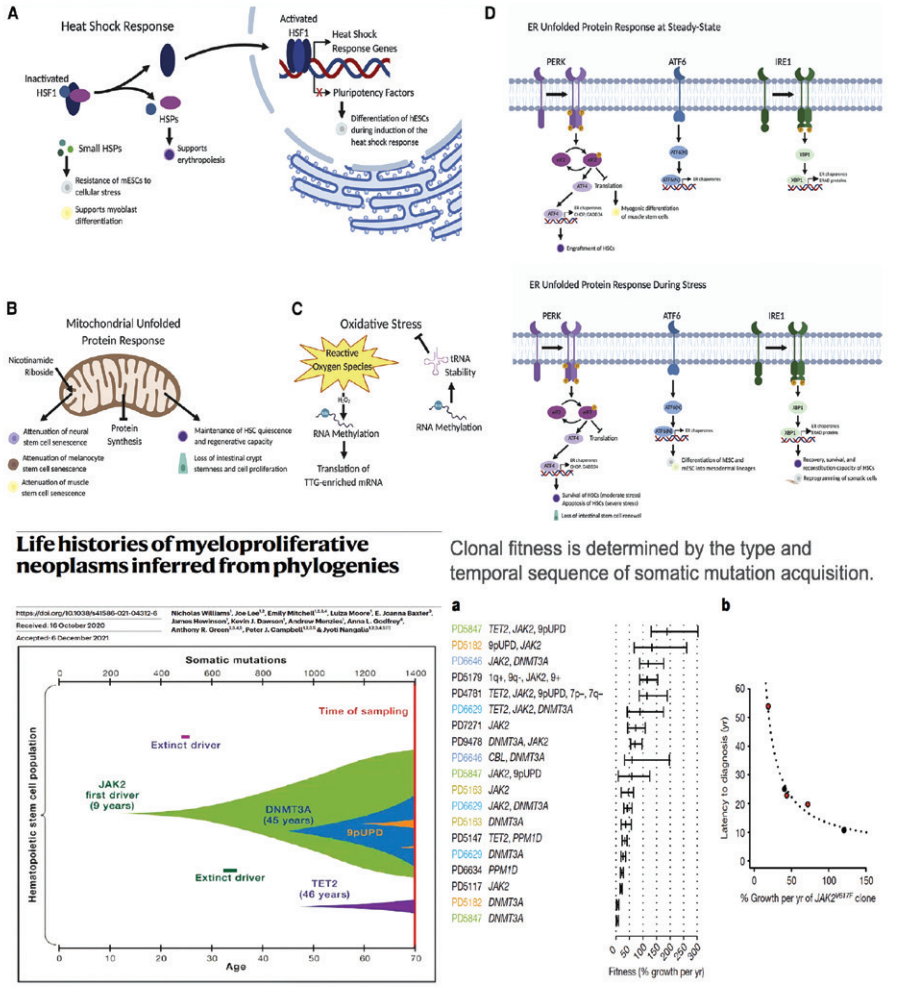

Figure 2. Molecular Determinants of Hematopoietic Stem Cell Identity and Clonal Hematopoiesis Under Conditions of Stress. Upper panel: In response to proteotoxic insults, the cell mounts adaptive responses to maintain protein quality control. These stress response pathways also regulate hematopoietic stem cells (reviewed in Chua, Van Der Werf, Jamieson, Signer. Cell Stem Cell 2020). Lower panel: whole genome sequencing reveals that clonal fitness is determined by the type and temporal sequence of mutation acquisition (Nangalia et al. *Nature* 2022).

Recently, both macroenvironmental and microenvironmental drivers of inflammaging in hematopoietic and other tissue-specific stem cells have come to the fore as major arbiters of pre-cancer stem cell generation and evolution to self-renewing cancer stem cells, which evade host innate and

adaptive immune responses. Seminal mouse and zebrafish model studies have demonstrated that HSC aging is typified by myeloid lineage bias, reduced dormancy and diminished regenerative (self-renewal) potential. Subsequent studies confirmed that the same is the case for human HSCs and spawned the field of clonal hematopoiesis (CH). While clonal somatic DNA mutations in epigenetic modifier genes, including TET2 and DNMT3A, in stem cell populations increase the risk of developing acute myeloid leukemia (AML) as well as cardiovascular death, there may be some protective effects with regard to Alzheimer's disease.[3] The complexity of clonal stem cell dominance has become apparent as a result of high resolution single cell sequencing that demonstrates that some mutations in splicing factor related genes emerge later in life and provide a greater clonal competitive advantage and potential for AML development than classic epigenetic modifier gene mutations.[15] However, the propensity to develop AML varies substantially between individuals, thereby suggesting that host environmental exposures and immune haplotypes may shape the trajectory of these stem cell clone wars.

Pre-Leukemic Evolution of Hematopoietic Stem Cells

Recent provocative data suggest that hematopoietic stem cell aging may be accelerated by acquisition of somatic DNA mutations early in life and that the usually indolent process of clonal hematopoiesis of indeterminate potential (CHIP) may be superseded later in life by more rapidly dividing, splicing factor gene mutated clones that form the apex of an oligoclonal and ultimately malignant hierarchy.[14-20] While there is substantial variation in the occurrence and clonal trajectories of CHIP between monozygotic twins thereby underscoring the importance of environmental exposures and epigenetic factors in the evolution of CHIP, those with CHIP have shorter telomeres.[15]

In addition to radiation and toxic exposure-induced somatic DNA mutagenesis, pre-leukemia stem cells (pre-LSC) generation from hematopoietic stem and progenitor cells may be driven by activation of primate-specific cytidine-to-thymidine (C-to-T) DNA editing enzymes.[21] As human longevity is extended by advances in precision medicine; the global spread of viral and bacterial pathogens induces acute and chronic inflammatory responses; and immune dysfunction is elicited by advanced age and stem cell stress-inducing environments, including low earth orbit (LEO) as space exploration expands, extrinsic exposures will start to gain even greater relevance with regard to accelerated human aging and pre-leukemia stem cell

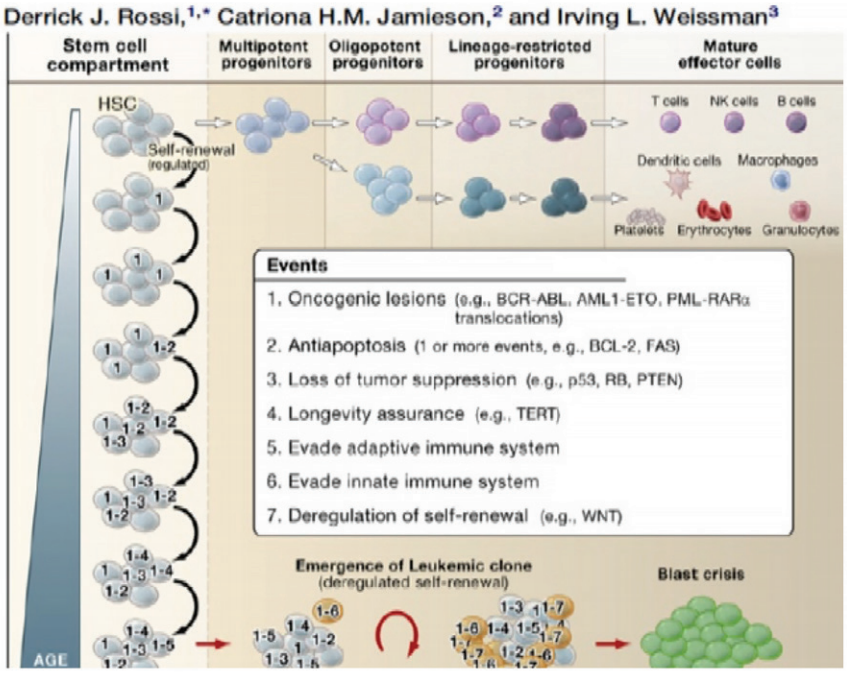

Figure 3. Hematopoietic Stem Cells and Pathways to Aging, Pre-Leukemia and Leukemia. During aging, normal HSCs acquire mutations that lead to lineage bias, apoptosis resistance, loss of tumor suppression, evasion of host immune responses that expand pre-leukemic progenitors that acquire deregulated self-renewal capacity (*Cell* 2008).

generation coupled with immune dysfunction.[22] These mutations result in cell differentiation (lineage) bias and evasion of apoptosis as well as evasion of host innate and adaptive immune responses (Fig. 3). Recent research performed by our group and others suggests that inflammatory cytokine-activated APOBEC3C induces C-to-T somatic DNA mutagenesis thereby fueling pre-leukemic stem cell generation in myeloproliferative neoplasms (Fig. 4).[21] This niche-dependent inflammatory cytokine milieu combined with evasion of host innate as well as adaptive immune responses enables pre-leukemic clonal escape and expansion.

Malignant Reprogramming of Progenitors into Leukemia Stem Cells

Cumulative data suggest that some clonal HSC mutations enhance sensitivity to inflammatory growth factor signaling, including JAK2 V617F, MPL and CALR. For the most part, these mutations lead to the generation

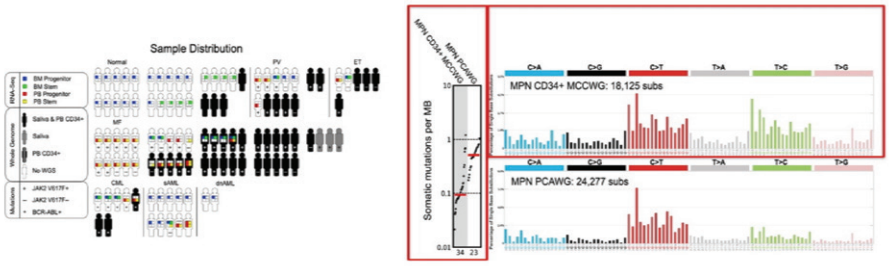

Figure 4. Inflammatory Cytokine Activated APOBEC3C Fuels Pre-leukemic hematopoiesis in HSCs. Whole genome sequencing of 38 myeloproliferative neoplasm CD34+ stem and progenitor cells revealed APOBEC3-induced C-to-T DNA mutagenesis patterns (*Cell Reports* 2021).

of myeloproliferative neoplasms (MPNs), which are initiated by pre-leukemic stem cells with myeloid skewed differentiation potential, loss of dormancy and a propensity to migrate to extramedullary niches, including the spleen, thereby resulting in myeloproliferative neoplasm development (Fig. 3, 4). Comparative whole genome sequencing of purified hematopoietic stem cells and mature cells in saliva from the same individuals with myeloproliferative neoplasms suggest that germline mutations may predispose individuals to chronic inflammatory cytokine signaling that enhances inflammaging, CH and pre-leukemic stem cell generation.[21] Protracted activation of primate-specific anti-viral DNA editing enzymes, such as APOBEC3 cytidine deaminase enzymes, in response to chronic pro-inflammatory cytokine signaling can induce cytidine to thymidine (C-to-T) mutations thereby promoting clonal somatic mutagenesis in stem cell populations.[21] Moreover, APOBEC3C fueled expansion of the progenitor pool triggers ADAR1 activation resulting in increased RNA editing, including of STAT3, and evasion of tumor suppression. Overexpression of ADAR1 and missplicing of GSK3beta fuel activation of beta-catenin thereby resulting in malignant reprogramming of myeloid progenitors into self-renewing leukemia stem cells that drive blast crisis transformation in chronic myeloid leukemia and myeloproliferative neoplasms (Fig. 5).

Reversal of Malignant Progenitor Reprogramming

Increased expression of the inflammatory cytokine inducible splice isoform of ADAR1, ADAR1 p150 (ADAR-202) has been linked to progression and therapeutic resistance of 20 different malignancies. Moreover, RNA splicing deregulation also promotes leukemia stem cell in both

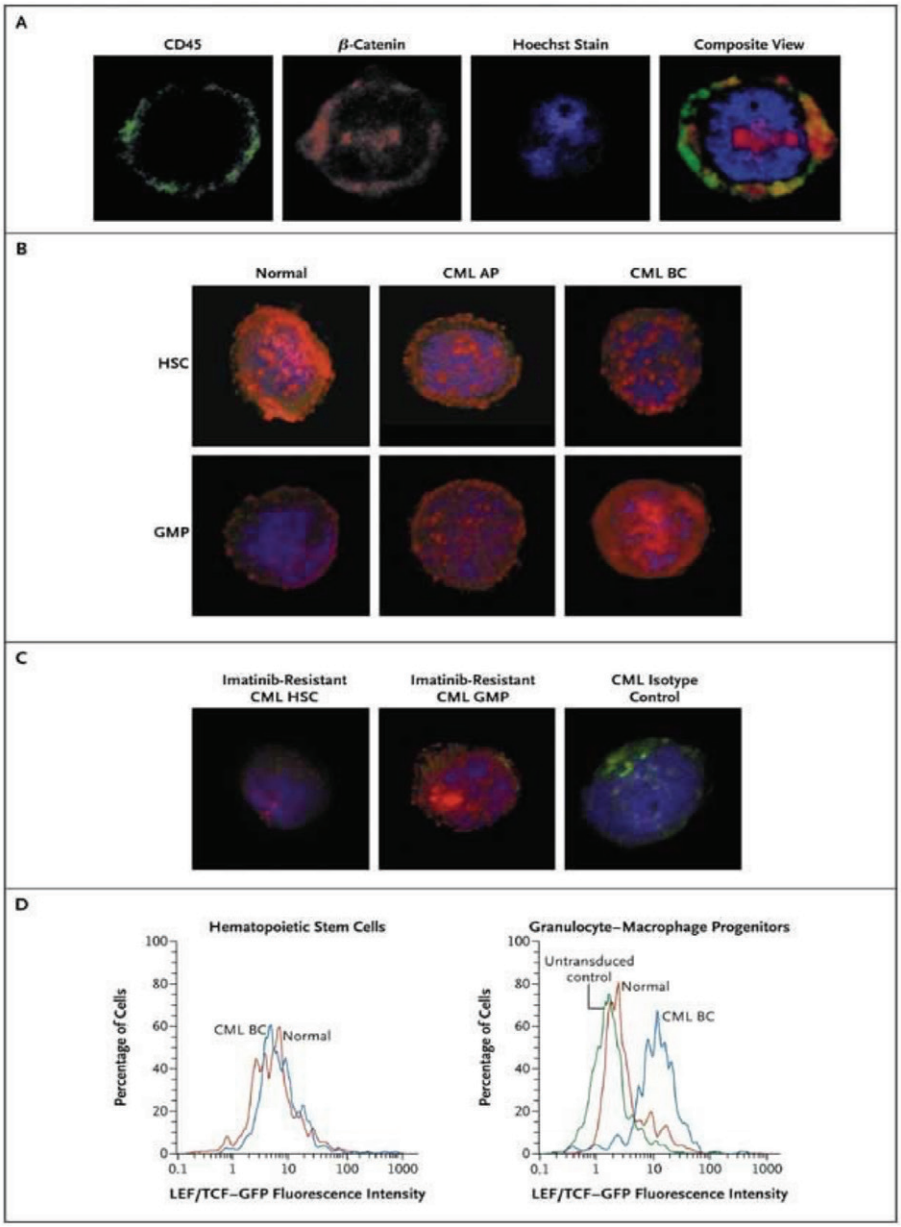

Figure 5. Malignant Progenitor Reprogramming Drives Blast Crisis Transformation of CML (*NEJM* 2004).

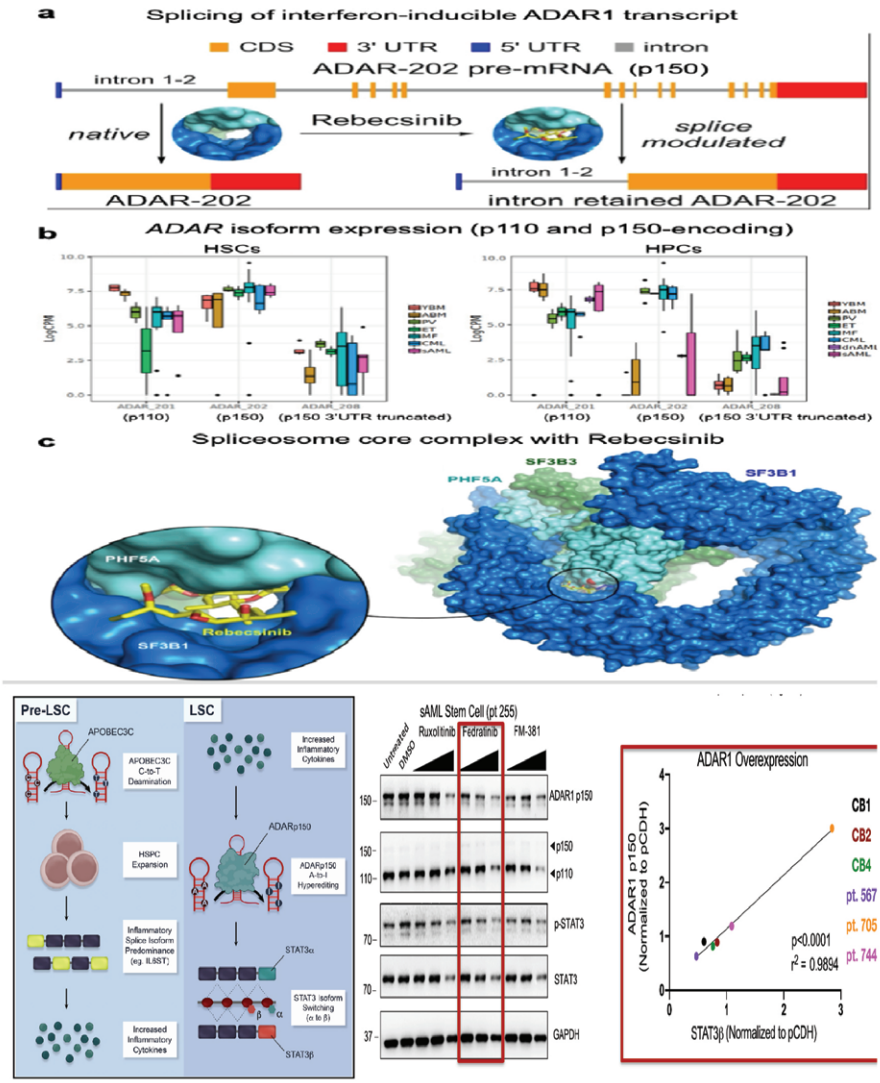

Figure 6. Two routes to ADAR1p150 inhibition. Upper panel: Rebecsinib prevents splicing mediated ADAR1 activation.29 Lower panel: Fedratinib prevents STAT3-mediated transcriptional activation and of ADAR1.[21]

adult and pediatric patients. While splicing modulators show some signs of clinical efficacy, including E7107 and H3B-8800, ocular toxicity or insufficient efficacy against LSC in AML, respectively, have limited their use. Recently, we completed CIRM and NCI-funded pre-IND studies with a selective splicing modulator, Rebecsinib (17S-FD0895), that prevents splicing-mediated activation of ADAR1 into pro-malignant isoform, p150 (Fig. 6).[29]

Other strategies for inhibiting LSC self-renewal are under development and include N^6-methyl adenosine RNA targeted therapeutics, small molecule APOBEC3 inhibitors, anti-sense oligonucleotide (ASO) targeting of upstream ADAR1 activators and cytokine signaling disruption with Cirmtuzumab and other LSC-targeted monoclonal antibody therapies.[34] Deregulation of sonic hedgehog signaling has also proven to be an LSC Achilles heel with glasdegib targeted small molecule inhibition resulting in doubling of survival for elderly patients with AML.[20,33] Also, deregulated programmed cell removal remains a key arbiter of LSC escape from host innate immune responses. Targeted inhibitors of the "don't eat me signal", including CD47 and its ligand, SIRP alpha, are currently undergoing clinical development for LSC eradication in AML. Additional strategies to enhance innate and adaptive immune eradication of LSC include inhibition of immune editing that leads to HLA class 1 loss and induced pluripotent stem cell as well as lipid nanoparticle derived NK cell activation and macrophage repolarization strategies.

In summary, human hematopoietic stem cell informed therapeutic development aimed at inhibiting pre-LSC and LSC propagation may reduce rates of therapeutic resistance and ultimately allow patients to emerge from the cave of uncertainty and see the light of treatment free remission.

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LOOKING TO THE FUTURE

Informational Molecules and Delivery Systems for Regenerative Medicine

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Abstract

Informational molecules regulate numerous processes in the body. However, most of these – if used therapeutically – are difficult to deliver safely and effectively to patients. Our work in this area started in 1974 with our studies on isolating the first angiogenesis inhibitors and has continued to this day and includes developing approaches to deliver substances that can regulate stem cell behavior. Applications range from cancer treatments, to Covid vaccines, to regenerative medicine, including possibly restoring hearing.

Introduction

I started my postdoctoral career working with the late Judah Folkman, attempting to isolate the first inhibitor of angiogenesis (new blood vessel growth). To do this, it was critical to develop a bioassay for angiogenesis inhibitors, nearly all of which were macromolecules. We conceived of using a rabbit cornea assay where we could directly visualize blood vessel growth (Langer *et al.*, 1976) through an ophthalmic microscope. However, that assay could take up to several months, so it was critical to develop a very small biocompatible controlled release system that would not cause inflammation in the cornea, and that could slowly and continuously release macromolecules (*e.g.,* peptides, proteins, and nucleic acids). When I started my investigations, it was widely believed that only low-molecular weight lipophilic compounds – but certainly not ionic molecules, peptides, proteins, or nucleic acids – could be controllably delivered from biocompatible materials (*e.g.,* polymers, lipids) (Langer, 2019).

Developing a method to control the movement of macromolecules and ionic species from biocompatible materials

Nonetheless, it was crucial to create such systems if we were going to isolate angiogenesis inhibitors. Thus, I began studying this problem by examining different materials with a known safety record in humans (Langer

et al., 1981) as well as different formulation approaches (Langer and Folkman, 1976; Langer, 2019).

After several hundred failures, I discovered an approach that enabled the continuous release of different molecules including nucleic acids and proteins for up to 100 days (Figure 1). This discovery was initially ridiculed by the scientific community. My first nine research grant applications were rejected. No chemical engineering department in the country would hire me as a faculty member. So I ended up joining the Nutrition and Food Science Department at MIT. But the year after I joined, the department head who had hired me left, so a number of members of the senior faculty told me I should leave, too. As my colleague, Michael Marletta recalled:

> One evening, I went to a faculty dinner with Bob Langer and some senior MIT professors. A senior scientist sat quizzing us while smoking a cigar. When the older scientist heard Langer's concept for ... drug delivery, he blew a cloud of smoke in Langer's face and said, "You better start looking for another job...".

Although much later, the National Academy of Sciences would cite this work as being "responsible for much of today's drug delivery technology" and *Nature* would cite this work for the "founding the field of controlled release drug delivery" these repeated rejections at this early stage in my career were devastating to me (Langer, 2019).

I recall Dr. Folkman suggesting we file a patent. In the 1970s, Boston Children's Hospital, where I started this research, had not filed a patent

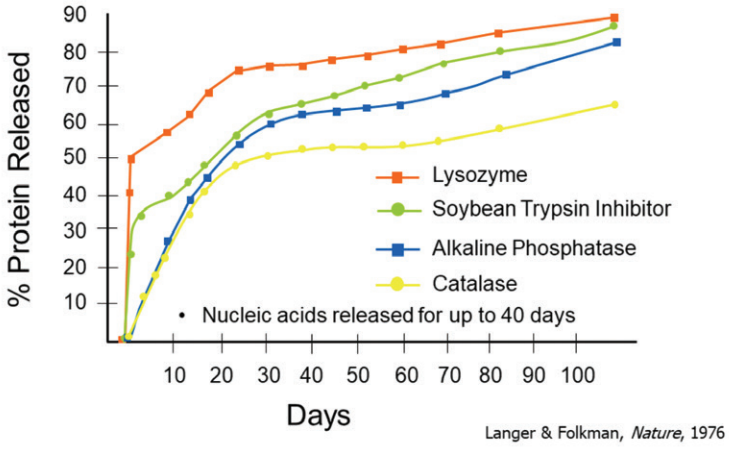

Figure 1.

before. However, they agreed to let us file one. However, for five years in a row, the patent examiner rejected the patent application. Then the head of the Hospital's Technology Transfer Office told me the patent would never be allowed and that I should stop trying to convince the examiner, since explaining the science was not working. However, I don't like to give up. As discussed earlier, when we started our research, many people told us that delivery of macromolecules from small particles was impossible – that it could never work. I wondered if anyone had written that down. So, in 1982, I did a citation search of our 1976 *Nature* paper, and I found many papers citing us. One of them, written by five of the leading materials scientists in the world, described the drug delivery field as follows:

> Generally, the agent to be released is a relatively small molecule with a molecular weight of no larger than a few hundred. One would not expect that macromolecules, e.g. proteins, could be released in by such a technique because of their extremely small permeation rates ... However, Folkman and Langer have reported some surprising results that clearly demonstrate the opposite (Stannett *et al.* 1979).

"Surprising" was a critical word for the patent examiner. When the examiner saw that, he said if I could get written affidavits from all five scientists that they really wrote that, he would allow the patent. So I wrote them and they were all nice enough to write back that they really wrote it, and so the examiner agreed to allow the patent (U.S. Patent 4391797). Over time, this discovery enabled the practical use of many peptides, charged low-molecular pharmaceuticals, proteins, and nucleic acids. Since such molecules have extremely short half-lives in the body (minutes in some cases), a controlled release system must often be used (Langer, 2019).

These controlled release systems enabled us to isolate the first substances that could inhibit the vascularization of tumors (Langer *et al.*, 1976). Using the rabbit cornea and the controlled release pellets as a bioassay for tumor-induced vascularization, we assessed the inhibitory effect of many partially purified macromolecule fractions. Pellets of drug delivery system and pieces of tumor (V2 carcinoma) were placed in the corneal pockets in over 1000 corneas (Figure 2a). Normally, the tumors grew as thin plaques, inducing vessels to sprout from the edge of the cornea 4-6 days after implantation. Vessel length and tumor diameter were measured every few days.

When drug delivery pellets were empty or if a macromolecule fraction was inactive (as was almost always the case), vessels appeared as a dense carpet sweeping over the pellets toward the tumor (Figure 2b). When vessels penetrated the tumor, it grew rapidly, into a large protruding mass occu-

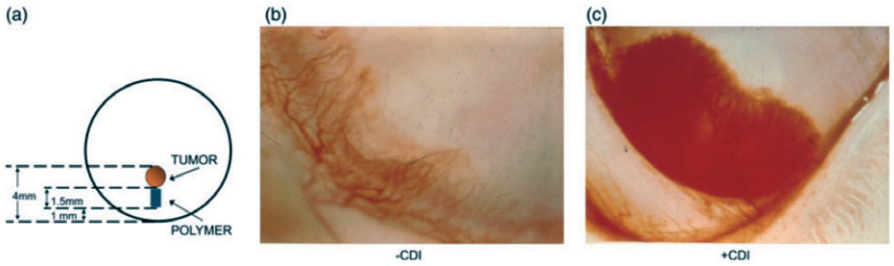

Figure 2.

pying nearly the entire cornea. Very similar results were obtained when pellets containing substances without inhibitory activity were tested. By contrast, when an inhibitor was present, vessels were sparse, grew slowly, and failed to grow in a zone surrounding the drug delivery pellet (Figure 2c). By the 4th week, many vessels were regressing. It was remarkable to see that the blood vessels were stopped in their tracks or even regressing with my own eyes every day.

This study (Langer *et al.*, 1976) established that angiogenesis inhibitors did, in fact, exist. Over many decades, the above controlled release systems have proven fundamental to the isolation and study *in vivo* of nearly all angiogenesis stimulators and inhibitors, as well as numerous other informational molecules in developmental biology studies (Langer, 2019).

There are also many controlled release systems used by patients worldwide that continuously release peptides for up to six months from a single injection (*e.g.* Lupron Depot©, Zoladex©, and Decapeptyl©). Similar or related microspheres or nanospheres, or other systems containing bioactive molecules have led to treatments of schizophrenia (Risperdal Consta©), alcoholism, opioid addiction (Vivitrol©), arthritis (Zilretta©), controlling bleeding (Floseal©, Surgiflo©), pituitary dwarfism (Nutropin Depot©), Type 2 Diabetes (Bydureon©), all drug eluting stents, and many other diseases. Nucleic acids like RNA are also protected and delivered from small particles. Examples are OnPattro© and all Covid mRNA vaccines (*e.g.,* SpikeVax©). (Langer, 2019; Dong *et al*, 2021).

One additional area where drug delivery may be useful is in tissue engineering/regenerative medicine. Every year millions of patients suffer tissue loss or end-stage organ failure. For the most part, physicians treat organ or tissue loss by transplanting organs from one individual into another, performing surgical reconstruction, or using mechanical devices such as kidney dialyzers. Although these therapies have saved and improved countless

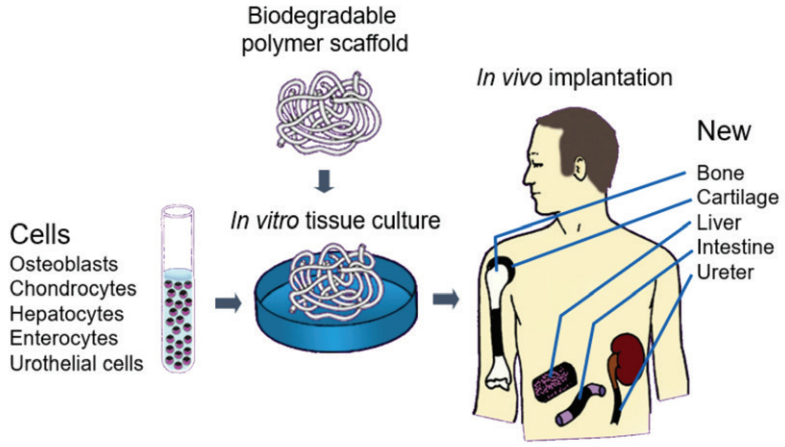

Figure 3.

lives, they are imperfect solutions. In the early 1980s, my colleague, Jay Vacanti, who was head of the liver transplantation program at Boston Children's Hospital asked me if we could create a new liver. Prior to this, several research groups had worked to try creating two-dimensional systems to form certain tissues. We started to use two-dimensional cell/material systems as well. However, after much work in trying to grow liver cells in two-dimensional structures (*e.g.,* Discs, Petri dishes) to test our prototypes, we realized that we could not get enough cells per unit volume to create tissue with enough liver function. One day, Jay was in Cape Cod and saw some seaweed. So, he called me and said, "Bob, could you make a polymer system that was three-dimensional, like seaweed, and if so, could that solve the surface-to-volume problem?" So, we did (*see* Figure 3) and these eventually led to new ways to create cartilage, spinal cords, blood vessels, and many other tissues and organs. It also helped lead to organs and tissues on a chip, which may reduce drug testing in animals and people (Langer, R., 2019., *Molecular Frontiers Journal*).

Regenerative Medicine, Hearing Loss, and Drug Delivery

One area of regenerative medicine where we combined novel drug delivery systems with discoveries in stem cell biology involves hearing loss. An estimated 1.1. billion people are at risk of disabling hearing loss worldwide for which there is currently no pharmacologic treatment. Chronic sensorineural hearing loss (SNHL) accounts for roughly 90% of this sensory

deficit and is likely caused by noise, chemical, viral, and aging insults with potentially debilitating effects. In people with SNHL, audibility (loudness of sound) and intelligibility (clarity of words) deteriorate due to the aforementioned auditory insults. Except for "retrocochlear" hearing loss, more than 80% of chronic SNHL is due at least in part to loss of cochlear hair cells. While many vertebrates such as birds and reptiles generate hair cells spontaneously to restore hearing after various insults, mammals do not. Mammalian progenitor cells that produce hair cells during embryonic development persist into adulthood but are quiescent. (McLean, W., 2021) It occurred to us that Lgr5+ stem cells exist in other parts of the body and are precursors to hair cells. Although Lgr5+ intestinal stem cells have been expanded *in vitro* as organoids, homogenous culture of these cells has not been possible thus far. Building on the work of Hans Clevers, who collaborated with us in our initial study (Yin *et al*, 2014), we discovered that two small molecules (CHIR99021 and valproic acid (VPA), synergistically maintain self-renewal of mouse Lgr5+ intestinal stem cells resulting in nearly homogenous cultures. We found that the colony forming efficiency of cells from these cultures is ~100-fold greater than that of cells cultured in the absence of CHIR99021 and valproic acid (CV), and multilineage differentiation ability is preserved. We used these homogenous cultures to identify conditions employing simultaneous modulation of Wnt and Notch signaling to direct lineage differentiation into mature enterocytes, globet cells, and Paneth cells. We then showed that the combination of CHIRS99021 and valproic acid (VPA) acts synergistically to activate proliferation of quiescent mammalian cochlear progenitor cells *in vitro* from mice, nonhuman primates, and humans (McLean *et al*., 2017). Additionally, a murine *ex vivo* study showed the application of CHIR99021+VPA (CV) following aminoglycoside ototoxicity induced supporting cells in the organ of Corti expressing the leucine-rich repeat-combining G-protein receptor 5 (Lgr5) to divide and regenerate hair cells (McLean, W. *et al.,* 2017).

Working with Jeff Karp, Will McLean, Chris Loose, David Lucchino, and others, we thought that applying compounds that regenerated hair cells could provide a novel approach to improve auditory function in subjects with chronic SNHL. CV was formulated for human use by developing a novel drug delivery system. In particular, we used a thermoreversible poloxamer named Pluronics that can be injected intratympanically as a liquid which will then transition to a gel in the middle ear to allow the prolonged diffusion into the cochlea. The combination of the gel and CV is called FX-322. It is important to note that this is the reverse of what

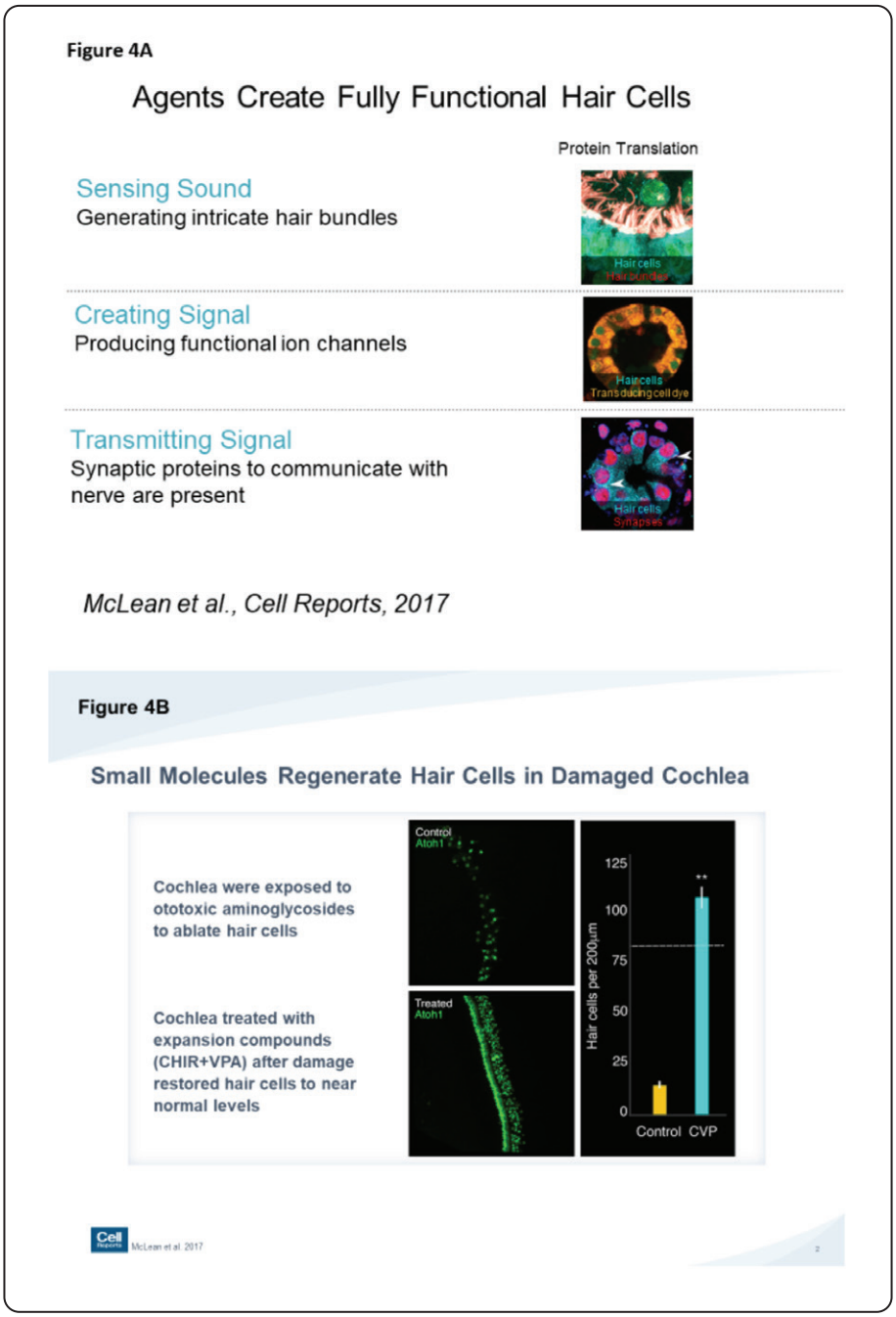
Figure 4A
Agents Create Fully Functional Hair Cells
Protein Translation
Sensing Sound
Generating intricate hair bundles
Hair cells
Creating Signal
Producing functional ion channels
Hair cells
Transducing cell dye
Transmitting Signal
Synaptic proteins to communicate with nerve are present
Hair cells
Synapses
McLean et al., Cell Reports, 2017
Figure 4B
Small Molecules Regenerate Hair Cells in Damaged Cochlea
Cochlea were exposed to ototoxic aminoglycosides to ablate hair cells
Cochlea treated with expansion compounds (CHIR+VPA) after damage restored hair cells to near normal levels
Control
Atoh1
Treated
Atoh1
Hair cells per 200μm
125
100
75
50
25
0
**
Control
CVP
Cell Reports
McLean et al. 2017

Figure 4.

normally occurs with gels (normally increases in temperature decreases gelation). We then conducted clinical cochlear pharmacokinetics (PK) and pharmacodynamics (PD) studies to examine FX-322 as a potential therapy for restoration of hearing in patients with SNHL. We first evaluated spatial and temporal drug distribution in guinea pig cochlea. Predicted concentrations were compared to those that showed activity in *ex vivo* mouse and human studies. Drug concentrations were measured for samples of middle ear contents and perilymph to calibrate the human PK model and validate measured and modeled values. A Phase 1b clinical trial was conducted to assess safety of intratympanically administered FX-322 in adult human subjects with chronic SNHL and to study its PD effect on hearing. (McLean *et al.*, 2021)

In humans, comparison of baseline and Day 90 pure tone thresholds showed no statistically significant differences between the groups at any frequency. However, Day 90 pure-tone assessment showed that 4/15 FX-322-treated patient ears had 10 cB improvement at the highest test frequency (9kHz), whereas no placebo-treated ears showed this level of improvement. Changes in individual word recognition (WR) performance were analyzed to determine if any clinically meaningful change had occurred based on parameters set forth by Thornton and Raffin's binomial distribution. Of the 23 participants, 10 displayed a deficit in WR performance (≤90%) before treatment so could be assessed for hearing improvement without a "ceiling effect". Of these ten patients, six were treated with FX-322 and four with placebo. Four of the six FX-322-treated ears showed statistically significant and clinically meaningful improvements from baseline to 90 days in the prespecified WR test, exceeding expected results of test-retest variability for this measure. In contrast, no placebo-treated ears showed statistically significant changes. The four FX-322-treated ears that had clinically meaningful improvements has an absolute mean (SE) WR increase of 35.4 (5.5) percentage points. FX-322-treated subjects' speech recognition improved over the duration of the study while placebo-treated subjects did not. In WR assessment, FX-322-treated ears showed a statistical improvement in percentage change from baseline scores versus placebo on average across all time points (p=0.029). The effects were sustained throughout the study, with the following least-square mean difference (SE)=18.3% (11.0); Day 30=14.2% (11.4); Day 60=s2.1% (11.4); and Day 90=21.9% (11.0) (McLean, W.J. *et al.*, 2021).

Speech recognition in a noisy background using WIN also improved over time for FX-322-treated patient ears but not placebo-treated patient

ears. Performance was quantified as the signal-to-noise ratio (SNR: 0-24 dB) consistent with 50% correct WR, with lower SNR values indicating better speech perception in background noise. Analyses showed a significant improvement in average SNR from baseline to Day 90 in FX-322-treated ears (-1.3 dB; P=0.012) but not placebo-treated patient ears (-0.21 dB, p=0.71) (McLean, W.J. *et al.*, 2021).

Individual responses across intelligibility tests for four FX-322-treated patient ears showed clinically significant improvements. Absolute improvements in WR from baseline to Day 90 range from 18 to 42% in these four patients. Two of these four ears showed substantial and clinically meaningful improvements in WIN testing from baseline to Day 90, with SNR improvements that exceeded the 3.1 dB threshold representing the theoretical difference exceeding expected test-retest variabilities established by Wilson and McArdle. Subjects from both etiologies and dose volume cohorts responded to treatment (McLean, W.J. *et al.*, 2021). A summary of clinical data is shown in Figure 5.

Conclusion

In summary, the totality of the above studies has led to the first delivery systems for administering macromolecules and has helped enable the discovery of new angiogenesis inhibitors, the development of Covid vaccines, and many other new therapies. These delivery systems and extensions thereof have also played a role in regenerative medicine and could possibly play a role in new approaches for treating hearing loss.

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Final Statement of the Workshop on Looking to the Future: Stem Cells, Organoids and Regenerative Medicine

Abstract

Stem cell therapies, once a dream, are now becoming a reality. The term stem cells dates back to the late 1800s at which time it referred to the fertilized egg, a pluripotent cell able to give rise to all of the complex cell types that comprise our body. In the 1960s and 1970s, scientists discovered that there are cells within adult tissues of the body that harbor many of the same special properties as *embryonic stem cells*. However, these adult stem cells have more restricted potential – they are specialized to replenish, rejuvenate, and repair the tissues in which they reside. As discussed in this workshop, recent advances in the development of cell and molecular biology tools have enabled the isolation, characterization and unprecedented manipulation of stem cells, heralding an era of breakthrough biological research and a revolution in the therapeutic application of stem cells in the clinic, known as "regenerative medicine".

Stem cell biologists now classify stem cells into two major types that exist during the lifecycle of multicellular organisms: embryonic stem cells, resulting from the early divisions of the egg, characterized by their "pluripotency", i.e. the capacity, that they share with the egg cell itself, to produce all the cell types found in the adult organism, and the *tissue-specific stem cells* present in the tissues and organs of the adult. The latter play an important role in renewing the cells of the various organs during the entire life. They are particularly active in tissues and organs in which the lifespan of the differentiated cells is short, like blood, skin and the inner cell layer covering the intestinal cavity, as well as in the repair of skeletal muscle after exercise of injury. These adult stem cells are highly specialized and can only produce the tissue in which they reside. They are "unipotent" or "multipotent".

Significant advances have been made in isolating, culturing and reintroducing adult stem cells into tissues. In addition, there has been remarkable progress in developing approaches to stimulate these tissue-resident stem cells *in situ* in the tissue. In addition, new stem cell technologies entail enlisting immune cells as anticancer agents. Induced pluripotent stem cells (iPSC) can now be generated from skin or blood of mice or humans by overexpressing four key transcription factors. Scientists have learned to

grow these iPSC in a dish and convert them into mini-versions of mouse and human organs. This so-called "organoid technology" opens new avenues for the study of development, physiology and disease and for personalized medicine. In the future, this technology coupled with advances in bioengineering, may yield cultured mini-organs that can replace organ transplants from donors which would constitute a breakthrough for regenerative medicine.

In this two day symposium, an international group of stem cell biologists, immunologists and engineers discussed advances in basic and clinical stem cell research that could lead to tractable solutions for patients with degenerative diseases, including Parkinson's disease, age-related macular degeneration, leukemia and other therapeutically recalcitrant malignancies, bone marrow failure syndromes, age-related muscle atrophy, epidermolysis bullosa, inherited valvular heart disease, and SARS-CoV-2.

The proceedings of this workshop will be dedicated to Professor Nicole Le Douarin without whose inspiration and dedication this meeting would not have occurred. Organized by PAS Academicians: Nicole Le Douarin, Elaine Fuchs, Helen M. Blau

Issues and Agenda

1. Advances in stem cell biology and regenerative medicine are accelerating at a remarkable pace. These advances are exciting as they promise to significantly impact human health and welfare. Currently, although the average human lifespan is increasing, healthspan is not. People are living longer, but this increase is associated with chronic disabling disease. There is a great need for interventions that improve the quality of life. Regenerative medicine offers the potential to accelerate and augment repair of damaged tissues by tissue-specific stem cells. Great strides have also been made of late in the production of "organoids", mini-organs from pluripotent stem cells that may serve as a replacement of organs. These advances promise to alleviate much suffering throughout the world.

2. Overcoming the need to find organ donors, who are limited in number, could save many lives and overcome ethical concerns. Many individuals wait years for a suitable transplant donor for a kidney, liver or heart. In addition, obtaining organs from healthy volunteer donors can lead to exploitation. Therefore, the ability to generate organs to replace those that have failed due to illness, injury, or combat would constitute a major medical advance.

3. Another ethical concern is the prevalence of Stem Cell Clinics that are not validated and promise therapies that are not proven to be efficacious. A prime example are mesenchymal stem cells that can be isolated from adipose tissues and then injected into the joints, for example the knee or hip. To date the benefit of such interventions remains to be proven. Indeed, the expanding clinical impact of stem cell research has motivated charlatans to exploit the public's belief in the potential of stem cell research by selling fake stem cell therapies to desperate patients. Thousands of companies have sprung up around the world, marketing products whose safety and effectiveness have not been proven in clinical trials and that are often implausible based on our understanding of the biology of the conditions they are claimed to treat. A critical challenge for the field is to enforce regulations that prevent the marketing of unproven stem cell therapies that victimize vulnerable patients.

4. Stem cell therapies are currently very expensive and this presents an obstacle to their dissemination globally. A way to reduce their price and make them available to needy populations throughout the world is of prime importance. One approach is to identify drugs that stimulate the endogenous stem cells that reside in many of our tissues and augment their function in situ. The cost of such treatments should be considerably lower than cell and organ-based treatments that require sterile environments and complex culture.

5. A major case in point is the global need for hematopoietic stem cells which can reconstitute the entire circulation. Human transfusions are needed for treatment of hemorrhage or severe anemia, for example after an injury, accident, or battle. Blood is in short supply worldwide and is a major life saving intervention. While blood and bone marrow donations remain life-saving and common practices in medicine, the blood supply is finite and unpredictable, prone to shortages, and subject to contamination with pathogens. While blood group matching is essential, the heterogeneity of human blood antigens and the wide variation in blood cell numbers in different donors means blood products lack the reliability of pharmaceuticals. The development of facile ways to generate these stem cells from human induced pluripotent stem cells of particular immune phenotypes could revolutionize treatments requiring delivery of blood and save lives worldwide.

6. An ethical issue that may arise in the future is the potential to generate human embryos in tissue culture. 10% of couples are infertile and in many

countries there is a shortage of embryos generated by in vitro fertilization and sometimes via incubation by surrogate mothers. Scientists are making significant progress in generating human sperm and oocytes from induced pluripotent cells in tissue culture. This suggests the possibility of fertilization in vitro. In fact, human blastocysts, an early developmental stage post fertilization, have been successfully produced in culture. While there are major gaps in our knowledge of early human development, and crucial scientific insights into human development could be gained that could enhance in vitro fertilization protocols, ethical issues arise. First, the technology is not sufficiently advanced to ensure the development of viable human embryos. Action has been taken by the International Society for Stem Cell Research (ISSCR) Guidelines for Stem Cell Research and Clinical Translation which concluded that human stem cell-derived embryos should never be transferred into the uterus of an animal or human and that the time in culture should be restricted to that required to address the specific scientific questions under study. With an ever-evolving frontier of stem cell science, embryology and genome editing, the philosophical and moral standards of professional conduct will continue to change and guidelines are needed from learned organizations such as the ISSCR that distinguish the permissible from impermissible realms of early embryo and embryonic stem cell research.

7. There remains a pressing need for developing more effective screening strategies that predict the propagation of malignantly transformed cells derived from stem cell products. While advances in stem cell technology have spawned a plethora of early stage therapies, two separate reports of leukemia development in patients who received lentiviral stem cell gene therapy for sickle cell disease have provided a cautionary note for the regenerative medicine field.

8. The remarkable pace of stem cell biology applications in regenerative medicine is in great part due to the foundational research performed in many cell types and organisms over the past 20 years in laboratories across the world. We are beginning to reap benefits from this global scientific effort and technologies like stem cell transplantation, induced pluripotent stem cells (iPSCs), and organoids are poised to usher in a new age in medicine. It is important to note that many themes of tissue homeostasis, and alterations in disease and age are shared among different tissue and organ types, and even across diverse species including model systems such as pla-

naria and axolotls. This includes an understanding of the interplay between epithelial cells, immune cells, mesenchymal supporting cells and the extracellular matrix they produce. Extracellular matrix is key in the regulation and emergence of transitional cellular states that may be "frozen" in disease conditions. To address future challenges, and make progress in curing the incurable, and developing yet unimagined new technologies, it is essential that robust investments continue to be made in foundational, curiosity-driven research: the source of all new technologies. Cross-species research highlights the importance of continued basic science experimentation.

9. Finally, interactions and conversations like those that took place inside the walls of the Vatican on May 5-6, 2022, are essential to further and enhance our understanding of stem cell biology and regenerative medicine and how it can benefit mankind.

Advances that are revolutionizing Regenerative Medicine

Induced Pluripotent Stem Cell (iPSC) Technology

10. The discovery by Shinya Yamanaka of induced pluripotent stem cells (iPSC) created an alternative to embryonic stem cells (ESC). Upon expressing four transcription factors, the skin or blood cells of any human being can be made into pluripotent cells that can be propagated indefinitely and that can be differentiated into essentially any given specialized cell type, as is true for ESC. For example, this technology is leading to the generation of retinal pigment epithelial cells as a treatment for age-related macular degeneration (AMD) that leads to blindness in a large proportion of aged individuals. IPSC are also being successfully differentiated to generate corneal epithelia cells to repair physical damage to the cornea, to restore the retinal pigment epithelium and counter retinal degeneration, to create dopaminergic neurons for the treatment of Parkinson's Disease, and as cancer immunotherapies.

Parkinson's Disease (PD) is the second most common neurodegenerative disorder and is associated with significant health and financial burdens. The key motor symptoms of PD are caused by the loss of dopamine neurons in the midbrain. A healthy individual has about 300,000-400,000 midbrain dopamine neurons and the loss of greater than 50% of those neurons is thought to trigger symptoms of Parkinson's disease. The rather discrete loss of dopamine neurons in a defined brain region makes PD an attractive target for regenerative medicine, as only a limited number of

new neurons might suffice to significantly impact motor function in an individual patient. Preclinical experiments in rats and early clinical trials using iPSC and ESC derived dopaminergic neurons in multiple countries appear promising for treating PD. An advantage is that challenges of mismatch and rejection by the immune system may not be an issue since the brain is relatively immune privileged. Only transient immunosuppression may be required.

11. A major hurdle for therapies outside the brain is the immune system which rejects cells that are of foreign origin, i.e. not 'self'. The generation of patient-specific cells that are not rejected by the immune system is prohibitive in cost. Efforts to automate the production of iPSC will help to reduce costs. In addition, a major effort is being devoted to the development of universal donor cells that can be used to treat everyone, or at least subsets of individuals with similar immune phenotypes. To reduce the risk of transplant rejection, gene editing technology is being used to alter genes involved in immune recognition, in particular HLA haplotypes.

12. **Blood.** Induced pluripotent stem cells offer the prospect of an inexhaustible, predictable, antigenically defined and pathogen-free source of red blood cells and platelets with precise dose-response properties. Such cells might one day become an appealing alternative to blood donation. Challenges that remain include producing functional red cells, platelets, lymphocytes and hematopoietic stem cells that mimic their native counterparts. In addition, improvements in cell manufacturing are needed to reduce costs and enable commercial viability. Considerable progress has been made to date from laboratories around the globe which has led to clinical trials testing platelets derived from induced pluripotent stem cells (iPSCs) for patients at risk of bleeding, as well as studies that have demonstrated marked anti-cancer activity for patients treated with iPSC-derived natural killer (NK) cells. There is still a great need for advances to generate more developmentally mature and robust T and NK cells for use in adoptive immunotherapies.

Model organisms

13. Scientific advances have always benefitted from the study of model organisms. Salamanders (axolotls) show remarkable regeneration of complete body part including limb, tails, jaws, heart and spinal cord and therefore represent an important model for studying how organs can be regenerated,

and what is missing or blocked in mammals, including humans. There is a cellular memory, or 'zipcode' that dictates which body part is efficaciously regenerated following amputation at different sites along the limb of the axolotl. This memory can be altered and expanded by interactions of specific cells that secrete particular protein molecules like sonic hedgehog and Fgf8. Another model organism, the planarian, a flatworm, is capable of remarkable regeneration and in fact has pluripotent stem cells that can rescue viability and regenerative capacity in animals devoid of stem cells. Once specialized, these cells remain stably differentiated, but this state is not terminal, as the cells are capable of plasticity and if transplanted into foreign sites, can induce regeneration of these cell types. The lessons learned about site specific molecular memory for regeneration and plasticity of highly specialized cells in these organisms are instructive to studies in mammals.

Tissue specific stem cells and the immune response

14. **In skin.** When the skin confronts pathogens, its stem cells record their experience within the chromatin of their nucleus. Like the axolotl limb, a molecular memory is retained long after the inflammation has subsided and the skin pathology has returned to normal. Moreover, upon exposure to a second inflammatory encounter, the inflammation-experienced stem cells recall this memory and respond faster and with heightened reactions relative to the first encounter. The mechanisms of how our cells harbor, retain and recall epigenetic memory of inflammation are being resolved at a molecular level. These findings not only impact our understanding of normal wound repairs but also explain why in inflammatory disorders such as psoriasis, atopic dermatitis, asthma and inflammatory bowel disease, episodic bouts of inflammation are often heightened in severity and occur in the same areas. They also explain how prior inflammatory experience can cause robust responses triggered by different pathogens and other stimuli that have not been encountered before. These insights into "epigenetic inflammatory memory" may inform therapeutic strategies to erase the bad memories and retain the good ones. Diet may be one, as high fat diets exacerbate inflammatory memory.

15. **Innate immunity.** There are parallels to be drawn between skin and the small intestine epithelium. Activation of a cellular circuit between epithelial tuft cells by parasite metabolites, a subset of tissue-innate immune cells and differentiating gut lining cells increases production of protective mucus that leads to greater gut motility, thus protecting the epithelium

from further injury. Knowledge of the products generated by these rare innate immune cells that are capable of altering stem cell generation in the intestine could lead to interventions that protect the intestinal barrier during inflammatory diseases. In addition, they could yield new approaches to cancers that result from progressive injury and host pathology.

Inflammation. Although transient inflammation is essential to wound repair, a chronic inflammatory state, a characteristic of many aged tissues, can impede tissue-specific stem cells. In the bone marrow, persistent inflammation leads to hematopoietic stem cell (HSC) depletion. This loss can be countered in the blood by Leptin Receptor-expressing stromal cells in the bone marrow, which constitute a source of adiponectin, an anti-inflammatory cytokine that promotes HSC quiescence and self-renewal. Adiponectin suppresses myeloid cell and T cell production of interferon gamma and tumor necrosis factor, which when chronically increased, persistently activate the HSCs, leading to their depletion. This discovery of adiponectin as a component of the niche that is key to HSC maintenance with aging suggests that similar molecules likely play a crucial role in protecting stem cells from inflammation due to pathogen infection or aging in other tissues.

Miniature Organs: Organoids

16. **Gut.** The inner lining of the gut is entirely replaced each week of our life. This happens through the activity of a large number of diminutive stem cells that hide at the bottoms of the crypts in the gut wall. Each day, these stem cells divide to create daughter cells which briefly divide, after which they mature to perform any of about 10 different tasks having to do with food digestion and uptake. After about a week they are extruded from the gut lining and die. Stem cell biologists showed how this vigorous stem cell-driven process can be recreated in a plastic dish: single stem cell – when given the appropriate growth factors – creates a mini-version of the gut or "organoid". Indeed, a large number of organs can now be grown as organoids for the repair of diseased organs by transplantation.

Organoids can also be grown from malignant tumors from cancer patients. This allows tailoring of cancer therapy to the individual patient, by testing multiple drugs on the cultured mini-tumors of that patient to find the best individualized therapy. Mini-lungs and gut organoids can also be used to study infectious diseases of human organs, such as COVID-19.

Organoid technology is not limited to mammalian species. The venom glands of poisonous snakes, responsible for 150,000 deaths worldwide each

year, can be grown indefinitely in the lab as snake venom gland organoids. This offers hope for the development of cheap and effective therapies for snake bites. Moreover, snake venom has already been used as a starting point for around a dozen effective heart-, brain- and diabetes medicines. Given that the world is home to more than 2,000 venomous snake species and that each snake produces around 20 different toxins in its venom, organoid technology may allow development of a large number of novel venom-based medicines.

17. **Lung.** Chronic and acute lung diseases are among the leading causes of morbidity worldwide. Organoids, tiny structures that can now be grown from stem cells in culture dishes that resemble the three key regions in the lung: the trachea (windpipe), bronchioles (airways), and the alveolar space (alveoli, where gas exchange occurs). These three-dimensional culture systems are able to mimic the lung niche and are advancing the understanding of how stem and progenitor cells regulate lung biology in development and in lung disease. For example, alveolar cell organoids can mimic tumor progression mediated by the oncogene *Kras* and recapitulate early-stage lung adenocarcinoma in patients. In aging, organoid cultures have uncovered mechanisms that entail changes in lysine methylation regulated by methyltransferases. Organoids offer hope as treatments for lung diseases in the future, depending on the injury and cell populations impacted. Alternatively, the cellular and molecular changes in lung disease that organoids allow us to understand may pave the way for treatments that involve small molecules or other biologics.

18. **Brain.** The human brain is unique in its size and complexity. It is made up of 87 billion neurons that need to be born at the right time and form the right connections in order to achieve the enormous intellectual abilities we have as human beings. This complexity comes at a price: Brain disorders are among the most frequent, most devastating and least curable diseases. Cerebral organoids, three-dimensional human cell cultures derived from iPS, can recapitulate the development of the human brain. By growing cerebral organoids from patients suffering from brain disorders, the cellular events leading to disease can be recapitulated, opening new avenues for prevention and reversion. Tuberous Sclerosis, a severe childhood abnormality caused by defects in the mTOR signaling pathway, provides an example. The disease is caused by a type of cell that is found in humans and does not exist in most animals like mice. Analyzing human

disease in organoid culture can lead to unexpected insights, to the discovery of events that occur only in humans and to the development of new therapeutic strategies targeting these human specific processes.

Stem cells to treat disease states

19. **Skeletal muscle and aging.** Aging is thought to bring wisdom, but for many individuals, their later years are characterized by decline and discomfort as body systems slow and eventually fail. This is a problem that impacts billions of people around the world. Throughout history, people have sought means to reverse aging, or to maximize healthspan, time spent free from ailments. Humans over the age of 50 lose 10% of their muscle mass per decade. All forms of muscle injury, from exercise to severe trauma, generate an inflammatory response. A recent highly promising approach to building muscle and countering the debilitating loss of muscle that accompanies aging (sarcopenia), capitalizes on mechanisms associated with this natural wound healing. Prostaglandin E2 (PGE2), an inflammatory metabolite generated from membrane phospholipids, is required and sufficient for muscle stem cell proliferation, survival, expansion and engraftment into injured tissues. In addition, PGE2 rejuvenates aged muscle fiber function, leading to tissue remodeling and a dramatic increase in mitochondrial biogenesis and metabolic function. This potent dual function of PGE2 was most clearly shown when PGE2 levels in aged mice were restored to levels found in young mice by small molecule inhibition of the prostaglandin degrading enzyme, 15-PGDH. A remarkable 15% increase in muscle mass and strength was observed after one month of treatment. These findings suggest that inhibition of 15-PGDH, now termed a "gerozyme" with a critical role in determining levels of PGE2, may serve as a therapeutic strategy to counter the loss of muscle mass and strength that plagues the ever-increasing proportion of aged individuals.

20. **The heart.** Heart disease remains the number one non-infectious cause of death worldwide in adults and infants. Survivors with heart disease are often left with damaged hearts due to the inability of the mammalian heart to regenerate itself. As a result, over 25 million people globally suffer from heart failure due to the inability of the cardiovascular system to support the body's normal activities. In addition, valve disease and congenital malformations result from genetic defects in 1% of all live births, but the etiology remains unknown and disease modifying therapies are lacking. To date, no bona fide cardiac stem cells have been identified in the heart.

One promising approach is to reprogram endogenous scar forming fibroblastic cells in situ to become functioning cardiac cells. Given its efficacy in mice and pigs, clinical trials are on the horizon. In another approach, iPSC derived cells were generated from patients' cells that recapitulate features of aortic valve disease, including generation of osteoblast-like cells that lay down the calcium leading to stenosis. Using machine learning to screen drugs that shift the entire dysregulated gene network in this patient iPSC-derived disease model led to the identification of a drug that has been effective in preventing and treating the valve disease in a mouse model. These examples highlight the potency of using iPSC to model human genetic disease. Further, they underscore how understanding the mechanisms by which stem cell fate decisions are regulated can lead to drugs that induce regeneration, yielding novel treatments.

21. **The skin.** These stem cells are currently the poster child of regenerative medicine. One of the most severe and life hindering blistering skin diseases, Junctional Epidermolysis Bullosa (JEB), was successfully treated in 2017 by genetically correcting a small number of a patient's epidermal stem cells (holoclones) in the laboratory, so that they could express the laminin protein (LAMB3) missing in JEB. When engrafted onto the boy, these genetically corrected, self-renewing epidermal stem cells took over and began producing healthy skin. Normally any trauma to the skin of junctional epidermolysis bullosa patients leads to painful skin lesions. This boy, who had not been able to leave the hospital for years before his treatment, now plays soccer and continues a life not thought possible prior to regenerative medicine therapy. The genetically engineered laminin is expressed in the appropriate location in the basal lamina and well-defined, organized hemidesmosomes comparable to those of healthy controls are apparent, indicative of a fully restored epidermal-dermal junction. There are other types of epidermolysis bullosa which harbor mutations in other structural genes and affect different layers of the skin. Although the epidermis can be regenerated and repaired by CRISPR corrected gene edited epidermal cells, challenges remain for regenerating the dermis and restoring proteins such as collagen 7.

22. **Leukemia.** Lentiviral reporters of HSC function and robust humanized mouse model systems have enabled a direct assessment of human benign, pre-malignant and malignant HSC aging and highlighted vital differences between human and mouse biology. APOBEC3C, a DNA editing

enzyme, if overexpressed, leads to myeloproliferative neoplasms. It also induces ADAR1 activation leading to RNA editing to evade tumor suppression as well as the innate immune system. Various isoforms of ADAR1 have been identified whose activation can be prevented by a splicing modulator, which shows promise as new anti-cancer therapeutic. Increasing our basic science understanding of stem cell biology and how pre-leukemia stem cells are generated from HSCs is informing novel strategies to surmount blood derived cancers.

Looking to the Future: informational molecules and delivery systems for regenerative medicine

23. Micro and nano particles have been instrumental in the delivery of large molecules including DNA and RNA to the body. This technology has enabled therapies for cancer and heart disease, and most recently proven invaluable for mRNA vaccine delivery for the prevention of COVID-19. In combination with cells, tissues and organs such as blood vessels and skin have been engineered. As organs on a chip, such constructs could reduce animal and human testing of drugs. A recent application of this technology entails generation of hair cells from stem cells in quantity delivered to the ear with novel solvents to restore the loss of hearing, which plagues a large proportion of the aged population. In early clinical trials, some patients were able to hear for the first time in over 30 years even in a moderately noisy environment. The applications of novel bioengineering technologies to enhance organoid maturation and function and to facilitate stem cell delivery in situ will be far reaching and stand to revolutionize regenerative medicine.

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