To create a regenerative medicine it is necessary to "reread evolution".

"…molecules as those of DNA are molecules with a history, and their structure tells us about the past in which they were generated. They are fossils, or if preferred, witnesses of the past,…
…How is time printed in matter? In essence, this is life, it is time that is printed in matter, and this is valid not only for life but also for the work of art."

Ilya Prigogine *"The birth of time"*

INTRODUCTION

We physicians know little or nothing of the so called "*evolutionary biology*" because it has developed separately and with scarce or no interaction with "medicine". This gap is due both to the slight or inexistent understanding biologists have of medicine and to the fact that physicians know little or nothing of evolutionary biology.

Evolutionary biology, far from the schools of medicine and their education, seems to be focused on the paleontological study of human evolution through the systematization of fossil records in dull museums, and lately has been employed as a tool of population genetics. (1)

However, we are now suddenly aware that the alleles causing falciform anemia, thalassemia and other hemoglobinopathies, which we would naturally classify as genetic disorders that produce diseases, were developed by evolutionary natural selection in territories infected with malaria. And why did this happen?; because this genetic condition conferred "resistance" against Plasmodium falciparum, the etiological agent of malaria. This means that at that time it protected from a prevalent infectious disease. Thus, what in our malaria free environment causes disease (anemia) in the earlier environment with malaria, it protected against a severe illness (malaria). Perhaps Dobzhansky was right when he expressed: "*nothing in medicine has sense except in the light of evolution*". (2)

The core of the problem is that medicine explains thoroughly the nearby mechanism of body disease, "*how*" it works (how increased LDL cholesterol generates atherosclerosis, how are the different fetal presentations at the time of birth, how…), and the forgotten evolutionary explanation refers to "why" natural selection, considering our history as a species, has left the body vulnerable to prevailing diseases (why our present arteries are prone to obstruction by atheromas, why we have a narrow birth canal, why…), with the few exceptions of some unique genetic defects.

The question for the "nearby mechanism" is: how does ontogeny or the development of our life cycle make this mechanism work? In turn, the question for "evolutionary selection" is: why has phylogeny, the trace of human evolutionary history, adapted and adopted the features that, in their interaction with the environment, confer a selective advantage or disadvantage? (3)

Let us consider another real problem about what we call "normal" or "abnormal". Traditionally, medicine speaks about the abnormal "*lactose intolerance syndrome*" and classifies it as an illness; but this concept could be questioned if we knew that in fact 70% of the world population is intolerant to lactose, and that in a population context where no lactose is consumed, this situation is normal and moreover, does not produce any illness.

We will learn something else of the ontogeny and phylogeny of lactose intolerance. The intestine of a child expresses the lactose gene to allow breast feeding, but stops its expression after weaning. Moreover, until the beginning of the Neolithic period, adult humans did not need to digest lactose for their nourishment. It was only approximately 8000 to 10000 years ago that, together with wheat crops, settled populations in the Middle East began raising domestic animals, including species from which milk could be obtained. At that moment, the mutation in the lactose gene that allowed the persistence of its expression throughout life started to provide an additional nutritional benefit to individuals who drank milk. The selective advantage conferred by this mutation led to a quick growth of the population, inducing a strong migratory pressure that produced a fast dispersion of the mutation throughout Europe 8000 years ago. As evidence of the adaptation caused by evolutionary selection, we also know that in Africa, a different though equally effective mutation appeared much later, 2000 years ago, when domestic cattle was developed there. As a result of these mutations, populations descending from African and European ancestors can digest lactose all their lives, whereas those descending mostly from Australian natives from Oceania and Asia which were not exposed to a high milk load after weaning, have gastrointestinal symptoms when they ingest lactose. (3) Sometimes it is necessary to be careful when making interpretations, since it could happen that the cause for the incidence of a "population disease" might go unnoticed if the factors inducing it are oversimplified, as Geoffrey Rose emphasized would occur if the disease were analyzed with case or "*people diseases*" study designs. Like Rose has pointed out, "*if everybody smoked 20 cigarettes a day, case control and cohort clinical studies would lead us to conclude that lung cancer is a genetic disease*" and

not, as it really is, a pandemic attributed to tobacco smoking. (1) This would be one of the reasons why current massive atherosclerosis and hypertension, generated by our globalized feeding habits producing extremely high cholesterol and sodium intake levels in most people compared to Paleolithic populations, may be underestimated in the analysis of "*people diseases*". (4) In this situation, these factors could only partially explain why people are going to become ill in a comparative analysis within the community, where differences are not so extreme. However, they would mask the evolutionary mechanisms of the high incidence of hypertension and atherosclerosis in the population, hence delaying the implementation of necessary collective measures.

But another thing that we must bear in mind is that the mechanism of "*natural selection*" described by Darwin and Wallace helped the reproductive capacity - the ability to survive until reproductive maturity to engender and sustain offspring – of our ancestors, which is not a biological mechanism to eliminate diseases, pain or death.

Natural selection shapes our "*lifestyle strategy*", since in the utilization of total body energy there is competition between the energy channelled towards the process of "*reproduction*" and that focussed on "repairing" the damage that time (i.e. old age) produces in protein molecules, cells and organs.

During development we accumulate excess physiological capacity, since it is well known that we have many more nephrons and more hepatocytes than are necessary for normal renal and hepatic function. A metaphor on body economy called "*physiological capital*" clarifies the evolutionary vision of old age. Because the repairing and maintenance processes are not perfect, we consume that capital during our lifetime and once it ends, we suffer diseases and eventually die. This metaphor allows us to understand that we incorporate two strategies in the development of our human life: on one hand, the increase of our physiological capital, achieved with a sound nutrition and prenatal hygiene during the first years of our lives, and on the other, the delay in the depletion of that physiological capital through the repairing capacity obtained with adequate adult nutrition, specially micronutrients, and the reduction in the exposure to agents that increase somatic injury (tobacco, alcohol, salt, lipid diet and a great amount of calories). (5)

ORGAN REGENERATION?

More than 240 years have elapsed since Spallanzani wrote the first scientific treaty in 1769 describing the "*Leg Reproduction in the Aquatic Salamander*" in an "Essay on Animal Reproduction", and even longer since Abraham Trembley observed that upon dissecting hydrae he had found swimming in a stream near The Hague in 1740, they regenerated under the microscope. Despite the significant advances in biology and medicine, many of the most remarkable aspects of limb regeneration outlined by Spallanzani are still unknown. (6)

Besides amphibian limb regeneration, as in the aquatic salamander, we also know that the zebrafish has the ability of regenerating various organs and even its damaged heart. The potential to renew limbs and damaged hearts seems a science-fiction story. (7) That is why the capacity of humans that can only regenerate large parts of the liver and pancreas and partially restore the skeletal muscle and peripheral nervous system pales when compared to the amazing ability of amphibians and fishes who, in addition to regenerating amputated limbs and fins, can restore most of their organs, including the crystalline, retina, cardiac muscle and the central nervous system

In mammals, like us, the liver is one of the few organs that has the remarkable capacity of completely restoring itself after a significant loss of hepatic tissue due to partial hepatectomy or acute hepatic injury. This extraordinary regenerative potential was possibly known thousands of years ago, as revealed by classical Greek mythology in Prometheus myth. Having stolen the secret of the art of fire from the gods of the Olympus and given it to mankind, Prometheus was punished by Zeus – and chained by Hephaestus to a rock in the Caucasus – to the torture of having his liver eaten each day by an eagle. Thus, as the liver regenerated during the night, while the eagle had an eternal feast, Prometheus suffered an eternal torture. (8)

Liver regeneration after partial hepatectomy is mediated by the proliferation of the different mature cells residing in the liver (hepatocytes, endothelial cells, bile duct epithelial cells, hepatic stellate cells, Kupffer cells) until the lost hepatic tissue is restored. However, only in the last few years, we have realized that in order to divide, even a liver cell has to dedifferentiate, to pass from a post-mitotic state to the mitotic cell cycle. (9)

At this stage, we could ask the following questions:

What can scientists learn from simple creatures, as the aquatic salamander or the zebrafish? Why have mammals not preserved, except in some tissues like the liver, this extremely useful property during the course of evolution? Can an evolutionary perspective on the mechanisms used by these "humble" beings give us a clue about the necessary strategy for the regeneration of human tissues? Could the generation of a great number of patient specific differentiated cells be utilized for cell therapy, to elucidate the mechanisms of disease or to search for new therapeutic drugs? Recent studies suggest that this is possible, and will be explained next. (10-13)

REGENERATION OF AMPUTATED LIMBS

The ability of amphibians, such as aquatic salamanders, of regenerating an entire limb after amputation has drawn the attention of scientists for ages, in the hope of reproducing this highly useful capacity in humans.

In the last years, pioneer studies in these species have provided many seminal perspectives. (12, 13)

Following amputation, the aquatic salamander's limb bleeds only for a brief period, immediately setting off the important process of wound healing that will lead to limb regeneration. In only 24 hours, if the skin is not sutured, the wound becomes completely enclosed by cells from the "wound epidermis", which proliferate and migrate to the surface of the stump forming an apical epidermal cap (APC). It has been postulated that this initial structure activates key molecular pathways necessary to stimulate and maintain the initial stages of regeneration, since if the skin is sutured no APC is formed and the limb remains as a permanent residual stump. Another evidence of the importance of this step derives from human medicine: small children with distal finger amputation may have a perfect regeneration of the fingertip, but only if the skin of the stump is not sutured. (6)

The following critical step is blastema formation, a mass of cells generated by cell proliferation from the distal end of the stump, which forms a cone-shaped transparent outgrowth below the APC from which the new limb will develop. For a long time, it was thought that the blastema consisted of a group of pluripotent cells that had to specialize again. Surprisingly, however, important recent studies on lineage tracking have shown that the cells are not pluripotent, but that each tissue produces progenitor cells with restricted potential. Therefore, a blastema is a heterogeneous collection of restricted progenitor cells, the positional identity is a specific property of each type of blastema cell and thus cells destined for cartilage, bone, nerves and muscle remain in their corresponding site. (14) A critical step in the process of limb regeneration is the attainment of proliferating potential, which is achieved by the return to the cell cycle of post-mitotic cells while preserving their specific identity. A similar development is observed in the cardiac regeneration of the zebrafish.

Blastema limb regeneration implies the renewed growth of a number of tissues into their appropriate proportions and positions. This complex process depends on innervation (in the denervated muscle blastema formation is insufficient and regeneration fails, though it can be restored replacing the nerve with a single nAG protein, a cell surface molecule which expresses gradually in the proximal-distal axis of the salamander limb). It also requires the coordination of dynamic cell interaction (12)

The process ends with the flattening and formation – as a painter´s palette – of the limb bud, with an emergent design that allows perceiving the future fingers or toes. The final development is the reconstruction of a limb equal to the severed one, with new vessels and delicate nerves connecting directly with the already existing structure in the stump, and a resulting morphology and function indistinguishable from the rest of the limbs. (6) If an animal looses a foot, only the missing foot and no more will grow, and if it looses a limb at the level of the thigh, all the missing part distal to the amputation will grow.

How is this perfect reconstruction engineering generated? Is it owing to the context where it proliferates or to a type of "cell memory? Some of these ideas have been clarified with "blastema implant" experiments.

When a "*thigh*" proximal blastema was implanted distally with a distal blastema "destined" to become a foot, it gave rise to a completely regenerated limb with two feet. Alternatively, if a proximal blastema is implanted in the proximal blastema of the host, the salamander will regenerate two perfect and complete legs. Therefore, it is as if each blastema "*remembered*" its role and the proximal-distal information were codified in the blastema genes.

There are other experiments which show that the blastema is beyond doubt an independent unit and that, once created, can only respond to the underlying information in the tissue and not to the contextual information, since if the proximal blastema of a limb is implanted in a receptive field such as the salamander's eye, a limb will grow from the eye socket.

Another interesting point is the well known capacity of the aquatic salamander of regenerating multiple organs during its lifetime. In comparison, a frog can only regenerate its limbs while it is a tadpole and gradually looses this capacity when it reaches the late stage of metamorphosis.

Understanding the molecular and cellular mechanisms that allow a salamander to generate and develop a blastema, or the simple comparison of a tadpole regenerating a tail in its regenerative stage vs. its inability in the late stage, would help in the development of therapies that improve the regenerative ability in animals, like us, who do not possess it.

CAN EVOLUTIONARY BIOLOGY EXPLAIN CELL CYCLE INHIBITION?

It has been suggested that mammalian loss of regenerative potential during the increased complexity brought by evolution would be due to the management of a vast number of cellular lineages, shape integrity and maintenance of a stable body plan, all of which would entail a trade-off to protect from the proliferation of disorganized cells producing cancer.

But, what would happen if, like aquatic salamanders or fish, human completely "*differentiated*" cells that do not divide and are therefore specialized in specific functions could become "*undifferentiated*" (dedifferentiated) by being pushed only one step backwards, thus entering again in a proliferation state while retaining their identity or "*sameness*"? These cells could produce precise copies of themselves, capable of regenerating the damaged tissues from

which they originated.

A crucial step would probably be to eliminate the inhibition of cellular proliferation, which would imply, as defined by an author, "lifting the brakes" in cellular division. However, the "brakes should be lifted" only temporarily to avoid uncontrolled proliferation with the potential formation of tumors.

Can transient blockade of tumor inhibitors play a role to achieve "dedifferentiation"?

Tumor suppressor Rb – codified by the retinoblastoma gene – is cited as the eukaryotic "gatekeeper" that inhibits the synthesis phase of the cell cycle in which DNA is replicated (G1-S). Rb inactivation allows limb regeneration in the aquatic salamander. (15) Conversely, Rb loss in mammals does not lead to cell dedifferentiation (e.g. the primary skeletal muscle cells). (11, 16)

Which is then the additional brake that blocks cell cycle reentry in mammals?

Scientists dedicated to cancer biology have already defined a critical function for the "*alternative reading frame*" (ARF) protein (also known as p19ARF in mice and p14ARF in humans), that is transcribed from the mammalian tumor suppressor INK4a/ARF locus.

The ARF function is to keep the cell cycle arrested when the Rb is inactivated to avoid tumor formation.

It is known that ARF is frequently inactivated in human cancers. (17) Even mature differentiated cells become undifferentiated when ARF is inactive, if they are also exposed to abnormally high growth factor signals. (18) In evolution, there is no homologous ARF protein in regenerative vertebrates. Effectively, ARF has not been identified in the evolutionary development of organisms lower than the chicken. (19)

Consequently, ARF has been postulated as the other culprit of the "*brake*" to cell cycle reentry. It is noteworthy that transfection of microRNA – short non-codifying ARN acting as a negative regulator of genetic expression –inhibits both Rb and ARF, allowing the cell nucleus to initiate DNA synthesis.

Thus, the double loss of these two tumor suppressors (Rb and ARF) overcomes cell cycle reentry blockade. (11)

Recently, the microRNA miR33 has been recognized the property of inhibiting the expression of "*cyclin-dependent kinase 6*" (CDK6) and "cyclin D1" (CCND1), causing the arrest of cell cycle progression in G1 and inhibition of cell proliferation. Conversely, inhibiting miR33 increases the expression of CDK6 and CCND1 mARN promoting cell proliferation. (20)

CAN AN ISOLATED MONONUCLEAR CELL REENTER THE CELL CYCLE, COMPLETE MITOSIS AND PROLIFERATE?

The critical question heading this section was tested in myocytes, differentiated muscle cells which cannot reenter the cell cycle despite being exposed to a battery of "growth factors". To definitively demonstrate that a post-mitotic differentiated cell can initiate division and proliferate, it was essential for researchers from the University of Stanford to create an instrument to track individual myocytes. (11)

For this purpose, myocytes were circumscribed and removed by laser microdissection. Then, the laser was reoriented to produce the necessary energy to catapult the individual myocytes off the membrane into a capsule from which the intact cell was harvested and seeded for culture

If the individual myocytes were not treated they survived and crawled off of membranes but never divided. Conversely, myocytes treated with inhibitory microRNA, oriented to transiently decrease Rb and ARF activities, divided, proliferated and originated colonies. Clones obtained by means of this temporary "*double loss*" preserved their identity and were functional. Later, cells differentiated in the culture and expressed again Rb and ARF.

In addition, this study proved that introducing these myocytes in the injured limb of a mouse, the cells repaired the injury. These findings suggest that "*terminally differentiated*" mammalian cells can "*undifferentiate*" to a proliferative state, preserving their identity and without producing tumors when they are transformed. (11)

WHY CAN THE INDUCTION OF TISSUE REGENERATION BY CELLULAR DEDIFFERENTIATION BE A USEFUL METHOD FOR HUMANS?

We have already discussed extensively that regeneration of most mammalian tissues is extremely limited. Even though the regenerative source of adult stem cells seems absent in some tissues, others harbor these quiescent precursors, which are stimulated to divide when they are needed to repair an injury. However, the population of adult stem cells is very small to support a significant regeneration.

For a long time it was believed that the adult heart, as other organs, was a post-mitotic organ with only completely differentiated cells which needed to survive throughout lifetime, without the possibility of forming new vascular or myocardial cells due to the absence of stem cells. But in last few years different types of cardiac progenitor cells (CPC) have been identified changing the concept of heart biology. Several studies have identified human cardiac stem cells (hCSC) as positively marked C-kitPOS cells, comprising 1.1 ± 1.0% of all the cardiac cellular population. (21) Embryonic stem cells or, interestingly, induced pluripotent cells have also been postulated as a substitute source of progenitor cells. (22)

On the other hand, cell dedifferentiation in the same damaged tissue could be a viable alternative, because cells would be abundant, know their identity, have the desired tissue properties and be located precisely where they are needed.

If dedifferentiation were applied to induce, for example, healthy cardiomyocyte proliferation in the vicinity of the infarcted myocardial tissue, it

would be potentially possible to produce cells with the appropriate identity, leading to the regeneration of true myocardial tissue, instead of fibrosis or angiogenesis. Could this theory be achieved by the transient suppression of Rb and ARF, reproducing a process that nature already applies in species that regenerate their organs and, hence, is known to work?

An uncertainty that should worry us is that nature has presumably good reasons to restrict promiscuous proliferation. Consequently, a controlled dedifferentiation is critical, entailing only a temporary loss of tumor suppression.

Another major application of dedifferentiation would be the provision of human tissue cells (cardiomyocytes, pancreatic islet cells, dopaminergic neurons and others) from biopsy or necropsy, which could be induced, by Rb and ARF suppression, to reenter the cell cycle, proliferate and thus faithfully replicate, in vitro, the phenotype of the original disease (Parkinson, Alzheimer, diabetes, certain cardiac diseases), as going only one step backwards keeps their identity. Furthermore, the potential of this in vitro disease model would allow, for example, the research of specific drugs.

Replication of a defined cell can be easier, in some cases, than trying to direct an induced pluripotent stem cell – iPS – to adopt the desired specialized phenotype. It would be possible for the dedifferentiation-derived cells described here, to complement, as sources of cell therapy, the utilization of pluripotent embrionary stem cells, iPS and stem cells from the adult specific tissue, and even in a sort of modern alchemy, the reconversion or reprogramming of mature somatic cells to other cellular destinies. (23)

Mixed mechanisms could also be possible. For example, in human cardiac progenitor or stem cell implant (CPC, hCSC), in addition to their direct establishment, the greatest part of regeneration could be due to the paracrine stimulus of resident stem cells or even to the entry in the cardiac cell cycle of differentiated cells, as will be seen in the discussion of recent phase 1 clinical trials with cardiac autologous stem cells or progenitor cells.

LATEST NEWS

While I was writing this article I was pleasantly surprised by the recent publication of the SCIPIO study and the discovery in The Lancet on line of the clinical trial CADUCEUS, both on the safety and for "proof of concept" of human cardiomyocyte implant and regeneration.

SCIPIO

(Stem Cell Infusion in Patients with Ischemic cardiOmyopathy) (24)

Patients with chronic ischemic-necrotic left ventricular dysfunction (EF ≤ 40%) were enrolled to control or treatment groups before coronary surgery. The right atrial appendage was resected during surgery and used to isolate and expand CSC. Almost 4 months later, the 16 patients assigned to the treatment group, received 1000000 autologous CSC, expressing surface tirosine kinase c-kit receptor, by coronary infusion. Seven patients served as controls. The ejection fraction increased from 30.3% to 38.5% ($p = 0.001$) at 4 months after infusion in the treated patients, while in the control group there were no changes (30.1% vs. 30.2%). The ejection fraction improved even more at 1 year in 8 patients, with 12.3% absolute increase (EF 42.5%; $p = 0.0007$). In the 7 patients in whom cardiac MRI could be done, infarct size decreased from 32.6 g, by approximately 7.8 g (24%) at 4 months ($p = 0.004$) and 9.8 g (30%) at 1 year ($p = 0.04$).

These initial results are very encouraging, as they suggest that coronary infusion of autologous CSC is effective in improving left ventricular systolic function and reducing infarct size in patients with severe chronic cardiomyopathy after myocardial infarction.

CADUCEUS

(CArdiosphere-Derived aUtologous stem Cells in reverse ventricUlar dySfunction) (25)

Patients with AMI – after 1.5 to 3 months – and ejection fraction of 25% to 45% were assigned in a 2:1 ratio to receive autologous cells derived from cardiospheres (17 patients) or standard care (8 patients) by coronary infusion in the infarct-related artery.

The injection during the AMI convalescence period is explained because an endomyocardial biopsy sample is taken between the second and fourth week after the onset of infarction and also because the "in vitro" cell culture takes almost 4 weeks to grow to the requested volume for infusion of 25 million cells. At 12 months, the size of the necrotic area by MRI decreased by 12% in absolute values vs. zero in control ($p = 0.007$) and the average necrotic mass diminished 13 g (42%) with respect to basal values in patients who received derived cardiac cells ($p = 0.003$), with a non-significant increase of 0.9g in the control group ($p = 0.02$ between groups). It is important to remark that the amount of viable myocardial mass increased (22g) around 60% more than the reduction of the necrotic scar, which suggests that lesion reversion had taken place and explains the restitution of part of the left ventricular mass in patients treated with cell therapy.

Eduardo Marbán, main investigator of this study pointed out: "Our significant finding in this study is very simple: this is the first study which has demonstrated therapeutic regeneration of the heart…. This had never been accomplished before, and it has certainly been our medical fantasy for decades".

Evidently, as indicated in the accompanying editorial, further research "involving more patients, longer follow-up, and a true placebo arm are needed to confirm the safety and efficacy of cardiospehere-derived cell therapy. (26)

Even though both studies were designed to assess

how autologous hCSC regenerate new myocardial tissue by direct differentiation, other experimental evidence, however, support the idea of an indirect mechanism, since both physical contact and paracrine factors stimulate and activate safe healing and regenerative pathways with possibly more enduring benefits. (27)

CONCLUSIONS

To conclude, we can confirm the truth of what is postulated in the title: "*to create a regenerative medicine it is necessary to reread evolution*".

We now realize that to transfer the powerful regenerative ability of the aquatic salamander and the zebrafish to humans it is necessary to transiently and simultaneously inhibit the critical obstacle of the two tumor suppressors, ARF and Rb.

The discovery of the mechanism previously designed and used by evolution in lower invertebrates and its adaptation to later evolved humans is a fascinating adventure, since this "*dedifferentiation*" mechanism might aid to better understand human diseases, probably lead to the discovery and development of new drugs and, above all, help to design new techniques to attempt the regeneration of our damaged tissues.

Finally, I am going to use the final comment of Blau and Pomerantz, (10) because it seems impossible to improve the ironic elegance of its double sense: "*Perhaps aquatic salamanders can give humans, as to themselves, a hand*".

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