Review

Human regeneration: An achievable goal or a dream?

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The main objective of regenerative medicine is to replenish cells or tissues or even to restore different body parts that are lost or damaged due to disease, injury and aging. Several avenues have been explored over many decades to address the fascinating problem of regeneration at the cell, tissue and organ levels. Here we discuss some of the primary approaches adopted by researchers in the context of enhancing the regenerating ability of mammals. Natural regeneration can occur in different animal species, and the underlying mechanism is highly relevant to regenerative medicine-based intervention. Significant progress has been achieved in understanding the endogenous regeneration in urodeles and fishes with the hope that they could help to reach our goal of designing future strategies for human regeneration.

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1. Introduction

The human mind has shown boundless capacity of imagination, but evolution has deprived the human body of any significant capacity to regenerate. We have heard and read about liver regeneration in the Greek mythical character Prometheus. The ten-headed Ravana in Hindu mythology can be a fit of imagination, but considering it synonymous to head regeneration in hydra may be farfetched. The ever multiplying hypostomes in hydra or regenerating a whole new body of flatworm are examples of varied regeneration which did not escape curious minds and created enormous waves of thought and imagination. Nature has an impressive array of organisms such as planaria, axolotl, hydra, zebrafish, Xenopus, Drosophila, etc., which are capable of regenerating their lost or damaged body parts. Scientists have been studying the fascinating problem of regeneration in these animal species for well over two centuries (Reaumer 1742; Trembley 1744; Spallanzani 1768; Morgan 1901; Lenhoff and Lenhoff 1986; see Dinsmore 1991). It remains a major challenge to explain the variable capability of regeneration among different animal species. However, these studies and their relevance to human health and disease have long been overlooked. This is because mammals, including humans, are considered to be regeneration-incompetent, where massive replacement of tissue or of entire body parts is not possible. Thus, for many years significant focus of mammalian, rather the human, regeneration research remained confined predominantly to using the 'can't do it' model rather than the 'can do it' model. Recent research has somewhat broadened the view, shifted the paradigm and caused a concomitant change in understanding hopefully to a more fruitful direction. Yet the prospect of creating new human tissues and organs and their day-to-day use in medicine still remain elusive and continue to be a formidable challenge for scientists and clinicians.

Our ambition to induce human regenerative potential has been rekindled by recent scientific advances in the field of biology that include tissue engineering and stem cell biology. These methods have future potential in regenerative medicine, but regenerative therapies based on them and having wide scope of application are yet to be adapted for routine use. On the other hand, a critical appraisal of the underlying basis of regenerative potential of different organs in regeneration competent models is an essential prerequisite in order to bring about any feasible medical treatment to humanity. The

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challenge is to understand why regeneration does happen in many lower vertebrates but starkly fails to occur in mammals. Unraveling the cellular and molecular mechanisms underlying these efficient regenerative processes in different model organisms could provide vital clues to develop future therapeutic strategies for inducing regeneration in higher vertebrates including humans. However, to achieve the important goal of inducing regeneration in higher vertebrates, several approaches could have been adapted. These are (a) transplantation of exogenous stem cells or progenitors into injured tissues or organs, (b) transplantation of cell-seeded scaffolds made of either synthetic, biodegradable or non-biodegradable materials and finally (c) induction of endogenous or natural regeneration.

2. Promises and challenges of stem-cell-based therapy

The discovery of adult and embryonic stem cells (ESC) in early 1980s (Martin 1981), and further studies (Thomson *et al.* 1998), brought the concept of cell-based regenerative medicine into the limelight. The first human ESCs were generated in the late 1990s. Subsequently it was shown that besides embryonic stem cells, the maintenance and repair of adult tissues can be achieved by niches of adult stem cells as well. The use of ESC remains controversial because of the ethical concerns involving the use of early embryos. There has been a continued interest in generating alternatives to ESC, which led to the discovery of several different multi- or pluripotent tissue-specific adult stem cells. Despite great progress in cell culture techniques and identification of tissue specific multi- or pluripotent cell types, lineage-committed cells got much attention from tissue engineers, and several lineage-committed cells were thought to be viable options for therapeutic use in the repair of different organs, such as liver, bladder, kidney and pancreas (Van de Kerkhove *et al.* 2002; Selden and Hodgson 2004; Atala *et al.* 2006; Streetz 2008; Opara *et al*. 2010; Roy *et al.* 2011). But these lineage-committed cells may not be a viable option, at least in some situations, due to their low proliferation rate, accessibility and limited numbers. Thus, predominant attention was paid towards the exploration of the benefits of multipotent stem cells that can generate both mesenchymal and non-mesenchymal tissues *in vitro* as well as *in vivo*. Several mesenchymal stem cells have been and continue to be used in clinical trials for different remedies with variable efficacies. Some of the promising stem cell sources are the umbilical cord blood, the umbilical cord itself, muscle and adipose tissue (Bosch *et al.* 2000; Zak *et al.* 2001; 2002; Fukuchi *et al.* 2004; Lee *et al.* 2004; Trounson 2009; Trounson *et al.* 2011). A rapid progress in another front of cell-based therapy includes the generation of neural stem cells (NSC). Vigorous efforts had been mobilized to produce neural stem/progenitor cells (NS/PC) that have led to the development of cellular therapeutic transplantation strategies targeting different CNS disorders and injuries (Okano 2010). These include fetal dopaminergic cells for Parkinson's disease (PD), human fetal neural stem cells for Pelizaeas Merzbacher disease (PMD), spinal cord injury (SCI), amytrophic lateral sclerosis and human ESC derived oligodendrocyte precursors in SCI. The risks and benefits of the effectiveness of stem cell transplantation in different central nervous system (CNS) disorders need to be calibrated using appropriate disease models. A greater understanding of the basic nature of neural stem cell (NSC), neural stem/progenitor cell (NS/PC) and how recapitulating the normal CNS developmental program contribute to the repair process, could provide important clues for possible innovative therapies in treating the damaged CNS.

The recent discovery of induced pluripotent stem cells (iPSC) and concurrent advancement of cell culture procedures for their cell-type-specific differentiation has opened another exciting facet in regeneration research with huge potential. There are several advantages of iPSC over ESC. Somatic cells of human origin can be reprogrammed into iPSC and these cells can be obtained by methods free from ethical riddles. Furthermore, the possibility of creating patient-specific human iPSC could revolutionize personalized medicine. Although the efficiency of obtaining appropriate cells in huge numbers, their storage and distribution still remains a challenge. These aspects need serious attention to overcome such hurdles. The worrisome features also highlighted by several recent studies is the occurrence of increased somatic mutation (Gore *et al.* 2011), increased copy number variation (Hussein *et al.* 2011), significant variation in DNA methylation and epigenomic event in iPSCs, when compared with their fibroblast origin and human embryonic stem cells (hESC). This raises some serious safety concerns that require further detailed study to address these issues. Hence, in comparison, the primary bone of contention in allogenic embryonic stem cell (ESC) or fetal oriented cells are immune reaction and ethical concern, whereas iPSC has higher potential risk of tumor formation and genetic instability. Needless to say, future approaches should explore the possibility of using dedifferentiated somatic stem cells, which could be obtained as an abundant source of stem cells (Tang *et al.* 2012; Cai *et al.* 2007). There are reports that several human tissues can be dedifferentiated into progenitor states *in vitro*; examples are human thyroid follicular cells, epidermal keratinocytes and islet cells (Suzuki *et al.* 2011; Sun *et al.* 2011; Hanley *et al.* 2011).

3. Relevance of tissue engineering to replace damaged tissues and organs

In the last two decades several bioengineered tissues have become commercially available to treat local wounds in

diabetic foot ulcers, cartilage grafts or bony voids (Lewandowska-Szumiel and Kalaszczynska 2013). Use of many biomaterials have been proposed in recent years for tissue engineering, but the prospect of creating a new functional human organ *in vitro*, like a whole arm or for that matter, even a hand or digit, is very limited at present. Tissue engineers believe, constructing a human limb in a highly sophisticated environment, called an 'ex vivo bioreactor', may be a realistic target within next two decades. However, this ambitious goal of regenerating human limbs has to confront numerous hurdles, such as the difficulties in preparing the cultured limbs in the first place, masking the interface with nerves or blood vessels, revascularization and integrating the finished product, i.e. the bioengineered organ with appropriate parts of the host (which would need high-tech surgical skills) and lastly to restore the function of the limb. Regenerating an entire limb is only one example of the complexity involved in generating new organs. Moreover, the strategy to develop *ex vivo* construction would further differ significantly from one organ to other. The choice of strategy to generate new organs *in vitro* thus remains a daunting challenge. One of the most principal difficulties in the area of regenerative medicine is to select the appropriate cell source for transplant. While using cell-seeded scaffold, implanting such a scaffold would require a steady supply of nutrient to the cells inside the scaffold, which is a difficult proposition and a viable route has not yet been found (Langer 2007)

Despite these startling discoveries in the field of stem cell biology, the ultimate goal of regenerating a functional organ is still a far cry. Since the incidence of stem cell engraftment is low and represents the behavior of a small minority of cells within the target tissue, it may not be adequate for regenerating an entire organ. Thus, re-growing a complex organ and restoring its full functionality poses challenge at multiple planes. It requires restoration of full structural tissue diversity and its spatial organization. Furthermore, increased emphasis must be given to integrate vital information from developing and regenerating organs to understand how these structures can be made and remade. For example, if growing an entire limb is not plausible in a bioreactor, generating an accessory limb *in vivo* by identifying appropriate positional information along with molecular cues involved in re-patterning could be a way out (Bryant *et al.* 2002; Gardiner *et al.* 2002; Endo *et al.* 2004). It has been shown that limb regeneration is dependent on the cells of connective tissue that retains positional memory and that information is being recalled during re-patterning. In this context it can be emphasized that some cells are plastic that can be reprogrammed to generate new positional information whereas others are stable. The stability of positional information is related with tissue organization, proliferation and cellular differentiation (McCusker *et al.* 2015).

4. Limited regenerative response in mammals

Unfortunately, adult humans have a very restricted capacity to regenerate. Only selective tissues like bone marrow, intestinal mucosa or superficial layers of skin can regenerate (Epstein and Maibach 1965; Chan and Yoder 2004; Oates and West 2006; Michalopoulos 2007). These are often referred to as physiological regeneration, where continuous replacement of old and damaged tissue occurs because of constant turnover and proliferation of resident progenitor cells. The term regeneration is being used loosely by many to describe both tissue and organ regeneration. In mammals the biological events underlying muscle, liver and bone regeneration actually refers to the capacity to replace the amount of tissue that is lost. Moreover, the extent of tissue regeneration as described above is variable and cannot generate whole organs with identical anatomical pattern of the original. To be more explicit, liver regeneration actually represents compensatory growth rather than regeneration wherein tissue hyperplasia occurs after partial hepatectomy. The remaining liver expands in mass to compensate for lost tissue without activation of progenitor cells (Michalopoulos and De Frances 1997). On the other hand, skeletal muscle repair is satellite cell mediated and works extremely well for limited damage, like tears, strains, toxin damage and smaller lesions, where fibrous scar tissue can be visible in the regenerated muscle. However, replacement of the entire excised muscle or replacement of volumetric muscle loss (VML) resulting from severe traumatic injuries is not possible (Grefte *et al.* 2007; Grogan and Hsu 2011). Again the epidermis of skin can regenerate efficiently but damaged dermis leads to fibrosis (Martin 1997; Harty *et al.* 2003).

5. Endogenous regeneration is widespread in lower vertebrates

Among vertebrates, urodele amphibians and teleost fishes are capable of extraordinary regeneration. As adults, they can efficiently regenerate a catalogue of organs like limbs, fins, jaws, retina, heart and spinal cord (Goss 1969; Oberpriller and Oberpriller 1974; Raymond *et al.* 1988; Geraudie and Singer 1992; Ghosh *et al.* 1994; Becker *et al.* 1997; Clarke and Ferretti 1998; Poss *et al.* 2002; Akimenko et al. 2003; Ferretti *et al.* 2003; Brockes and Kumar 2005; Hui *et al.* 2010). These are examples of reparative regeneration and the mechanism underlying this process is far less understood. On the other hand, the anuran frog (*Xenopus laevis)* can only regenerate its limbs and tail as larvae (Goss 1969; Antos and Tanaka 2010; Poss 2010).

Regeneration is often referred to as reactivation of development and appropriately defined as 'regeneration renaissance', where recreation of embryonic environment occurs in adult tissue (Bryant 1999). For example, in the urodele

limb, a widely studied model for vertebrate appendage, regeneration proceeds through the formation of blastema, a mound of proliferating mesenchymal progenitors, that resembles morphologically and in gene expression profile to a limb bud (Knapp *et al.* 2013). There is a creation of embryonic/undifferentiated state for redevelopment to take place, which is achieved once the cells have been differentiated. The proliferating blastemal cells redifferentiate primarily into their parental phenotype of derivation as understood by genetic marking and eventually restore the missing structures. The proliferating mesodermal tissue in blastema arises through a process of dedifferentiation, described as the reversal of cell fate to a more primitive state, as these tissues lose their differentiated morphology. Early grafting experiments suggest that the tissues that contribute to blastema are muscle, dermal fibroblast, cartilage, Schwann cell and connective tissue. Myofibre dedifferentiation contributes to the limb blastema, as is evident from earlier histological and electron microscopic analysis, as well as from the radioactive tracer studies (Thornton 1938; Hay 1959; Hay and Fischman 1961). A detailed analysis revealed that the amphibian blastemal cells are locally derived and are not pluripotent.

Dedifferentiation is a process involving cell cycle re-entry with loss of the most differentiated character of the cells taking part in this process, as observed in various regenerating organs like limb, jaw, tail, etc. (Ferretti and Ghosh 1997; Brockes and Kumar 2002). A thrombin activated serum factor can induce differentiated salamander muscle fibre to reenter into the S-phase. This factor is also important in transdifferentiation of retinal pigment epithelial cells (PEC) to lens (Thitoff *et al.* 2003) as well as in heart regeneration in zebrafish and salamander, where cardiomyocytes re-enter cell cycle (Poss *et al.* 2002).

Retinoblastoma protein (Rb) plays an important part in cell cycle re-entry. Inactivation of hyperphosphorylated Rb allows mature cells to dedifferentiate. In contrast, mouse myotubes are firmly withdrawn from cell cycle re-entry and remain refractory to the growth factors when differentiated. This differential response between newt and mouse myotubes had been corroborated with the state of phosphorylation of Rb and it stays unphosphorylated in mouse myotubes. It has also been shown that newt limb blastemal extract not only can induce dedifferentiation of newt myotubes but also can induce dedifferentiation of mouse C2C12 myotubes into proliferating mononucleated cells (Odelberg *et al.* 2000). Another important component in this dedifferentiation saga is the genes of the Msx family. Msx1, a transcriptional repressor, is required for myotube cell cycle re-entry *in vitro*. Both in urodeles and mammals Msx1 can induce differentiated, multinucleated myofibre to proliferating mononucleate cells *in vitro* (Tanaka *et al.* 1997; Odelberg *et al.* 2000; Kumar et al. 2004). Members of Msx family are expressed in many regenerating systems like urodele limb (Simon *et al.* 1995; Koshiba *et al.* 1998; Odelberg 2002), fish fin (Murciano *et al.* 2002) and spinal cord (Hui *et al.* 2015), anuran tail and limb bud (Beck *et al.* 2003) and neonatal mouse digit tip (Reginelli *et al.* 1995). In limb, Msx is known to be expressed in early stages of development during an epithelial-mesenchymal transition, where the expression allows cells to be maintained in an undifferentiated state. The above-mentioned observations are quite encouraging because these evidences highlight the existence of a common mechanism of dedifferentiation in a variety of regenerating tissues and the feasibility of inducing mammalian cellular dedifferentiation.

6. Endogenous regeneration involves both resident stem cell and dedifferentiation

There has been a long-standing debate on the origin and fate of the regenerative cells in various regenerating organs. The most critical issues are the identification and characterization of the source of proliferative blastemal cells contributing to the formation of the regenerating tissue and the underlying mechanism controlling it. Cellular strategies used during regeneration in newt and salamanders have been studied for long. Following amputation of limb, for example, there would be wound healing by rapid migration of epithelial cells. Underlying the wound epidermis, mesodermal cells accumulate to give rise to a blastema, which is often considered to be an equivalent of limb bud. One important property of the blastema is the presence of proliferating mesodermal cells that arise by a process of dedifferentiation. Differentiated stump cells lose their characteristic genetic program such as myosin synthesis or collagen synthesis and become undifferentiated into a stem cell like state and proliferate rapidly followed by redifferentiation. This process of dedifferentiation can be observed histologically and electron microscopically (Hay 1959) where multinucleate myofibres get fragmented into single cells and enter into the blastema. Furthermore the intrinsic abililty of newt muscle fibres to dedifferentiate, to fragment and to proliferate was also confirmed *in vitro* (Tanaka *et al.* 1999).

In recent years, the advent of new technologies like lineage tracing analysis and use of transgenics in fish and amphibians allowed us to understand some of the key issues common to the natural regenerative event. The generation of transgenic axolotl line expressing green fluorescent protein in targeted tissue demonstrated fragmentation of myofibres and its contribution to blastema (Kragl *et al.* 2009). This study also confirmed dedifferentiation of tissues, other than muscle, and their contribution to the blastema. Cells derived from dedifferentiated tissues retain memory of their developmental origin and these are restricted progenitors, which contribute to the formation of very limited spectrum of tissues. For example, axolotl limb Schwann cells and muscle

were labeled with embryonic pre-somitic mesoderm and neural crest transplantation, when amputated labeled Schwann cells gave rise to Schwann cells and muscle regenerated into muscle but not into cartilage. So, blastemal cells are not multi-potent but have restricted differentiation potential. Studies using genetic-labelling (Cre/loxP)-based fate mapping demonstrate that in regenerating zebrafish heart and fin, cardiomyocytes and osteoblasts indeed dedifferentiate and contribute to blastema (Jopling *et al.* 2010; Kikuchi *et al.* 2010; Knopf *et al.* 2011). Fin regeneration involves expansion of lineage restricted progenitors generated by dedifferentiation of mature cells and heart regeneration primarily involves pre-existing cardiomyocytes rather than progenitors.

Although experimental evidence suggests an overall lineage restriction in axolotl limb blastema, it is important to resolve the fact as to whether the blastema formation involves activation of resident stem cells/progenitor populations or it involves dedifferentiation of post mitotic stump cells. Almost all studies in larval urodeles demonstrate myofiber dedifferentiation, the exception being one species of adult urodele *Notophthalmus viridescence*, where muscle satellite cells contribute to blastema. However, the presence of stem cells have also been demonstrated in other regenerating organs such as, in the ependymal lining of salamander brain and zebrafish spinal cord (Berg *et al*. 2010; Hui *et al*. 2015). Morrison *et al.* (2006) isolated satellite cells in regenerating limbs expressing markers of mammalian satellite cells Pax7 and M-cadherin. Evidences suggests that, dedifferentiating muscle fibers do not contribute to blastemas of either *Xenopus* tadpole tail and limb or zebrafish tail (Gargioli and Slack 2004; Cavaco-Rodrigues *et al.* 2012). On the contrary, satellite cells do contribute to *Xenopus* tail regeneration (Chen *et al.* 2006), suggesting stem cell activation rather than dedifferentiation. Recently, Sandoval-Guzman *et al.* (2014) highlighted differences in dedifferentiation and stem cell recruitment during muscle regeneration in two different species such as newt and axolotl. They reported relevance of Pax7 positive satellite cells to muscle regeneration and referred to two different scenarios. In newt, myofiber dedifferentiation is an essential part of limb regeneration where myofibre fragmentation result in proliferating, Pax7-negative, mononuclear cell population in blastema and that contributes in formation of the new limb. In case of axolotl, myofibre do not generate proliferating cells but Pax7-positive cells give rise to regenerating limb tissue. These results, in effect, highlight the possibility of implementing multiple strategies to induce regeneration even in mammals. Finally, the question remains: could it be possible to induce dedifferentiation in mammalian tissue and could it be a procedure for inducing regeneration in near future? To explore this possibility, studying the potential differences between cells that dedifferentiate in response to injury and those which do not is a line of research that should be vigorously pursued.

7. Endogenous repair mechanism: Potential for repair and regeneration

An impressive range of different strategies were adapted to regenerate various tissues and organs such as dedifferentiation of mature cells, for example, heart, limb, fin; transdifferentiation where cells dedifferentiate and subsequently natural developmental program is activated allowing these cells to redifferentiate into a new lineage, for example, Wolffian regeneration of lens in urodeles, anuran frog and cobitid fish (Henry and Tsonis 2010; Henry *et al.* 2013). Lens regeneration takes place via the transdifferentiation path. It involves dedifferentiation of pigmented epithelial cells (PEC). The dorsal iris cells re-enter the cell cycle and eventually redifferentiate into a new lens.

The ultimate goal of replacement of lost or damaged cells due to trauma or disease can potentially also be achieved by reprogramming, aimed to induce differentiated mature cells reverting into pluripotent stage. It has been observed that the oocyte type linker histone B4, which is associated with reprogramming, is also required for newt lens transdifferentiation (Maki *et al.* 2010). This implies that transdifferentiation may share reprogramming as there is a lineage switching similar to what is seen in somatic cell nuclear transfer into oocyte.

In the previous section we have discussed the complex biological process that takes place during regeneration of many organs in lower vertebrates. Although it is far from clear how blastema formation is achieved. Dedifferention and reprogramming seem apparently similar since in both cases a differentiated cell is induced to revert to a less differentiated state. The notion that the process of dedifferentiation of fibroblast cell to iPSC and dedifferentiation occurring during regeneration *in vivo*, may be regulated by similar mechanism is strikingly enigmatic yet remain unexplored in its full gamut. Some reports are coming into picture in the recent years which reveal that blastemal cells are not pluripotent but have strong similarities with iPSC. Christen *et al.* (2010) hypothesized that even the low level of expression of the transcription factor(s) may trigger partial reprogramming wherein the cells become multipotent during epimorphic regeneration. Such intermediary stages are commonly observed during reprogramming among iPSC, and others attempt to understand mechanism of reprogramming by interpreting these intermediate stages (Hochedlinger and Plath 2009). Direct reprogramming can be mediated by a forced expression of only four fate determining transcription factors (such as Oct4, Sox2, c-myc and KLf4), as expressed in ESC. Some examples are the derivation of iPSC from adult somatic cells, reprogramming of pancreatic beta cells

from exocrine cells and cardiac or dermal fibroblast to cardiac muscle cell (Taub 2004; Takahashi and Yamanaka 2006; Zhou *et al.* 2008). Up-regulation of these reprogramming factors has been reported during the dedifferentiation of regenerating newt limb and PEC (Maki *et al.* 2009), zebrafish spinal cord (Hui *et al.* 2015) and zebrafish retina (Goldman 2014). Thus different tissues regenerate using one or more of these abovementioned strategies but more information is required to understand similarities and differences of these three routes (Jopling *et al*. 2011).

8. Summary

Our goal was to study the events involved during the regeneration of different organs in lower vertebrates and to understand the cellular and molecular basis underlying these regenerative processes. This would allow us to apply regenerative strategies to address different diseases and injuries in mammals, including humans. So far, the focus has been on a few regenerating organs like limb, fin, heart and CNS. Identification of cell sources for regeneration in several lower vertebrate model organisms have elegantly elucidated the existence of multiple avenues by which progenitor cells are being produced during regeneration, namely dedifferentiation of mature cells or tissue stem cells. Furthermore, decoding the modes and mechanisms underlying endogenous regeneration in different model organisms would permit us to intensify our understanding of the scope of repair and regeneration in humans. Equally important is to understand as to how mechanisms of regeneration compare to embryological development. This would shed light to validate the long-standing dogma that regeneration is a mere recapitulation of development or clarify if it is made possible by independent novel mechanism in some organisms. Substantial work is still required to elucidate the molecular basis of dedifferentiation, initiation of regenerative response, positional identity and re-patterning. Recent results demonstrate the importance of studying regenerative events in not just one but different model organisms as each system tells us about different but distinct concepts which could be exploited for inducing endogenous regeneration in higher organism like human. We are beginning to answer some of the key problems of regeneration and may soon be able to replace our organs when they are injured or aged or diseased.

The ability to use endogenous stem cell for tissue regeneration has a lot of appeal and expectations. It could ideally avert many concerns associated with stem cell based therapies and bioengineered scaffolds and prosthetics. However, the approaches like tissue engineering and exogenous stem-cell-based therapy cannot be made redundant overnight. We still need greater understanding of the tissues that harness adult stem cells, the physiologically relevant niches like growth factors and hormones to recruit endogenous stem cells in appropriate direction, to identify the agents that can mobilize or expand the endogenous stem cells and lead to integration of the same into appropriate tissues. At the end of the day such approach would provide enormous hope for many human disorders.

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