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Keywords: Lizard; Regeneration; Comparative genomics; Dedifferentiation; Stem cells

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Title: Spot the difference: Solving the puzzle of hidden pictures in the lizard genomes for identification of regeneration factors

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Running Title: Identification of regeneration factors in lizard's genome.

Keywords: Lizard, Regeneration, Comparative genomics, Dedifferentiation, Stem cells

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ABSTRACT

All living things share some common life processes such as growth and reproduction, and have an ability to respond to their environment. However, each type of organism also has its own specialized way to manage the biological events. Genetic sequences of the organisms determine their phenotypic and physiological traits. Based on the genetic information, comparative genomics have been widely applied to delineate the differences and similarity between various genomes, and significant progress has been made in understanding of regenerative biology by comparing genomes of a variety of the lower animal models of regeneration such as planaria, zebra fishes and newts. However, genome of lizards has been relatively ignored until recently, although the lizards have been studied as an excellent amniote model for tissue regeneration. Very recently, whole genome sequences of the lizards have been revealed, and several attempts have been made to find regeneration factors based on their genetic information. In this article, recent advances in comparative analysis of the lizard genomes are introduced, and their biological implications and putative applications for regenerative medicine and stem-cell biology are discussed.

INTRODUCTION

No matter whether they believe the evolutionary theory or creationism, nobody would argue against the idea that humans are the most superb and clever creature among all living organisms in the world. However, the humans are still functionally incompetent or totally deficient of certain abilities that the lower animals possess; the humans cannot fly like the eagles, cannot swim like dolphins, and cannot run as fast as cheetahs. Thus, humans have been longing for such abilities, and the clever humans could develop various tools or the machines such as airplanes, ships and cars.

The desires to mimic the animals facilitated the development of biomimetics, a study of designing human-made apparatus that imitate the nature, and humans can now manufacture a variety of useful things such as solar cells mimicking plant's leaves, steel fibers inspired from spider's net, infrangible ceramics like clam shells, and computers sending the signals like living cells. Although the biomimetic approaches led to the invention of various tools, and could have humans imitate the external behaviors of animals or the plants, there are still many things that humans, by themselves, cannot physiologically mimic other living things. Among those, regeneration capability has been thought as a thing that humans can never possess.

Many lower animals have the capacity for spontaneous tissue regeneration after injury, a property that is lacking in humans except for limited liver regeneration (1). It is likely that the lower the evolutionary status is, the higher the regeneration ability is. As an example, many invertebrates such as planaria can reform its whole body from as little as one tenth of its original parts (2). Lower vertebrates such as zebrafish, newts, and lizards can also regenerate almost every tissue (3), while humans and other mammals cannot although limited regeneration in mammals have been reported (1, 4). As a matter of fact, lizards are the only

amniotic vertebrate among the animals that have the self-regenerative capacity. Thus, it seems that such regeneration abilities might have been lost during evolution, probably when the birds and the mammals appeared from early reptiles, although direct evidences are not enough to support above statement. The lack of the evidences might, at least partially, due to the insufficient information about genome sequences of the lizards up to date despite the development of modern genomic technologies. Very recently, whole genome of the lizards have been sequenced, and several genomic or proteomic attempts have been made to identify the regeneration factors. The studies of genetic sequences of the organisms have produced valuable information of the things of interest that could be applied in various fields including agriculture, industrial biotechnology, and medicine. Thus, in this article, recent advances in lizard genome sequencing and the comparative analyses of regeneration mechanisms in lizards will be introduced, and the possible applications of their strategies to the development of regenerative medicine for humans are discussed.

Evolutionary and genomic characteristics of the lizards

Reptiles first evolved from amphibians in the late Carboniferous period about 320 million years ago when they reached an evolutionary parting of the ways with other amoniotes such as the birds and mammals (5). Evolution is driven by a change in the gene pool of a population of organisms over time. Ultimately, these genetic changes constitute the unique genomes of the organisms and give them specific features, genetically and physiologically. Up to date, genome sequences of a variety of organisms, from simple microorganisms to higher vertebrates even including humans, have been revealed. However, those of reptiles, especially lizards, seem to have been ignored until recently, although they are regarded as important models for developmental biology, neurobiology, endocrinology,

behavioral biology and cancer biology (6-9).

The whole genome sequence of the lizard was first discovered in by Alfoldi *et al.* in 2011 (10). They sequenced and assembled the genome of the green anole lizard *Anolis carolinensis*, which have 18 chromosomes including 6 pairs of macrochromosomes and 12 pairs of microchromosomes with a total size of 1.78 Gb, while the chicken genome contains 28 pairs of microchromosomes (11, 12). The microchromosomes are the structures mainly found in birds, and they are also found in certain reptiles, fishes and amphibians whereas they seem to be absent in mammals. The presence of microchromosomes have made genomic study of the lower animals difficult due to their small size and poor condensation (13). Despite the small size of the microchromosomes however, they have been estimated to contain majority of the genes in certain species such as a chicken (14, 15), and comparative genomic analysis have shown that genetic information in the microchromosomes are conserved across multiple classes of chromosomes (16, 17). Thus, the genomic characterization of lizard microchromosomes may provide a valuable resource as an important experimental model for comparative analysis of their unique traits including tail regeneration. In this context, Alfoldi *et al.* not only provided the whole genome sequences of the lizard, but also revealed that *A. carolinensis* microchromosomes are highly syntenic with chicken microchromosomes, although they exhibit little regional variation of GC contents and low repeat contents in contrast to the avian microchromosomes. They suggested that synteny of microchromosomes between *A. carolinensis* and chicken may imply that these animals might have arisen in the common reptile ancestor, while other remaining microchromosomes in the chicken are unique in the chicken lineage. The nucleotide composition of the genome in *A. carolinensis* is as homogenous as the frog genome and this homogeneity of the genome is a distinctive property of the lizard compared to avian

amniotes. In fact, Fujita *et al.* also showed that *A. carolinensis* have the most compositionally homogeneous genome of all amniotes with a homogeneity exceeding that for *Xenopus* (18). They also found that *A. carolinensis* genome contains reduced isochores in size and numbers compared to those of human or chicken. Since the vertebrate genomes are the mosaics of isochores and the major changes among the amniotes are associated with the appearance of GC-rich isochores in avian and mammalian genomes (19), further study of these structural and compositional differences between lizard and other amniotes may give an evolutionary hint of tissue regeneration. In addition to compositional homogeneity and reduced isochores, another feature of *A. carolinensis* genome is a high level of transposons. The transposons are the parts of DNA that may move themselves to new locations within the chromosomes of each cell, creating mutations by insertions, deletions, and translocations of the genes. By doing this, transposon can create or reverse mutation in the genomes of the organisms, and accumulated transposition event produces interspersed repeats within genomes, ultimately facilitating the development of new genes by blocking gene conversion (20). Therefore, the high level of transposons in lizards might give them the flexible genome that can efficiently respond to unexpected environmental changes.

Comparative analyses of the genetic elements in tail regeneration of lizards

Availability of *A. carolinensis* genome sequence will play an important role in understanding the evolution of mammalian genomes and the possible explanations for important branch of the evolutionary tree of vertebrates. However, the genome sequence itself may not give direct evidences for their unique traits such as adhesive setae, nocturnal vision and natural limb regeneration, and bioinformatic comparative analysis involving genomics, transcriptomics, proteomics, should be performed to investigate those mechanisms

in lizards.

Very recently, Liu *et al*. reported the full genome of *Gekko japonicus*, another species of lizards, and investigated their genetic elements related to their unique physiological processes including tissue regeneration (21). They obtained genome sequence of 2.55 Gb, and identified total of 22,487 genes from *G. japonicus*. Among the genes, they selected 155 genes for possible candidates as the regeneration factor by comparing the homologues among different species background, which are known to be involved in the biological processes necessary for tissue regeneration such as wound healing, cellular proliferation and migration. Although functional analysis of each gene should be performed, their positive selection for tail regeneration in *G. japnoicus* seems to be feasible since the selected genes include reliable examples previously proven to be involved in regeneration of various tissues in different species, such as prostacyclin synthase (PTGIS) and prostaglandin-endoperoxide synthase (PTGS1) (22, 23). These are the enzymes involved in prostaglandin biosynthesis, and recent report showed that inhibition of prostaglandin-degrading enzyme accelerates repair of various tissues such as bone marrow, colon, and liver, suggesting the key role of prostaglandin in tissue regeneration (24). Thus, their data will be of great value in studying tissue regenerating events, although further genomic sampling of other reptiles and amniotes should be clarified. Still, however, it may not be sufficient to reveal detailed regeneration mechanism just by categorizing the gene pools based on their genomic sequence itself. In order to discover the key regeneration factors, it should be required to identify the genes that are actually differentially expressed during regeneration period.

For such purpose, the first transcriptomic analysis of regenerating tails has been recently attempted by performing RNA-Seq with *A. carolinensis* (25). They obtained 326 genes that are expanded or reduced in RNA level associated with tail regeneration.

Bioinformatic analysis showed that the genes are categorized into regeneration-involved processes such as myogenesis, cellular adhesion, and immune response. As an example, the selected genes include the members of Wnt and MAPK/FGF pathway, which are the key molecules in tissue regeneration of a variety of organisms (26-30), giving clue on feasibility of their data. In addition to the well-known regeneration factors, they also obtained 22 novel genes that are differentially expressed during regeneration. Together with the well-known genes, characterization of these novel genes may provide valuable data sets for studying regenerative processes and their signaling pathways.

In addition to above genomic and transcriptomic approach, proteomic analysis has also been made to study the mechanisms in lizard's tail regeneration. The first gene identified as a dedifferentiation factor from the proteomic study in lizard is a lactoferrin, reported by Bae *et al.* in 2014 (31). Many case of tissue regeneration in lower animals involves cellular dedifferentiation to form a blastema, followed by redifferentiation and subsequent regenerative proliferation (32). In fact, the first step in tissue regeneration is formation of a mound with specialized wound epithelium over the site of amputation (32, 33). This tissue undergoes dedifferentiation to make blastema at the injury site and subsequently re-enter the cell cycle, thus reconstructing complex structures of the original tissues through the cellular proliferation and specialized differentiation.

According to the previous report, tail regeneration of the lizard (*Eublepharis macularius*) are divided into wound healing phase, dedifferentiation phase and redevelopment stage (32). For identification of dedifferentiation factor, Bae *et al*. isolated the proteins from the collected tail-tip tissues at each stage, and analyzed the differential expressions of the protein by 2D-electrophoresis. Among total of 292 proteins of which expressions are differentially expressed during tail regeneration, they initially selected 18

proteins, which indicated increased expression during the dedifferentiation period and decreased expression in redifferentiation phase. Protein homology analysis showed that these proteins are functionally categorized into cellular metabolism, protease inhibitor, cytoskeleton, and cellular differentiation. Among them, they identified lactoferrin as a dedifferentiation factor based on its high frequency and expression pattern. Functional studies with lactoferrin showed that it was specifically expressed in embryonic stem cells and induced pluripotent stem cell (iPSC) of both mouse and human, and the addition of lactoferrin promoted the efficiency of iPSC generation. Furthermore, it was found that lactoferrin induces the expression of *Klf4*, a well-known factor for iPS generation (Figure 1). Thus, it seems that lactoferrin promotes the dedifferentiation by induction of endogenous *Klf 4* expression. Lactoferrin is a multifunctional protein involved in various processes associated with wound healing including cellular proliferation, migration, and survival (34-36). Interestingly, lactoferrin has been known as a sole natural tryptase inhibitor in humans. Tryptase is a trypsin-like serine protease secreted exclusively from the secretory granules of activated mast cells (37). The mast cells have been implicated in regenerative wound healing processes (38), and their proteases and inhibitors have been shown to contribute to the coordinated healing of cutaneous wounds (39). In fact, lactoferrin has been reported to be involved in regeneration of various tissues such as bone, cornea and skin (40-43). Thus, it will be of interesting to study the role of lactoferrin in dedifferentiation as a tryptase inhibitor in mammals.

Discussion and Perspectives

It is likely that all the animals have the capability of regenerating their damaged body parts, although the degree of regeneration capacity seems to be different among the groups.

Regeneration is more vigourous in the invertebrates than it is in the vertebrates. Indeed, many invertebrates, such as hydra, planarians, and starfishes, have the bidirectional regeneration capcity, so that they can generate two sets of the same animals by regenerating their missing parts, while regeneration processes in the vertebrates occur unidirectionally, by which the animal reproduces only damaged parts at the site of injury (Figure 2). In vertebrates, fishes and amphibians have the greatest regenerative capacities, and the aminoites such as reptiles, birds, and humans, seem to lose the regeneration capability, although many lizards can reproduce their tails. In the lower vertebrates, natural regeneration occurs mainly by virtue of their intrinsic plasticity of mature tissues, which involves cellular proliferation, migration of remaining parts, and redevelopment of the damaged or missing parts. Among them, the most prominent event in tissue regeneration in the lower vertebrate may be formation of a blastema. The blastema shares many characteristics with stem cells, and can eventually redevelop into various tissues including muscle, skin, bone, and blood vessels, that were originally present in the damaged site. The blastema is formed through dedifferentiation process, and this step is omitted in the higher vertebrates such as birds and mammals. Thus, it could be possible that the lack of regeneration capability in the birds and mammals might be evolutionarily related to loss of the dedifferentiation capacity. In fact, mammals share many key factors for regeneration with the lower animals, such as fibroblast growth factor (FGF), Wnt/beta-catenin and bone morphogenic protein (BMP)/Msx signaling, which are known to be involved in wound healing and cellular proliferation (26-30, 44). By these processes, mammals can repair their damaged tissues to some extent. Nevertheless, the mammals have little regeneration capability compared to the lower animals, probably due to the lack of dedifferentiation capability. Damages at human organs such as heart, brain, and liver, often lead to serious pathological conditions. Although stem cell-based transplantation would be

clinically performed, still additional strategies may be required for proper treatment of organ injuries in humans. Thus, study of the mechanisms in blastema formation and the development of protocols for mammalian dedifferentiation will be a breakthrough for regenerative medicines and stem cell biology. As a matter of fact, mammalian cells have been to known to undergo dedifferentiation *in vitro* by enforced expression of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* (45, 46). Although this iPSC strategy is an innovative tool in human tissue regeneration and stem cell therapeutics, it still has apparent problems including low efficiency and uncertain safety (47). For example, use of oncogenes such as *Klf4* and *c-Myc* in iPSC generation raised concerns about the safety of iPSCs for practical applications. Although other substitutes such as *Nanog* and *Lin28* have been suggested (48), these oncogenes may be regarded as indispensable to the efficiency of dedifferentiation (49, 50). However, recent discovery by proteomic approach showed that lactoferrin can substitute *Klf4*, even with higher efficiency for dedifferentiation of human fibroblast. Although lactoferrin by itself was not enough to replace all the oncogenes for dedifferentiation of human cells, and thus further identification of other factors should be performed, this finding might provide a clue that comparative studies of lizard would be a promising strategy to reveal regeneration mechanism.

Now, we have a big picture of lizard genome in hand. Elucidation of the differences and similarities between genomes of regenerative and non-regenerative models will facilitate understanding of regenerative biology, and bring a new perspective to development of regenerative medicine, and clinical strategies for the treatment of injured human organs. In addition, the data from these approaches may contribute to various fields including cosmetic industry and anti-cancer drug discovery (Figure).

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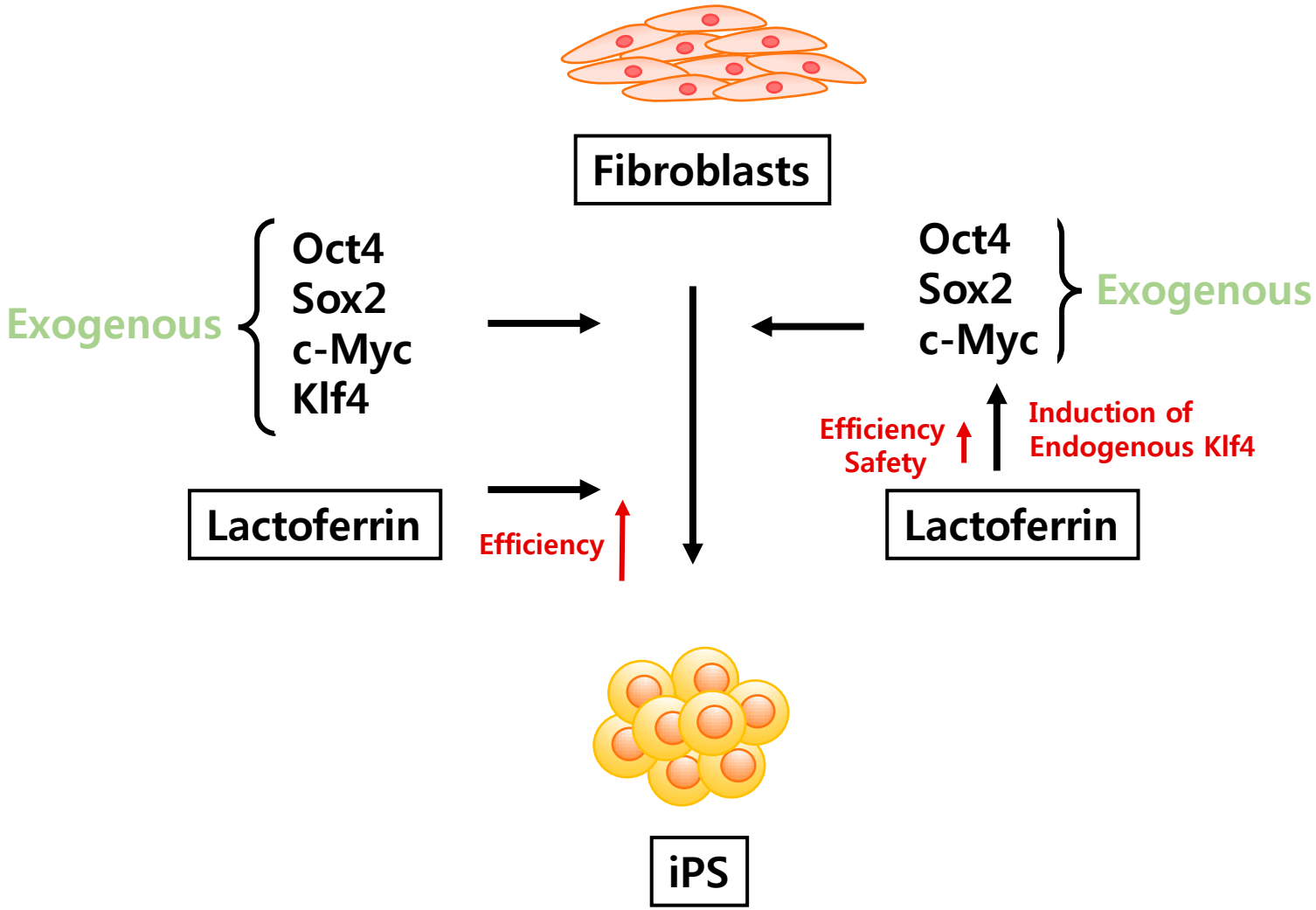
Figure legend

Figure 1. Functional effects of LF on iPSC generation. Addition of LF to the culture medium during in vitro dedifferentiation increase the efficiency of iPSC generation compared with conventional method in which only four genes (*Oct4, Sox2, Klf4,* and *c-Myc*) are introduced. Latoferrin can increase the efficiency of iPS generation even without forced expression of *Klf4* by induction of endogenous expression of *Klf4*.

Figure 2. Natural regenerations in the lower animals. Regenerative invertebrates, such as hydra, planarians, and starfishes, have the bidirectional regeneration capcity, so that they can generate two sets of the same animals from both side of damaged site. Regeneration processes in the vertebrates such as frog, zebrafish, and salamander, occur unidirectionally, by which the animal reproduces only damaged parts at the site of injury.

Figure 3. Applications of lizard biology. Comparative analysis of lizard genome may provide new insights into the evolutionary strategies of the lower animals in the tissue regeneration, and may contribute as a valuable source to the development of various fields including iPSC technology, medicinal biology and industrial biology.

Figure 1


Fibroblasts
Exogenous
Oct4
Sox2
c-Myc
Klf4
Lactoferrin
Efficiency
Oct4
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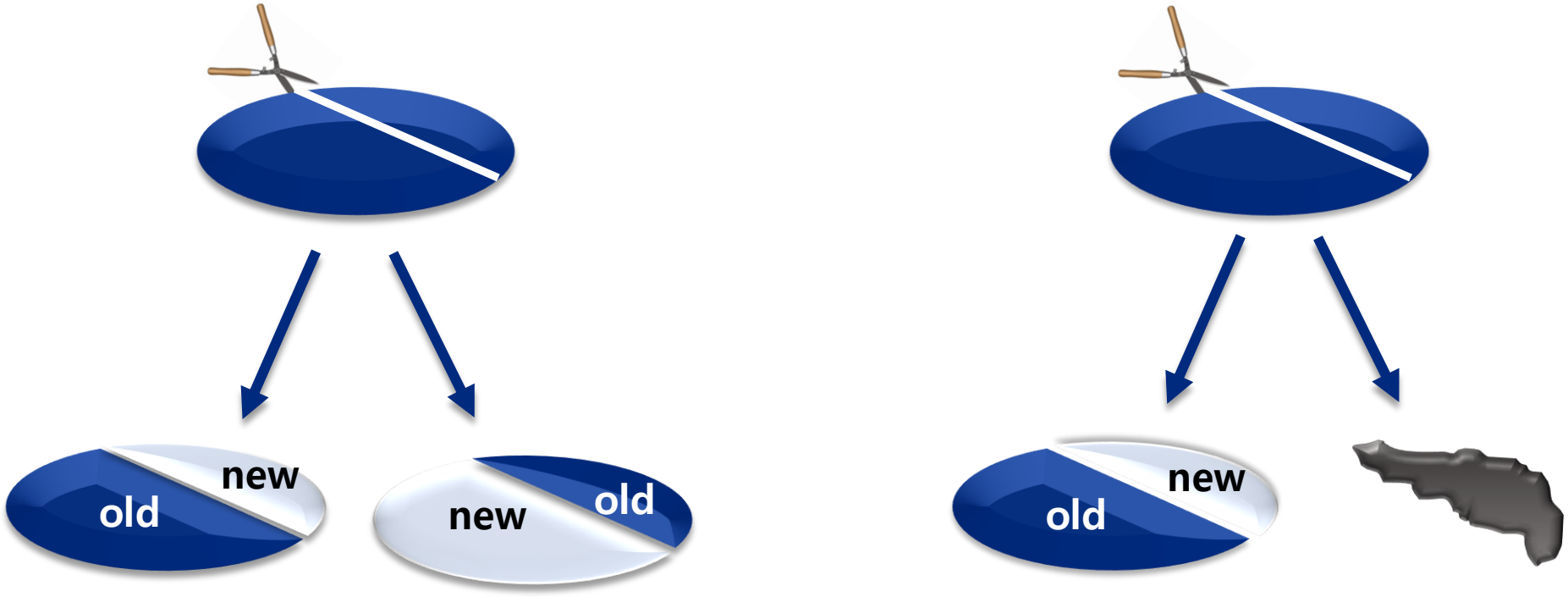
Exogenous
Efficiency
Safety
Induction of
Endogenous Klf4
Lactoferrin
iPS

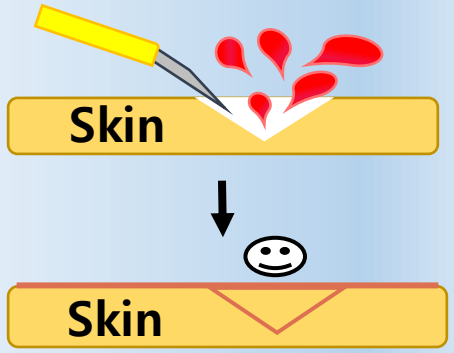
Figure 2



[Bidirectional regeneration]

[Unidirectional regeneration]

Figure 3



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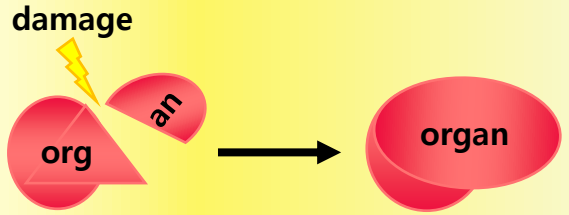
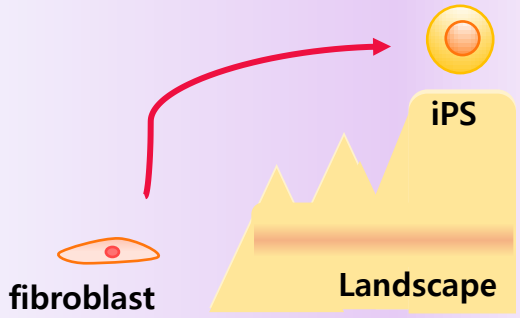
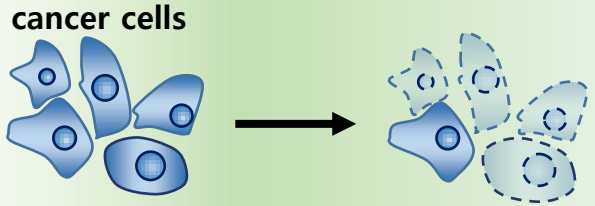


Tissue Regeneration



Dedifferentiation



Anti-cancer
Drug Discovery