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Development of hybrid-type artificial immune organ by means of experimental evolutionary research method using bioceramics

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The phylogenetic transition of branchiae into the lung as well as the migration of hemopoietic activity from the spleen to the bone marrow cavity demonstrate the concurrent evolutionary changes of organ structures and functions in vertebrates in response to environmental changes, such as the effect of gravity during terrestrialization from a water environment [1,2].

The present study introduces a hybrid-type artificial bone marrow chamber that uses sintered hydroxyapatite (HAP) and tricalcium phosphate (TCP) which is able to induce hemopoietic nest formation in conjunction with osteogenesis heterotopically in mammalian muscle, though not subcutaneous tissue [3–9]. This process is believed to proceed in biochambers by heterotopical hemopoiesis that are induced by the streaming potential of sintered HAP or TCP, which trigger genetic expression of mesenchymal cells to drive cytocaine of the bone morphogenetic protein (BMP). In order to investigate this hypothesis, the following preliminary experiments were carried out:

1. Transplantation of an HAP biochamber with BMP into the subcutis [10].
2. Transplantation of an HAP biochamber with a 10 μA current.
3. Measuring the streaming potential of sintered HAP using a physiological saline solution [10].
4. Transplantation of a titanium (Ti) mesh with a 10 μA current into the subcutis [10].

The preliminary results demonstrated that biomechanical stimuli were converted into streaming of the organic body fluid, ultimately resulting in the induction of a streaming potential [10]. Therefore, the author concluded that heterotopical hemopoiesis in the biochamber is induced by genetic expression of the mesenchymal cells. Based on this assumption, Ti artificial biochambers with a 10 μA current were developed. To further investigate this theory from a biomechanical perspective, a trilateral research method that integrates methodologies in morphology (Goethe), molecular biology (Delbrück) and physiology (Bernard) was developed. Based on this research method, the mechanisms associated with mor-

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phogenesis and function appear to originate from genetic expression, which are driven by biomechanical stimuli such as environmental factors.

An experimental evolutionary study, in which the trilateral research method is employed, was first proposed by the present author in 1995 [5,6]. In this study, biomechanical stimuli were applied to biomaterials in sintered hydroxyapatite chambers that have been implanted into various animal muscles during different phylogenetic stages. A hybrid-type artificial immune organ that induces hemopoietic bone marrow tissue was developed successfully not only in mammals, but also in chondrichthyes (sharks), which are relicts of the archetype that do not possess bone marrow tissue in their internal skeletons [11]. Using this method, an in vivo culture of an autogenous liver placed in a hydroxyapatite chamber which is connected to the femoral artery could also be established successfully in dogs [11].

The use and disuse theory proposed by Lamarck has been revived as a result of recent molecular genetic studies. Alberch reported that the recapitulation theory (Haeckel) could be explained by the heterochrony of genetic expression [12]. The use and disuse theory can also be explained by heterochrony, because all the functions of muscle cells and osteocytes are controlled through gene expression of mesenchymal and neural cells, which are triggered by physicochemical stimuli (also considered to be biomechanical stimuli) that affect the topical cells of the organism [10]. Based on this trilateral research, evolutionary changes in vertebrate morphology can be understood as a series of biomechanical events, as described by Lamarck [10]. Thus, evolutionary changes can be induced at the cellular level in the mesenchyma of heterospecies through biomechanical stimuli. This approach is referred to as an experimental evolutionary study [10].

The substance that defines the vertebrate structure is the hydroxyapatite-collagen complex skeleton that forms bone and teeth [5,6]. Vertebrates can be further distinguished by the presence of hemopoiesis in the bone marrow cavity of higher reptiles and mammals. This is not seen in lower vertebrates such as chondrocytes and cyclostomata. Structural differences also allow mammals to be distinguished from lower vertebrates. These consist of a highly evolved viscerocranium with gomphotic teeth and jawbones [5,6]. Combining the understanding of the importance of HAP in vertebrates with the effects of biomechanical stimuli, an experimental evolutionary research model can be developed at the cellular level [10]. Using such a model, a new technique in tissue engineering called the hybrid-type artificial immune organ has been developed [9]. If evolution is the product of biomechanical stimuli as proposed by Lamarck, heterospecific and heterotopical evolutionary changes should be possible to induce at the cellular level in the mesenchyma [9]. This paper investigates the correlation between the immunity system and evolutionary changes. Furthermore, this paper presents a new concept of immunology that involves a cytological digestion system [11], a new theory of biomechanics-responsive evolution [10] and a simple theory to explain biological reactions.

Materials and Methods

The following experimental evolutionary studies were carried out using artificial skeletal organs:

1) development of several kinds of artificial biochambers using:
   – artificial bone marrow chambers made of conventionally sintered HAP by Asahi Optical Co.,
   – artificial bone marrow chambers made of high-pressure sintering collagen-HAP composite [13] (by the National Institute for Research in Inorganic Material; collagen was extracted from cow skin),
   – artificial bone marrow chambers made of Ti mesh with a 10 μA current by the National Institute for Research in Inorganic Material [10], and
   – arterial biochamber for autogenous hepatic tissue culture,
2) implantation of the artificial bone marrow chambers into cyclostomata (hog-fish), chondrichthyes (dochishark), amphibian (xenops), aves (chicken) and mammals (dogs and Japanese monkeys),
3) based on the results of experiment 2, xenotransplantations between cyclostomata, chondrichthyes, amphibian, and mammals were carried out [14], and
4) transplantation of Ti biochamber with a 10 μA current into muscles of sharks and dogs.

Results

Artificial induction of hemopoiesis was carried out successfully in each of the newly developed chambers for all of the species. Figure 1 shows the leukocyte and lymphocyte induction around the Ti biochamber with a 10 μA current in a dog 4 months after implantation. Leukocyte production induced from undifferentiated mesenchymal cells by the electrical current around the Ti was observed.

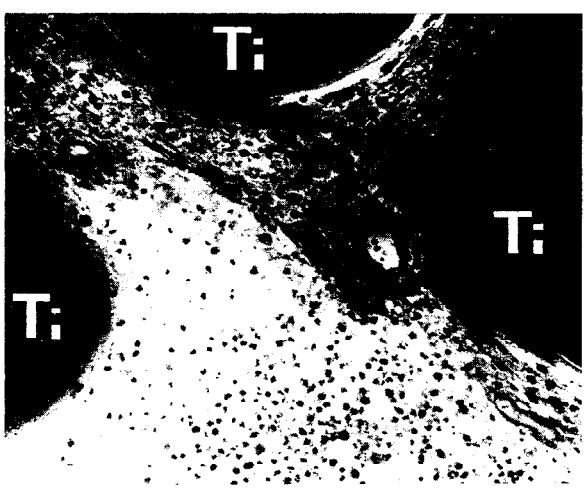

Fig. 1. Lymphocyte and leukocyte induction around Ti biochamber with 10 μA current. Ti: titanium.

Fig. 2. Hemopoiesis with osteogenesis in HAP biochamber 12 months after implantation in the muscles of Japanese monkey. HA: hydroxyapatite; HEM: hematopoiesis; OS: osseous tissue.

Figure 2 shows induction 12 months after surgery of hemopoiesis in conjunction with osteogenesis by the HAP biochamber implanted in a Japanese monkey. Osteogenesis as well as hemopoiesis could be observed around the chamber. Figure 3 shows the autogenous liver tissue 2 months after surgery, cultured in the HAP biochamber connected to the femoral artery of a dog. However, in dog

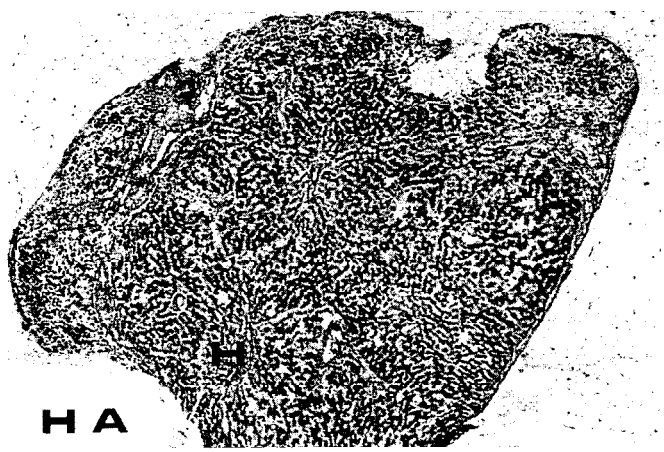

Fig. 3. Autogenous hepatic tissue cultured 2 months postoperation in HAP biochamber connected to dog femoral artery. HA: hydroxyapatite biochamber; H: hepatic tissue.

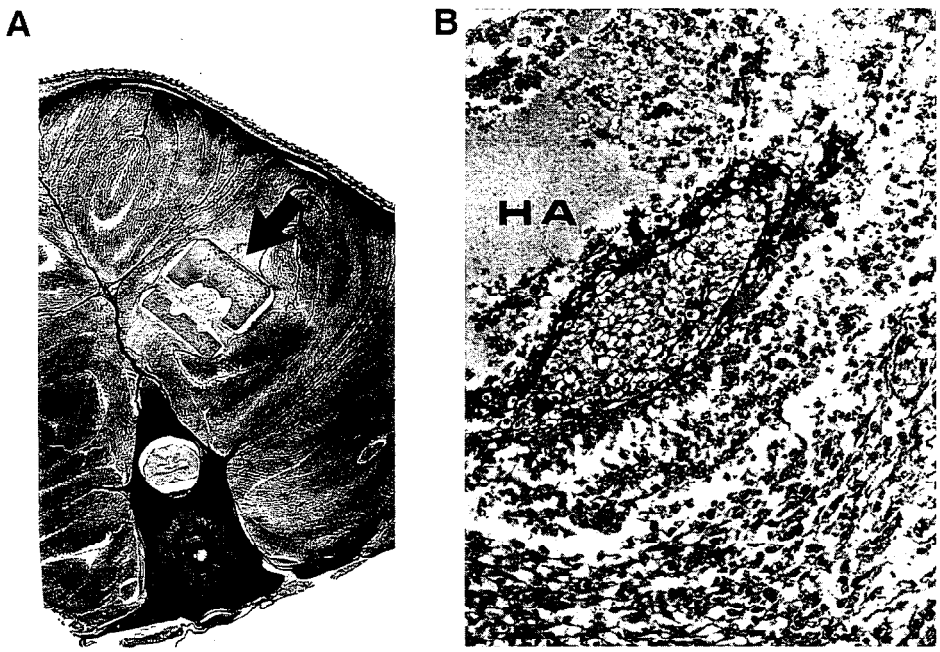

Fig. 4. **A:** Biochamber (arrow) of newly formed high pressure sintering composite collagen (cow skin)-HAP implanted in shark muscle. **B:** Osteoid formation with hemopoiesis is observed around biochamber.

muscles around the collagen-HAP biochambers, implanted marked tissue differentiation that resembled digestive tract formation could be observed. Figure 4A shows the collagen-hydroxyapatite artificial bone marrow chamber (arrow) implanted in shark muscle. Figure 4B shows hemopoiesis and osteoid formation 4 months after surgery around the hydroxyapatite implanted in the shark muscle. Figure 5A shows a cross-section of a shark (dochi). No bone marrow in the cartilaginous tissue around the spinal cord (arrow) is evident. Figure 5B shows a cross-section of a shark with a Ti biochamber with a 10 μA current. Figure 5C shows the histopathological findings associated with the dorsal cartilage with hemopoietic marrow induction (arrow) by the adjacent Ti biochamber. Reciprocal implantation of muscles between cyclostomata, chondrichthyes and xenops, mouse, and dogs was carried out successfully.

Discussion

Based on the phylogenetic transition of branchial smooth muscles into facial and mastication striated muscles, evolutionary changes in vertebrate organs are considered to reflect responses to environmental changes in biomechanical stimuli. Similarly, evolution of the branchiae into the lung and migration of hemopoietic activity from the spleen into the bone marrow cavity appear to coincide with changes in respiration from water to air and response to gravity associated with terrestrialization from a water environment (Fig. 6).

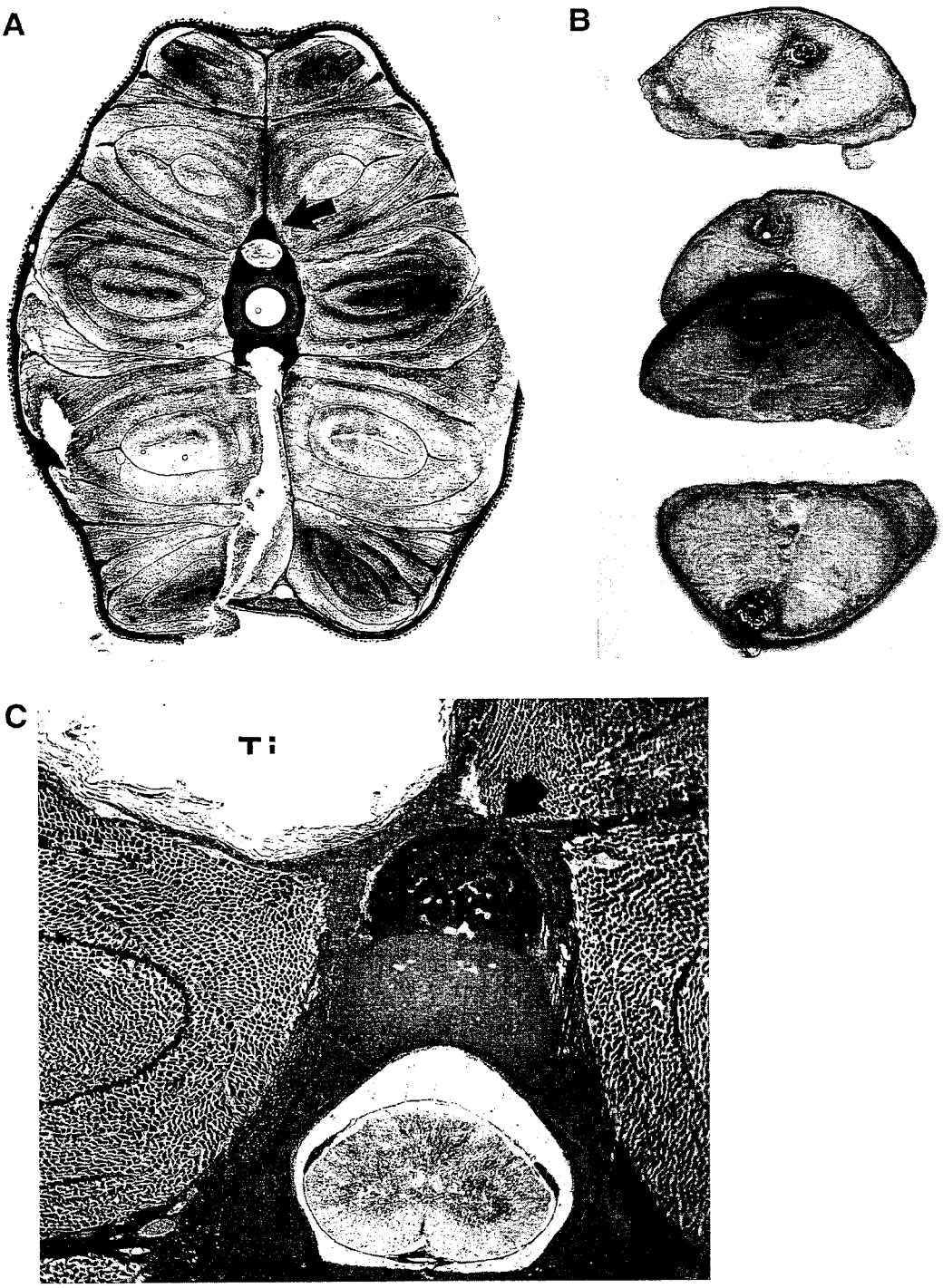

Fig. 5. **A:** Cross section of shark (dochi) for control, no hemopoietic marrow is seen in cartilage (arrow). **B:** Cross section of shark implanted with biochamber of Ti with current. **C:** Hematopoietic nest (arrow) induced by biochamber resembling bone marrow in shark vertebrate cartilage. Ti: biochamber of Ti with current.

The present series of studies were conducted based on the scientific categorization of vertebrates by Linne, the evolutionary theories of Lamarck, the morpho-

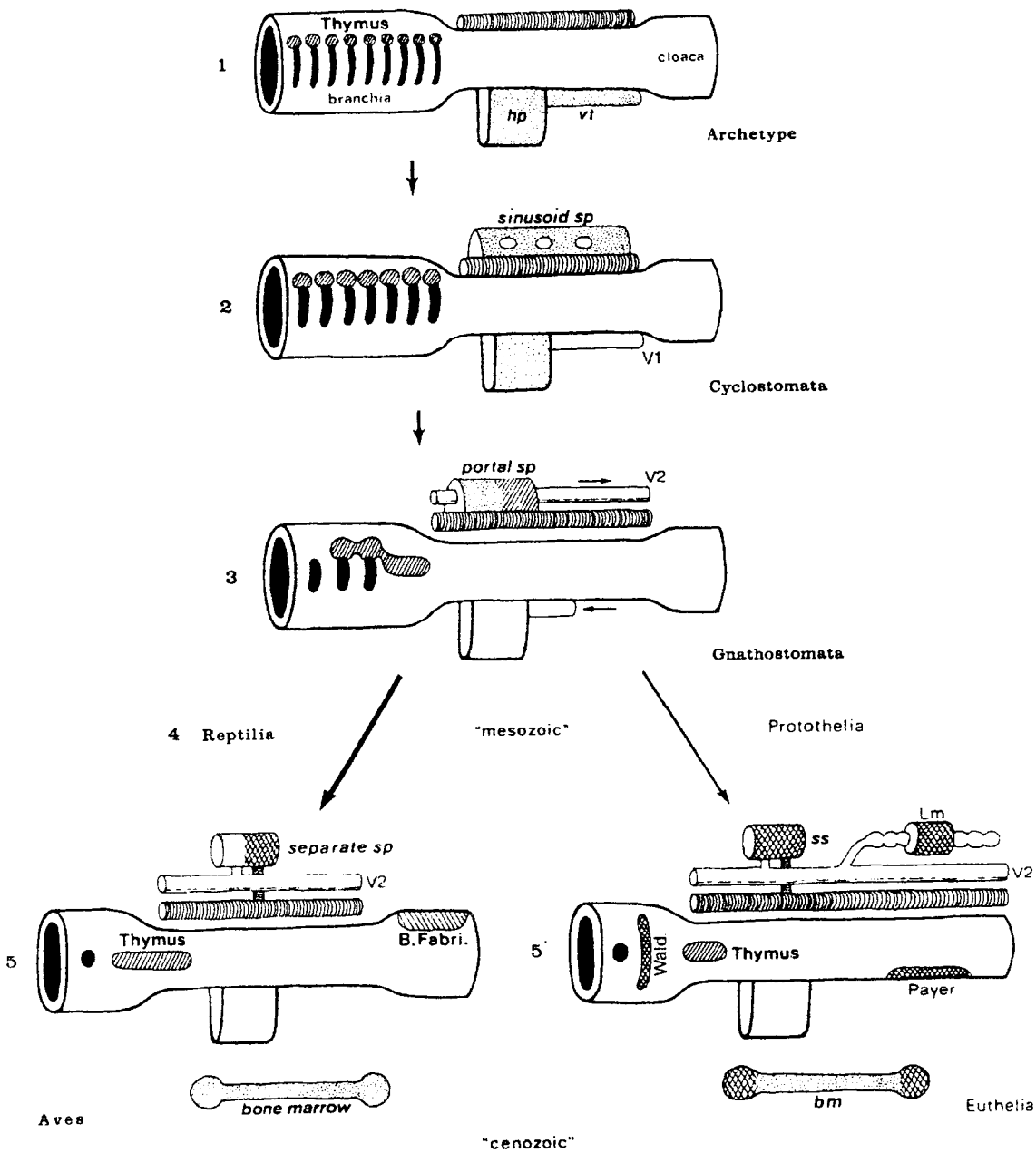

Fig. 6. Schematic diagram of evolution in vertebrate hemopoietic organs (from [17]).

logical studies by Goethe, the biogenetic law proposed by Haeckel, the biomechanical principles outlined by W. Roux [15], the morphology of organisms presented by S. Miki [16,17] and the molecular genetics developed by Delbrück [18]. The application of bioceramics to tissue engineering for therapeutic use was based on studies of phylogenetic transformation of osseous skeletal tissue in conjunction with hemopoiesis, i.e., the evolution of hematopoiesis in bone marrow. In the present study, morphology, physiology and biochemistry, and molecular genetics of remodeling were integrated with biomechanics to establish a trilat-

eral research method for studying life sciences using biomaterials [5]. This trilateral research approach was used to perform an experimental evolutionary study of the mechanisms involved in evolutionary changes in vertebrates by applying bioceramics to archetype vertebrates that possess no inner HAP skeleton.

Conventional research has only studied the effects of biomaterials on the tissues surrounding the implanted artificial material. No marked histological changes appear to occur around the porous sintered HAP implanted into the subcutis. However, if the HAP is implanted into the muscle, marked heterotopical hemopoiesis in conjunction with osteogenesis occur around the HAP implant [19,20]. Thus, the response in subcutis appears to differ from that in muscle. Biomechanical stimuli tend to differ between the subcutis and muscle. Therefore, not only the material effects, but also the shape and functional effects, referred to hereafter as biomechanical stimuli, should be examined because the organism is a closed system in which internal pressure exists [21,22]. The stress distribution pattern around the implant is dependent on the shape of the implanted biomaterials. Cells with higher functions induced from undifferentiated mesenchyma are dependent on stress distribution patterns as well as the functional characteristics of the applied stress. In order to effectively use biomaterials in clinical applications, the material, shape and functional effects must be clearly understood [21,22]. Through a trilateral approach using biomaterials, tissue engineering of hybrid-type artificial immune organs can be developed. Furthermore, by introducing gravity as well as low-current energy, a simple theory to explain biology can also be established. New immunological concepts that consider cytological respiration, metabolism and digestion in conjunction with cell differentiation can also be introduced [11].

Based on the successful induction of hemopoiesis and osteoid formation by a collagen-HAP biochamber of cow skin, chondrichthyes and cyclostomata do not appear to demonstrate any major histocompatibility antigen. Autogenous hepatic tissue culture was carried out successfully using a HAP biochamber that was connected to the femoral artery of a dog (Fig. 3). All epithelial organs derived from the endoderm and mesoderm showed epithelial-mesenchymal interaction. Due to HAP being a product of the mesenchyma, the use of a HAP chamber for in vivo culture of an autogenous endodermal epithelial organ appears to be the most appropriate biochamber [11].

In 1965 T. McCulloch developed the colony forming a unit-spleen method during lethal-dose irradiation exposure experiments using mice, one of which he did not irradiate on the tail. That mouse did not die, despite exposure to a lethal dose of radiation over the whole body except the tail [23]. The mouse appeared to recover due to proliferation of stem cells that migrated from the tail bone marrow. Thomas successfully applied the same method to human bone marrow transplantation in order to treat hematopoietic disorders [24], for which he was awarded the 1990 Nobel prize in medicine and physiology.

Based on the colony forming unit-spleen method as well as research on bone marrow cells [25–28] and phylogenetic research on evolution of the hemopoietic

organ [16,17,29] (Fig. 6), the concept of a hybrid-type artificial bone marrow chamber was developed [30,31]. Because all mesenchymal cells that comprise the body of vertebrates have common genetic codes, undifferentiated mesenchymal cells can differentiate into various types of cells that comprise the skeleton, i.e., osseous and cartilaginous tissue, muscle, blood, reticuloendothelial, lipid, tendon, endothelial cells, and fascia.

The present results suggest that the trigger for hemopoiesis induction in conjunction with osteogenesis is a streaming potential [31—34] which is converted from fluid flow that occurs due to repeated optimal loading. Cellular functions can be categorized as follows: 1) metabolism, 2) skeletal construction, 3) muscle activity, 4) planocytes and phagocytes for cytological respiration and digestion, 5) signal transmission of neural cells, 6) absorption of nutrients and oxygen, 7) excretion of catabolites, and 8) support of metabolites for epithelial cells.
Almost all cells are capable of cell division and remodeling. Osseous cells function as a support against mechanical stress. They form the basic skeleton of the organism in response to biomechanical stimuli. Almost all cellular functions are ultimately carried out through genetic expression of each cell.

Morphology, metabolism and remodeling are all products of cellular function. These cellular functions are also products of genetic expression of cells. Through trilateral research that integrates morphology, metabolism and molecular genetics of remodeling with responses to biomechanical stimuli, these three categories can be studied as the same molecular genetic phenomenon with three different features.

Thus, genetic expression appears to be under the control of the general biomechanical stimuli. The present results demonstrate that the migration of hemopoiesis from the spleen into the bone marrow cavity occurred as a result of gravity that became a factor during the terrestrialization period of evolution. The only difference between archetype vertebrates and higher vertebrates is blood pressure. Mammals have substantially higher blood pressure than sharks. By loading a mechanical stress of 1 g, blood pressure should dramatically increase in sharks that have very low blood pressure in water, with approximately 1/6 g.

During terrestrialization, archetype vertebrates (sharks) initially experienced difficulty in breathing due to the lack of respiration without water and writhed in response to the suffocation. As a result, blood pressure increased. During surgery, bleeding was not apparent in most areas of shark muscle. Thus, gravity appears to be the primary influence on the elevation of blood pressure during terrestrialization. This in turn induced a higher streaming potential [31] than that in the water environment. Therefore, hemopoietic nest migration appears to be a direct consequence of the introduction of gravity. Furthermore, these studies demonstrate that evolutionary changes in morphology result from biomechanical-physicochemical stimuli such as gravity and calcium ions. Figure 7A shows a schematic diagram of the neoteny of the ascidia. Figure 7B shows the artificially induced neoteny of the maboya (ascidia) in artificial sea water with a low density of calcium ions or with gadolinium ions instead of calcium [35]. The role of grav-

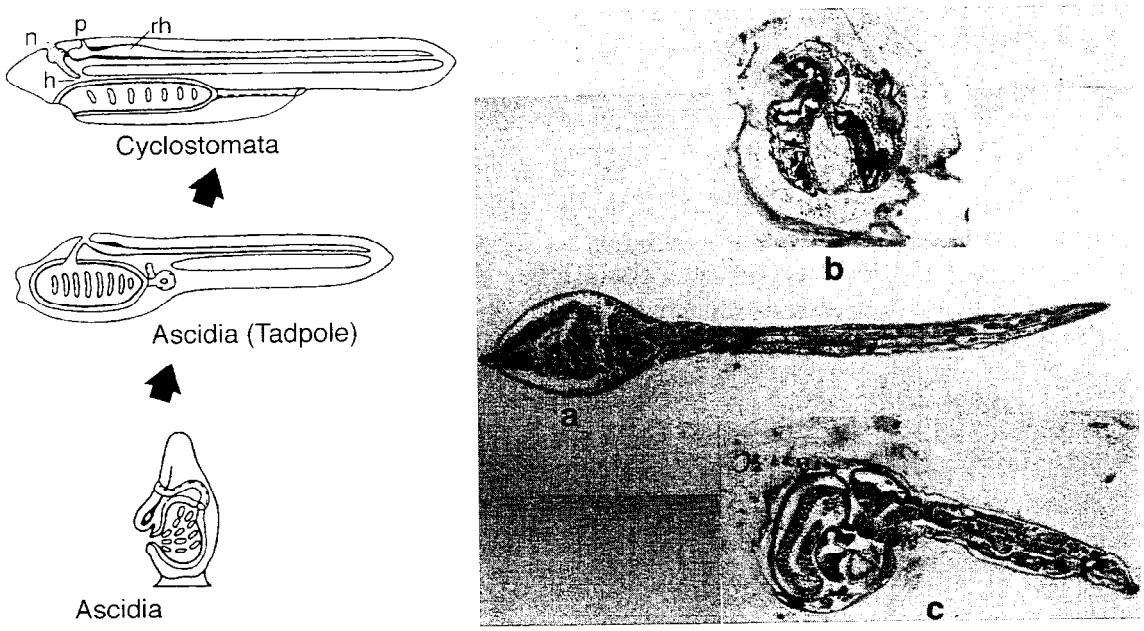

Fig. 7 **A:** Schematic diagram of neoteny of ascidia to vertebrate. **B:** Artificial induction of neoteny of maboya (ascidia). a. Larva (tadpole type); b. imago; c. imago with tail.

ity in the induction of major histocompatibility antigens is further demonstrated through experimental transplantation between sharks, hogfish and xenops, mice, rats and dogs [14]. Therefore, not only hemopoietic nest migration, but also major histocompatibility antigens appear to be induced by gravity through the genetic expression of mesenchymal cells [14].

Summary

The present experimental evolutionary study investigated the artificial induction of bone marrow hemopoiesis in heterospecies using chondrichthyes and cyclostomata, which have no osseous bone in their inner skeleton, and therefore represent the phylogenetical stage of archetype vertebrates. The major histocompatibility antigens appear to have differentiated concomitant with migration of hemopoiesis into the bone marrow during terrestrialization. Based on these results, this study presents a new concept of the immune system, a new theory of biomechanics-responsive evolution, as well as a simple theory to explain biological reactions.

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