Eunice Kennedy Shriver National Institute of Child Health and Human Development

Opportunities for Rapid Advancement of Limb Regeneration: From Animal Models to Humans February 17–18, 2021

Day 1: February 17, 2021

Welcome and Introduction

Mahua Mukhopadhyay, Ph.D., NICHD

Dr. Mukhopadhyay started the workshop at 10:00 a.m. and welcomed attendees. She said that the workshop was sponsored by NICHD to convene expert scientists and clinicians to help develop a road map and establish effective collaborations for the rapid advancement of limb regeneration research. The 2-day workshop would have two keynote talks and four scientific sessions and highlight basic science, translational science, and clinical regeneration research. Presentations and discussions would focus on the state of the science and on challenges and opportunities in the field. The workshop was recorded and will be made available to the public with closed captioning.

Introductory Remarks

Alison Cernich, Ph.D., Deputy Director, NICHD

Dr. Cernich said that NICHD is focused on leading research and training to understand human development, improve reproductive health, enhance the lives of children and adolescents, and optimize abilities for all. The institute recently updated its mission and strategy. In implementing its strategic plan, NICHD will focus on practical steps to advance its scientific research themes and objectives, scientific stewardship, management and accountability, and aspirational goals. Over the next 5 to 10 years, the institute will work toward the aspirational goal of advancing the ability to regenerate human limbs by using emerging technology to activate the body's own growth pathways and processes.

Each year, about 120,000 babies in the United States are born with limb reduction defects. Additionally, 1.6 million people are living with amputations of varying severity. The goal of the current workshop is for attendees to provide insights to help NICHD plan its road map to improve the quality of life of babies and children with limb differences and adults living with amputations. For example, regenerative techniques could help extend limbs or improve the fit of prostheses. NICHD will support *in vitro* and *in vivo* approaches to regeneration research and will encourage communication and collaboration among basic, translational, and clinical researchers. NICHD has a wide range of expertise from its Developmental Biology and

Structural Variation Branch and its National Center for Medical Rehabilitation Research. The institute will harness this expertise to gain momentum on limb loss and regeneration research and research to optimize post-amputation function. The workshop will help NICHD gather ideas for ambitious and innovative research and will initiate dialogues and spark collaborations across research domains. Additionally, the workshop's discussions will inform a future NICHD document on limb regeneration.

Keynote

Historical Perspective on Limb Regeneration Research

Ken Muneoka, Ph.D., Texas A&M University

Dr. Muneoka presented a summary of the history of limb regeneration research and current opportunities to use animal models to learn more about regeneration in humans. Salamanders have long been used to study regeneration, because they can regenerate complex limbs made up of multiple tissue types. After an amputation in a salamander, a blastema forms at the wound site through cell proliferation and migration. The blastema, which is critical for regeneration, is made up of mesenchymal cells with embryonic characteristics. These cells undergo developmental programs in an adult environment to reform a limb.

A key component of the regeneration response is the formation of a specialized wound epidermis, which is transformed into the apical epithelial cap. The apical epithelial cap drives the outgrowth of the blastema. Because precise limb regeneration in salamanders occurs after either proximal or distal amputations, the blastema appears to have positional information on what tissue was lost through amputation. This information helps organize the regeneration response.

Several past experiments have provided valuable information on regeneration in salamanders. Experiments by Marcus Singer, Ph.D., M.S., and his colleagues showed that removing the nerve supply to an amputated limb inhibits regeneration. Other work by Jeremy Brockes, Ph.D., and his colleagues showed that regeneration can be induced experimentally by redirecting a nerve to a lateral wound. Dr. Brockes and other researchers also showed that the regeneration response can be induced or rescued experimentally through the nAG-Prod1 pathway and other signaling pathways. Later studies showed that the Prod1 receptor evolved independently in urodeles, which have an extensive ability to regenerate. This suggests that regeneration is under selective pressure and has changed with time, and the various steps of the regenerative response might not be conserved across vertebrate orders.

Transplantation studies by Susan Bryant, Ph.D., and her colleagues provided evidence to support the presence of positional information in the regenerating limb. Other experiments showed that creating limbs without positional disparity inhibits regeneration. Also, Dr.

Muneoka and Dr. Bryant showed that there are significant similarities between patterning mechanisms in developing and regenerating limbs. Studies by Bruce Carlson, M.D., Ph.D., showed that shifting the skin around on a regenerating limb influences patterning, and Malcolm Maden, Ph.D., showed that retinoic acid can change positional information along the proximal–distal, anterior–posterior, and dorsal–ventral axes.

Other studies have shown the importance of fibroblasts within the dermis. These cells have been shown to be multipotent and to have positional information. Also, similar to the effect of redirecting a nerve, researchers can induce ectopic regeneration by grafting tissue to the site of a lateral wound. Nerve function can be replaced by activating the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signaling pathways. The effect of skin grafting can be reproduced by using retinoic acid to alter positional information. These results have helped develop a mechanism for regeneration involving an FGF8–sonic hedgehog (SHH) feedback loop. Nerve function during regeneration is likely to be responsible for transforming the wound epidermis into an apical ectodermal cap.

Mammalian models of regeneration are an important tool for improving our understanding of regenerative failure in humans. In mice, amputation of the tip of a digit leads to a regeneration response. In neonatal mice, a distal digit tip amputation regenerates well, but a proximal amputation regenerates poorly. Like salamander limb regeneration, mouse digit tip regeneration involves the formation of a structure that resembles a blastema and the differentiation of cells to form various tissue types. In adult mice, regeneration takes longer than in neonates and involves a long period of histolysis of the bone involving osteoclasts. Ultimately, cells from the blastema undergo differentiation to form the digit tip. Regeneration of bone in the digit involves direct ossification rather than ossification of cartilage as seen during development.

Several researchers are exploring the mammalian regeneration response, including the various cell types involved, pro and antiregenerative factors, and genetic programming. Many groups have focused on the differences between amputations through the P3 bone, which is the most distal phalanx and shows robust regeneration, and amputations through the P2 bone, which is more proximal and regenerates poorly. Work by Mayumi Ito, Ph.D., and colleagues has proposed that stem cells in the nail bed of amputated digits and the Wnt signaling pathway are important for regeneration in this model.

The Muneoka laboratory has found that in embryonic mice, digit regeneration is associated with the re-expression of the homeobox containing gene *Msx1.* Transplantation experiments have shown that embryos can regenerate more elaborate phalangeal elements and not just the digit tip. Regeneration is blocked in *Msx1* mutants, and the expression of *Msx2* and *Bmp4* is impaired. Regeneration in *Msx1* mutant mice can be rescued in a dose-dependent manner by treatment with BMP4. In neonate mice, inhibiting BMP signaling with the antagonist Noggin

blocks regeneration. Regeneration can be rescued by treatment with BMP7 and BMP2 but not BMP4. In experiments testing regeneration after proximal amputations, researchers have found that BMP signaling can induce regeneration of the P2 bone, and the regenerated bone is integrated within the stump. BMP-induced proliferation of chondrocytes creates an endochondral ossification center and leads to the building of new bone on existing bone. The polarity of endochondral ossification is reversed in the P3 bone, showing that cells of the amputation stump show positional polarity with respect to gene expression.

Researchers are also examining the genes that regulate regeneration downstream of BMP signaling. Results have shown that BMP signaling, through BMP2, induces the expression of *Sdf1.* Blastema cells, which express *Cxcr4,* migrate in response to *Sdf1* expression. BMP signaling therefore provides both migration and differentiation cues and might also provide positional information. The amputation site is most responsive to BMP signaling at particular times during the wound healing process, and this signaling pathway is sufficient to induce bone regrowth at a healed wound site.

Another BMP signaling molecule, BMP9, is normally expressed during digit joint development and is associated with the cavitation response. Treatment with BMP9 causes nonregenerative wound cells to regenerate a complex joint that includes cartilage, but using a mechanism that is different from regeneration induced by BMP2. Additionally, the expression of proteoglycan 4 (*Prg4*) is essential for cavitation and articular cartilage development during both embryonic development and regeneration. The combined effects of BMP2, BMP9, and PRG4 can initiate cavitation and cartilage condensation to direct regeneration ectopically.

To advance the field, researchers need to consider the regenerative potential of amputation wounds and the factors that inhibit regeneration. The formation of a blastema is an obvious important step in regeneration. However, studies from the Muneoka laboratory have shown that cell differentiation can be induced experimentally after wound healing, suggesting that it might be possible to bypass blastema formation. Researchers need to investigate the exact factors in a blastema, including progenitor cells, that induce cell differentiation.

Several clinical studies have reported that humans can regenerate distal parts of digits. This regenerative process is cosmetic and most often does not include bone regeneration. In a rare case where the nail bed and remnants of the periosteum remained after amputation, bone regeneration occurred. Although these regenerated digit tips are not aesthetically perfect, they are functional. In laboratory experiments, human fetal digits in culture showed a regenerative response similar to that observed in mice. This response also involved *msx1* expression. Nonhuman primates also show a cosmetic repair of amputated digit tips, and this response does not involve a blastema or bone regrowth. In goats, hoof tip amputation leads to the regeneration of the hoof wall without the formation of a blastema. These examples prove that amputation wounds in mammals are quite responsive to regeneration even without a

blastema. Researchers might be able to induce extensive cell differentiation to generate the various lost tissue types and structures without blastema formation. This work may lead to the development of effective methods to induce a stronger, clinically significant regenerative response in humans.

Discussion

- Larry Suva, Ph.D., asked how important nerves are during mouse regeneration. Dr. Muneoka said that the exact role of nerves in mouse regeneration is not well understood. He said that many of the studies to answer this question involve the sciatic nerve and amputation of a digit tip. Because sciatic nerve damage causes paralysis, it is difficult to determine whether regeneration defects in this model are due to nerve damage or loss of mechanical loading.
- Alberto Esquenazi, M.D., asked whether Dr. Muneoka expects a robust regeneration response in proximal amputations in mammals. Dr. Muneoka said that in adult amputations, BMP2 can cause patterned cell regrowth. This shows that regeneration in proximal amputations is possible, and it is important to find effective ways to deliver growth factors to wound sites.
- Ashley Seifert, Ph.D., asked whether mammalian embryos can regenerate a digit after a P2 amputation. Dr. Muneoka said that this is possible in embryonic mice.
- Catherine McCusker, Ph.D., asked whether other cells apart from connective tissue cells respond to BMP signaling during digit tip regeneration. Dr. Muneoka said that this is unclear.
- Ralph Nitkin, Ph.D., asked whether there are endogenous systems that attempt to correct limb patterning in cases where supernumerary structures are induced experimentally. Dr. Muneoka said that such systems have not been identified.
- A participant asked whether the epiphyseal center of the bone plays a role in bone regeneration. Dr. Muneoka said that this is unknown.
- David Butler, Ph.D., asked whether there were genetic similarities between the formation of extra digits and the formation of nonfunctional digits. Dr. Muneoka said that both cases likely result from events during development and not as a result of injury and repair.

Session 1: Limb Regeneration Response and Blastema in Lower Vertebrates

Overview of Limb Regeneration in Lower Vertebrate Systems and the Role of Blastema

Jessica Whited, Ph.D., Harvard University

Dr. Whited gave a presentation on the role of the blastema in limb regeneration. She said that historically, salamanders have contributed significantly to regeneration research because, although their internal limb structures are similar to other vertebrates, they maintain the ability to regenerate whole limbs without scarring throughout their lifetime. The blastema, which is essential for limb regeneration in salamanders, has information on how much tissue has been lost and needs to be replaced. In contrast, although tadpoles have some ability to regenerate limb tissue, adult frogs have very limited regenerative capabilities.

After limb amputation, salamanders form a wound epidermis under which the blastema forms. The formation of a blastema distinguishes regeneration in salamanders from regeneration in other animals. Research in the Whited laboratory is geared toward understanding blastema formation and how the blastema acquires all the necessary instructions for successful regeneration. Past experiments have shown that transplanting a blastema leads to the formation of an ectopic limb at the transplantation site. Experiments have also shown that the blastema has proximal and distal information, and a nerve supply is thought to stimulate cell proliferation and, along with the wound epidermis and macrophages, is essential for regeneration.

Some progress has been made in understanding the factors needed to form a blastema and promote regeneration. Upon injury, some cells are cued to re-enter the cell cycle, and this cell activation process might be occurring throughout the organism. Some of the activated cells are cued to transition into blastema cells. These cells migrate to the site of injury and group together, and the cells likely communicate with one another. It is possible that, depending on the type of salamander, these cells might originate from different parts of the body. Also, the cells might undergo de- and redifferentiation or stem cell activation.

Recent improvements in molecular biology and genetic tools have led to advances in determining the genes and cell types involved in regeneration. Several candidate factors have been identified and tested across model systems. Such experiments have confirmed the importance of SHH signaling and *Hox* genes in regeneration. Using RNA-Seq technology to analyze gene expression, the Whited laboratory has identified candidate genes that might regulate blastema formation. This technique has helped identify several cell types, including fibroblasts, in the blastema. Fluorescent labeling experiments have provided insights into the origins of these various cell types. Results have shown that there is some plasticity in dermal

cells that allows this cell lineage to contribute to other tissue types, such as tendons and cartilage, during regeneration. Muscle cells, however, produce only other muscle cells during regeneration. The new tools available will help researchers make significant progress toward understanding the genes and gene regulatory networks that control blastema formation and limb regeneration.

How Are Tissue Patterning and Tissue Scale Coordinated During Limb Regeneration?

Deneen Wellik, Ph.D., University of Wisconsin

Dr. Wellik gave a presentation on the role of *Hox* genes in patterning during limb regeneration. She said that *Hox* genes are known to influence skeletal patterning during embryonic development in mice. This has been shown by both the loss- and gain-of-function experiments. In mice, *Hox9* and *Hox10* are involved in patterning the upper limb, and *Hox11* is involved in patterning the lower limb. *Hox13* is involved in establishing the pattern of the paws. The Wellik laboratory focuses on the function of *Hox11,* which is expressed in stromal cells in the developing limb bud, but not in cartilage or osteoblasts. *Hox11* expression is maintained in an undefined stromal population in the developing lower limbs of adult animals. Further studies showed that *Hox11* is expressed in progenitor-enriched mesenchymal stem cells. Experiments using inducible fluorescent reporter constructs showed that upon induction, the *Hox11* construct extensively labeled cells of all parts of the skeleton, including osteoblasts, cartilage, and bone marrow fat. This expression remains localized and stable throughout the animal's life.

When the mouse skeleton is injured, there is a significant expansion of the population of *Hox11*-expressing skeletal stem cells. Using fluorescent-activated cell sorting, the Wellik laboratory has shown that these cells are most likely progenitor-enriched mesenchymal stem cells. The *Hox11*-expressing cells give rise to both cartilage and bone in the repairing skeleton. The *Hox11*-expressing cells therefore maintain extensive stem and progenitor activity and are able to repair the bone.

In vertebrates that are capable of regeneration, *Hox* gene expression patterns are similar to patterns in the mouse limb bud. Future work to determine the roles of embryonic patterning genes such as *Hox* genes during regeneration would help researchers better understand regeneration in mammals. It would be helpful to identify how much expansion of *Hox*-expressing cells occurs during regeneration, as well as whether stem cells with open *Hox* loci can be transplanted to more proximal positions on an amputated limb so that these cells recapitulate the expression of posterior *Hox* genes. This change in gene expression might be essential for regeneration.

Discussion

- James Monaghan, Ph.D., asked whether inducible *Hoxa11* cell ablation in adulthood stops fracture healing. Dr. Wellik said that experiments to address this are ongoing, and early results show that this impedes, but does not completely eliminate, fracture repair.
- Caroline Dealy, Ph.D., asked whether *Hoxa11* expansion is accompanied by cell proliferation. Dr. Wellik said that cells expressing *Hoxa11* are highly proliferative during the early repair response.
- Dr. Suva asked whether removing the periosteum changes the repair process and related gene expression profiles. Dr. Wellik said that it is likely that periosteal cells contribute significantly to regenerating bone.
- Joshua Currie, Ph.D., asked whether mouse cells from different digit segments might have self-associative properties. Dr. Wellik said that this is possible because cultured limb bud cells expressing a *Hoxa13-gfp* construct form distinct colonies.

How Are Genetic Programs Coordinated Throughout Limb Regeneration?

Kenneth Poss, Ph.D., Duke University

Dr. Poss gave a presentation on the genetic regulation of regeneration in zebrafish. He said that because they can regenerate their heart, spinal cord, fins, and retina, zebrafish can provide valuable insights into regeneration in other vertebrates. Fins are different from limbs because fins are made up of several segments of bone called limb rays. However, fin regeneration can provide insights into the fundamental principles of regeneration, such as the formation of a wound epidermis and a blastema, dedifferentiation, and positional information. Researchers have not identified the full complement of genes that control fin regeneration. However, evidence suggests that fin regeneration is controlled not by novel genes in the zebrafish but by novel methods of regulating genes that are common to vertebrates.

Experiments have shown changes in the expression levels of hundreds of genes during fin regeneration. The spatial and temporal dynamics of gene expression are impressive and precise. It is likely that specialized enhancer elements and promoters play an important role in creating these precise gene expression patterns. Recent studies have shown that some genes in an uninjured fin or limb are inactive and that the relevant section of chromatin is closed. Upon injury and regeneration, the chromatin opens up and activation and transcription occur, after which that area of chromatin closes up again. This concept has been shown to be true for the regulation of expression of leptin b*,* a gene that is expressed at the tip of the regenerating fin. Using a fluorescent reporter construct, the Poss laboratory has shown that a 7-kilobase region of regulatory sequence upstream of this gene is necessary to recapitulate the endogenous expression pattern of leptin b as seen in a regenerating fin. Such regulatory sequences are called tissue regeneration enhancer elements (TREEs) and can be identified by routine chromatin profiling comparing uninjured and regenerating tissue.

TREEs have also been identified in regenerating tissues in several model systems. Their presence has helped identify key transcription factors involved in regeneration and can help clarify the evolution of regeneration. The Poss laboratory has shown that TREEs can be used to recapitulate gene expression and rescue fin regeneration. Although not highly conserved across species, the sequences of these enhancers are often recognized and can direct gene expression across different species. TREEs could therefore be employed to boost regeneration in species that lack a robust regeneration response after amputation.

Discussion

- Luis Garza, M.D., Ph.D., asked about the possible identities of proteins and signaling pathways that interact with TREEs. Dr. Poss said that activator protein 1 (AP-1) has been identified in many species. He said that genome-wide analyses often identify numerous proteins associated with TREES, although these candidates have not yet been validated.
- Lea Goentoro, Ph.D., asked whether TREEs are conserved between animals with and without a robust regenerative response. Dr. Poss said that TREEs are present in mammals in tissues that show effective regeneration, such as skeletal muscle. TREEs are not highly conserved between mammals and zebrafish.

How Does Limb Regeneration Recapitulate and Differ from Initial Limb Development?

Ashley Seifert, Ph.D., University of Kentucky

Dr. Seifert gave a presentation on the molecular regulation of limb regeneration and the similarities and differences between limb regeneration and embryonic development. He said that limb regeneration and embryonic development share the greatest similarity during the formation of a limb bud, blastema expansion, and morphogenesis. Researchers have made significant advances in determining the molecular regulation of limb regeneration. Several research groups have found that *Fgf8* and *Shh* are expressed in the anterior and posterior parts of the regenerating limb, respectively. These genes work in a positive feedback loop to regulate limb regeneration in the salamander. Also, two signaling locations arise at the amputation site: the apical ectodermal cap and the zone of polarizing activity. The expression of *Hox* genes across the proximal–distal and anterior–posterior axes, along with other signaling molecules, helps establish polarity in the regenerating limb. The FGF signaling pathway and *Hox* genes are also important during limb development in chicks and mice. However, salamander embryos do not have the same early structures, gene expression patterns, or genetic regulators of development as mice and chicks. For example, blocking FGF signaling in salamander embryos leads to only slight defects in patterning. The function of SHH signaling during embryonic development, however, is conserved among salamanders, chicks, and mice.

To accurately compare regeneration and embryonic development, it is essential for researchers to develop new tools to understand limb development in salamanders. It is possible that, rather

than other vertebrates having lost the ability to regenerate limbs, salamanders have evolved the ability to regenerate limbs. Early processes during limb regeneration, such as inflammation and wound healing, are not present during limb development. However, it is possible that these processes have an impact on later events during regeneration, such as cell migration and differentiation, that are comparable to embryonic development. It would be helpful to determine whether the early events during the wound healing phase are permissive or instructive for limb regeneration.

Panel Discussion: Lessons Learned from Limb Regeneration in Lower Vertebrates

Chair: *Susan Mackem, Ph.D., National Cancer Institute*

Panelists: *Catherine McCusker, Ph.D., University of Massachusetts, Boston; James Monaghan, Ph.D., Northeastern University; Sandra Rieger, Ph.D., University of Miami*

Dr. Mackem introduced the panelists and asked that speakers and attendees share their thoughts and questions. Panelists first discussed the factors required for dedifferentiation of mature cells to produce progenitor cells. Dr. Monaghan said that opinions on the role of dedifferentiation during regeneration are constantly changing. Researchers now think that cells in a regenerating limb, especially fibroblasts, are able to change their differentiated states to become generic limb bud cells. Researchers do not know how these cells dedifferentiate and redifferentiate to facilitate regeneration, but the process involves the recapitulation of developmental programs to facilitate regeneration. It would be useful for researchers to consider the definition of dedifferentiation, whether this process is required for human limb regeneration, and how to harness the potential of dedifferentiation to improve regeneration.

Dr. Wellik said that because a subset of fibroblasts is set aside as stem cells during development, there might be no need for dedifferentiation during wound repair. In dedifferentiation, individual cells would have to step backward in development to replace cells that have been lost. Stromal cells can recapitulate earlier expression patterns of *Hox* and other genes in the context of tumor formation, and it is interesting that a few specific factors can have such a significant impact on gene expression. Similarly, it is possible that a single factor could have a monumental impact on bone regeneration.

Dr. Muneoka said that researchers have been exploring dedifferentiation, especially in muscles, for some time. He said that it is challenging to define dedifferentiated states in fibroblasts, and a single-cell sequencing approach might help address this. Dr. Whited said that it is likely that fibroblasts are a very heterogeneous cell population in mice, and it would be interesting to identify the types of fibroblasts responsible for specific aspects of wound healing and regeneration both in mice and in salamanders. Dr. Wellik said that researchers will need to discuss more specialized cell types instead of saying “fibroblasts” in reference to such a heterogeneous population of cells. Dr. Mackem asked whether there is evidence of overlap between cells that are involved in regeneration and cells that undergo fibrosis. Dr. Wellik said

that this is likely. Dr. Whited said that studies that compare human and axolotl single-cell sequencing data would help clarify humans' capacity for extensive regeneration. Dr. Wellik said that researchers need ways to observe or trace cell behaviors and gene expression.

Dr. McCusker said that it is clear that fibroblasts are contributing to regeneration in a way that they would not be able to do in an uninjured environment. The wound healing response and nerves downstream of this process might play a role in creating a permissive environment for regeneration. Dr. Seifert said that the response to injury across all organisms depends on cell regeneration. He said that it would be helpful for researchers to compare cycling versus noncycling cells and to determine how these cells produce collagen and how this affects scarring. Dr. Monaghan said that experiments to identify the epigenetic factors at play during regeneration would be useful. Dr. Wellik said that it might be challenging to develop epigenetic tools for a system that is poorly understood.

Dr. Rieger asked that panelists discuss the molecular mechanisms underlying the key roles of the wounding and inflammation response, wound epidermis formation, and neural signaling. She said that researchers need to consider whether the wound healing process can differentiate between a regular wound and an amputation. Some research groups are comparing the genes involved in wound healing and regeneration, and early signals in either setting could lead to different interpretations of cues. One early signal after the influx of calcium after an injury is hydrogen peroxide, which is produced as a reactive oxygen species (ROS) and has an effect on several cell types. In zebrafish larval fin regeneration, hydrogen peroxide induces the expression of at least 1,500 genes. Hydrogen peroxide promotes the migration of immune cells toward the wound, epithelial cell migration, and the regeneration of sensory axons that innervate the wound. Hydrogen peroxide is well conserved across organisms, and differences in how organisms respond to this molecule might affect their ability to regenerate. Compared with wound healing, ROS are produced for a longer period and at higher concentrations during regeneration. Because the effect of hydrogen peroxide lasts for only an hour, it would be interesting to know whether the length of response of a signaling molecule can lead to different downstream signaling events. Innervation of sensory axons in the wound epidermis can also induce signaling responses.

Dr. Rieger said that researchers can learn about regeneration by studying cancer models. Also, several studies have shown that an augmented nerve supply is sufficient to induce regeneration. It would be interesting to discover whether nerve quantity is actually the important factor and what the essential signals are for this process. Dr. Muneoka said that the number of axons growing into digit tips during regeneration is seven times lower than during endogenous innervation, and digit denervation delays regeneration but does not block the process. In experiments where the limb is paralyzed by denervation, the lack of regeneration might be due to unloading and not the absence of neurons.

Dr. Monaghan asked whether researchers could increase the number of Schwann cells to replace the influence of nerve cells during regeneration, as these cells might be secreting regeneration-inducing factors. He asked whether the regeneration of ear hole injuries is nerve-dependent. Dr. Muneoka said that Schwann cells can rescue regeneration that has been blocked by denervation. He said that it is possible that Schwann cells play a role in the nerve response during regeneration, but their exact function in this process is unknown. Dr. Rieger said that regeneration in zebrafish is dependent on nerves and not Schwann cells. In mice, Schwann cells appear to have a modulatory function during regeneration. Published data have shown that denervation causes defects in bone regeneration in mouse digit tips.

Dr. Poss said that there is an aneurogenic limb model that is very interesting: A limb that is denervated early in development can grow normally, and when this limb is amputated in adulthood, it can regenerate without a nerve supply. Dr. Monaghan said that it is challenging to replicate this experiment genetically. Dr. Rieger said that a published study has shown that limb innervation during development influences gene expression. Dr. Whited said that experiments in which mitogens are used to rescue defects caused by denervation do not prove that the nerve produces that mitogen during normal development. Dr. Rieger said that it is important not to directly compare results across species or across developmental stages, because there might be significant differences in development and regeneration. Yong Li, M.D., Ph.D., and Dr. Nitkin asked whether electrophysiological stimulation plays a role in pattern formation and limb regeneration. Dr. McCusker said that studies have shown the activation of electrophysiological signaling in wounds soon after amputation, and this does not happen in lateral limb wounds.

Dr. McCusker asked that panelists discuss how patterning is coordinated during regeneration and how regenerating structures are integrated into existing proximal tissues. She said during limb regeneration, regenerating cells must generate a unique pattern across the proximal–distal axis, the cells involved must receive and respond to new positional cues, and the newly formed pattern has to integrate seamlessly with the existing tissue. Cells from the connective tissue control patterning during regeneration, and cells in the very early blastema can acquire new patterning information. Retinoic acid, FGF signaling pathways, and BMP signaling pathways help guide patterning, but researchers do not know how these signals lead to the formation of an accurate pattern. Because many of the same signaling molecules are present during limb bud formation and regeneration, studying limb development can help inform regeneration research.

One key aspect of patterning is how the blastema knows to replace only tissue that is lost. Studies have found that a gradient of retinoic acid along the proximal–distal axis is essential to this process. However, researchers do not know whether this gradient contributes to patterning along the anterior–posterior axis. Additionally, there is little focus on how regenerative structures integrate into an existing limb. It is possible that regeneration and integration are not coupled, and three factors are likely to play a role in tissue integration: The

pattern of regeneration has to align with the stem, redifferentiation needs to be appropriately timed, and tissue-specific mechanisms are needed for the integration of each tissue type.

Dr. Wellik said that her research group has observed that, in addition to providing patterning signals, regional expression of *Hox* genes extends into tendons, muscle, and connective tissue. Mice deficient in *Hox* gene expression but without skeletal defects still have problems with their muscles and tendons. *Hox* genes might therefore play an essential role in integration. Dr. Muneoka said that little is known about the role of histolysis and degradation of host tissue during regeneration. Existing tissue has to be modified to integrate a regenerating limb. After amputation of a digit tip, there is an osteoclast response that degrades almost twice the volume of bone that was amputated. The level of tissue degradation is also an important factor to consider in integrating regenerated tissue. Past experiments have shown that treating amputation sites with hyperbaric oxygen extends the period of osteoclast activity and increases bone degradation after amputation. In these cases, regeneration starts from a more proximal location. Dr. Wellik asked whether bone degradation might help establish a field for regeneration. Dr. Muneoka said that this is possible.

Dr. Muneoka said that because removing the periosteum impairs digit regeneration, the periosteum could have stem cells that are essential for integration of regenerated structures. This is in line with observations that regeneration is improved when the periosteum is left behind in a human digit wound. Dr. Mackem asked whether bone removal could stimulate the proliferation of stem cells. Dr. Muneoka said that there is not much correlation between the proliferation response and the activity of the osteoclasts. However, active degradation is correlated with blastema formation. Dr. Seifert asked whether it is important to further investigate the role of *hox* genes in establishing patterns during regeneration. Dr. Wellik said it is very likely that *hox* genes are involved in this process. Dr. Muneoka said that cell sorting experiments using chick limb buds have produced some information on *hox* genes.

Dr. Mackem asked which comparative studies would most help researchers understand the molecular basis for loss of regenerative capabilities in some animals. She asked about the similarities and differences between early embryonic limb development and wound healing and regeneration, and how these processes diverge in animals with or without extensive regenerative capabilities. Extensive regeneration seen in salamanders might represent an evolutionary novelty, and this might affect the types of comparisons that could help inform regeneration in humans. Some animals, such as frogs, have extensive regenerative capabilities during development but not as adults. Also, axolotls can lose the ability to regenerate due to aging or repeated wounding. It would be informative to analyze the reasons for such differences. Additionally, some mammals, such as the spiny mouse, can regenerate extensively, and it would be interesting to compare regeneration and wound healing in this animal and in similar mammals which do not regenerate effectively.

Dr. Seifert said that a comparative analysis of animals that do or do not have extensive regenerative capabilities would be beneficial. He said that using the ear hole model, his research group has found that there are significant differences in the cell types involved and the extent of fibrosis during regeneration in closely related species. An immune response is an integral part of any wound healing response, and it follows a similar pattern in all model systems. It is likely that the immune response is permissive for regeneration.

Dr. Poss said that comparative studies are informative, and several factors, such as sex and age, can affect regenerative capacity. These differences should be explored in as many contexts as possible. Dr. Wellik said that it is unlikely that animals with extensive regenerative capabilities evolved completely new processes for regeneration. The pathways used for regeneration might therefore exist in animals that do not regenerate limbs effectively. Dr. Whited said that understanding how regeneration can be stopped in animals that do regenerate limbs will help researchers understand the mechanisms blocking regeneration in other animals. Dr. Mackem said that combining experimental, genomic, and genetic approaches would help improve comparative studies. Dr. Whited said that currently, researchers are not focused on systemic influences on regeneration. Some research has shown a link between heart regeneration and thyroid hormones, and other studies might elaborate on this in the future.

Dr. Rieger asked whether there are intriguing connections between cancer and regeneration and whether similarities in regulation would pose barriers to regeneration. She said that some developmental genes play a role in both regeneration and cancer, and some cancer genes do not play a role in development but are upregulated during regeneration. Information on how these genes are regulated could help advance research into regeneration and cancer biology. Dr. Mackem said that salamander cells have a very slow rate of cycling, and she asked whether this could be relevant to regeneration. Dr. McCusker said that salamanders have a large genome that limits the speed of their cell cycle. She said that cells involved in regeneration have faster cell cycles. Dr. Whited said that tumors and blastemas have some features in common but have very different outcomes. In experiments where newts were given known carcinogens, the newts developed extra limbs but not tumors. Such phenomena could help clarify the link between regeneration and cancer.

Session 2: Potential for Limb Regeneration in Higher Vertebrates

What Is the Evidence for Tissue and Organ Regenerative Potential in Higher Vertebrates?

Mayumi Ito, Ph.D., New York University

Dr. Ito gave a presentation on the regenerative potential of higher animals. She said that humans and other mammals have a diminished capacity for regenerating complex structures, which is called epimorphic regeneration. For example, in humans, regenerated skin lacks some

important complex structures, such as hair follicles and sweat glands. Mice are able to regenerate hair follicles and digit tips. Hair follicle regeneration in mice takes about 80 days and appears to be accomplished through a process similar to hair follicle development in the mouse embryo. The fact that mice can regenerate such a complex structure shows the dynamic nature of epimorphic regeneration.

Mice can regenerate digit tips after distal amputations, including reforming bone and nail tissue. This regeneration involves blastema formation and is therefore similar to salamander limb regeneration. Several research groups have conducted studies using genetic tools to label regenerating cells for lineage-tracing experiments. Results have shown that lineage conversion is not required for regeneration. Stem cells and progenitor cells from the germ layers, and lineage-restricted stem and progenitor cells, regenerate the mouse digit tip. However, recent studies have shown that some lineage conversion occurs during regeneration.

Researchers have also used transplantation experiments to study the origin of blastema cells. For these experiments, fluorescently labeled fibroblasts were transplanted into a digit, after which the digit tip was amputated. These experiments have shown that fibroblasts help form the blastema and later differentiate into osteoblasts. The nail epithelium appears to play an important role in digit tip regeneration, because regeneration does not occur when the entire nail is absent. Researchers have shown that nail transplantation after amputation induces bone growth and that the effects of the nail epithelium are dependent on the Wnt signaling pathway. A better understanding of the mechanisms underlying interactions between multiple cell types and signaling pathways during digit tip regeneration will help extend the limb regeneration potential of higher vertebrates.

Discussion

- Dr. Dealy asked whether hair follicles could confer growth-promoting signals to regenerating skin tissue. Dr. Ito said that hair follicle regeneration occurs after wound healing is completed.
- Dr. Garza asked whether digit joint regeneration is possible in mice. Dr. Ito said that her research group has not observed digit joint regeneration.

What Is the Role of the Regenerative Response After Amputation in Humans?

Paul Marasco, Ph.D., Cleveland Clinic

Dr. Marasco gave a presentation on the importance of regeneration in advancing the field of prosthetics. He said that his research group works with a regenerative neural–machine interface to create advanced prostheses. This involves reassigning nerves from amputated limbs to other sites on the skin to preserve touch, feeling, and motor control of a complex prosthesis. This work has shown that humans have a significant capacity for regeneration in the brain and nervous system. After amputation, cortical and peripheral channels in the brain are

intact for years, and bidirectional control and feedback improves the function of a prosthesis. Also, nerve cell regeneration can happen quickly and with high specificity. Limb ownership— a person's ability to accept the prosthesis as a part of his or her body—is critical to a successful neural–machine interface.

In re-establishing functional representational architecture, the sensory cortex is activated when sensory input is provided. This triggers regeneration of nerve cells, and the regenerated nerve cells get more efficient with repeated sensory input. The brain therefore maintains nerve cells that processed information from a limb prior to amputation, and these cells are able to efficiently process information once sensory input is provided. It is very interesting that the brain has a remarkable ability to regenerate even though limb regeneration does not occur in higher vertebrates.

Ideally, a regenerated or prosthetic limb will be aesthetically and functionally as close as possible to the natural limb. However, researchers need to consider how much similarity in appearance and function is acceptable and would help improve the quality of life of people living with amputations. In advancing limb regeneration from a scaffold, researchers would have to consider several of the same issues as with prosthetic limbs: structural fixation, rewiring motor control, and rewiring sensory feedback.

Discussion

- Dr. Dealy said that a patient's satisfaction with a prosthetic or regenerated limb might depend on social and cultural perceptions. Dr. Marasco said that for prostheses, patient satisfaction is often based on limb ownership.
- Connor Dolan, Ph.D., asked how the level of amputation affects the efficacy of a prosthesis and whether prostheses for distal injuries work better than prostheses for proximal injuries. Dr. Marasco said that successful use of complex prostheses with neural–machine interfaces depends on the efficacy of the mechanics of the physical prosthesis. It can be difficult to fit a prosthesis for a very proximal injury.
- Jennifer Simkin, Ph.D., asked whether some neuron circuits are harder to regenerate than others. Dr. Marasco said that neural sensation circuits appear to be intact in his model.

Strategies to Reactivate Regenerative Potential in Higher Vertebrates: Potential for Stem Cells for Repair and Regeneration of Limb Tissue

Caroline Dealy, Ph.D., University of Connecticut Health

Dr. Dealy gave a presentation on stem cells and regenerative potential in higher vertebrates. She said that stem cells are pluripotent cells that arise during development or from reprogrammed somatic cells. These cells can differentiate into all cell types in the body. Stem cells develop into lineage-restricted multipotent progenitor cells that will form ectoderm,

mesoderm, or endoderm cells. These cells become lineage-restricted “committed” progenitor cells and, later, differentiated cells. Several rounds of cell division separate lineage-restricted multipotent progenitor cells and differentiated cell types. Blastemas are made up of a mix of lineage-restricted multipotent progenitor cells, and researchers have not identified all the progenitor cell types present. Also, researchers do not know whether these progenitor cells can transdifferentiate to form tissues of a different lineage.

In the mouse model of digit tip regeneration, distal P3 amputations lead to complete regeneration. Proximal P3 amputation leads to partial regeneration, and P2 amputation leads to weak regeneration. However, researchers can induce complete regeneration after a proximal P3 amputation and partial regeneration after a P2 amputation by activating various signaling pathways. Exogenous BMP2 after a P2 amputation causes bone growth, but more distal structures such as joints do not form. Exogenous BMP9 leads to the regeneration of joint structures. The sequential application of BMP2 followed by BMP9 leads to a more intricate regeneration program that requires PRG4, a protein necessary for cartilage development.

The Dealy laboratory is currently studying the role of epidermal growth factor (EGF) signaling in regeneration. Results have shown that implanting beads soaked with various EGF family ligands at wound sites can induce bone growth, cartilage formation, and improved regeneration in P2 amputations. The combined effects of applying both the EGF ligand and BMP2 led to more advanced regeneration of the P2 bone and signs of joint formation. As more evidence is acquired, researchers are observing that the human body has a greater capacity for regeneration than previously thought, so it is important to determine the genes necessary for regeneration in other higher vertebrates and to identify their distinct roles. This work can also inform research into bone and cartilage morphogenesis and repair in other fields.

The development of new molecular tools is helping researchers find the factors necessary for complete limb regeneration. Single-cell RNA-Seq technology has enabled researchers to identify several blastema progenitor cell types and specific genes that are expressed in a blastema but not in an intact digit. Also, loss-of-function experiments have been used to eliminate specific progenitor populations. Cells in a blastema are known to have positional information that guides regeneration, and transplantation experiments have shown that cells from a P3 blastema have a higher innate capability for regeneration than cells from a P2 blastema. Also, the stump environment influences the regenerative ability of transplanted cells.

The extracellular matrix can influence the environment in which regenerative cells are placed. Amputated limbs can be decellularized in a way that retains the ultrastructure of tissues and the vascular system. Such treatments can be used to create grafts to facilitate regeneration without the risk of tissue rejection, and the grafts might maintain some positional cues essential for regeneration. When decellularized mouse limbs are repopulated with various cell

types and skin is grafted over them, these limbs are structurally similar to an intact limb and can contract and relax in response to external stimulation.

Future experiments using epigenetic profiling to identify switches, apply growth factor signals, and localize programming within the limb could help researchers recapitulate blastema formation and advanced regeneration in higher vertebrates. There is ongoing work on reprogramming adult cells using chemical cocktails. Such an approach could be used to create blastema cell banks to seed appropriate matrices for regeneration.

What Is the Role of Mechanical Loading in Muscle Regeneration and Repair?

Lisa Larkin, Ph.D., University of Michigan

Dr. Larkin gave a presentation on improving outcomes for tissue regeneration and repair. She said that mechanical loading is critical for maintaining healthy muscle. Muscle atrophy can be reversed in some cases if the time of unloading is not prolonged. In other cases, muscle loss is permanent. The molecular mechanisms that regulate muscle mass during atrophy and rehabilitation in humans are complex and can vary with the cause of atrophy. In general, muscle atrophy occurs because of a change in the normal balance between protein synthesis and protein degradation. Methods such as physical therapy and electrical stimulation can help reduce atrophy during times of unloading. Because it is critical for maintaining healthy muscle, muscle loading is likely to play an important role in the complete regeneration of muscle structure and function during repair.

Although skeletal muscle has a high regenerative capacity, muscle regeneration is inefficient in cases where more than 30% of the muscle is lost. This leads to a significant loss of muscle function and cosmetic deformity. Treatments for muscle loss include muscle grafts, muscle flaps, and injectable fillers and prostheses. These treatments are limited due to donor site mobility, tissue availability, size or shape mismatch, and potential complications such as immune rejection. Many treatment options lead to functional deficits and patient dissatisfaction. The Larkin laboratory is developing a tissue engineering skeletal muscle construct to treat volumetric muscle loss.

Experiments to explore muscle regeneration in the Larkin laboratory involve replacing 30% of the leg muscle of a sheep with several types of tissue. Researchers are exploring how this tissue can be anchored to actively contract without causing damage to the scaffold tissue or the muscle and how to innervate and vascularize the grafted tissue. Current results show that nerves that are rerouted into the grafted tissue are able to stimulate this tissue, and gait and force analyses yield comparable results in control and experimental animals 3 months into recovery. However, histological analyses show extensive fibrosis after grafting.

Several laboratories are studying new ways to control fibrosis during muscle repair, including keratin hydrogel fillers, small molecules, and other antifibrotic drugs that might prevent the

formation of fibrotic tissue in healing muscles. Also, the improvement of noninvasive imaging methods and other techniques for monitoring success are helping to advance muscle regeneration and repair research to improve quality of life in humans.

Panel Discussion: Potential for Mammalian Limb Regeneration

Chair: *Luis Garza, M.D., Ph.D., Johns Hopkins University*

Panelists: *Erica Crespi, Ph.D., Washington State University; Jianjun Guan, Ph.D., Washington University in St. Louis; Jessica Lehoczky, Ph.D., Brigham and Women's Hospital; Mimi Sammarco, Ph.D., Tulane University*

Dr. Crespi said that regeneration appears to happen across an organism and to be controlled by specific essential factors. She said that because they play an integral role in timing events during development and reflect the state of an animal, it is likely that hormones might contribute to regeneration and introduce individual variations to the process. She asked whether any of the attendees were communicating or collaborating with endocrinologists to determine whether hormones could be used to enhance regeneration. For example, myostatin inhibition is used to prevent muscle wasting, and this hormone could help improve muscle regeneration. Dr. Larkin said that several researchers are exploring myostatin's role in muscle repair, and some companies are developing compounds that mimic myostatin. She said that she is open to establishing collaborations to analyze blood samples for muscle repair experiments conducted by other groups.

Dr. Garza said that it would be helpful for more researchers to explore the role of hormones during regeneration, as there is evidence that hormones might be relevant to this process. Dr. Crespi said that factors that inhibit regeneration might be good targets to explore to better understand plasticity. Dr. Marasco said that his laboratory has observed substantial changes in metabolic events that are tied to mechanosensation and nerve regeneration in limbs. Dr. Crespi said that leptin, which promotes wound healing in mammals and is an important epidermal growth factor, might be involved in regeneration. Other endocrine factors working across the entire organism, especially growth factors, could also influence regeneration.

Dr. Guan said that the use of decellularization to preserve tissue structure, microvasculature, and extracellular matrix components would be a significant advancement in limb regeneration. The use of bioreactors to engineer a limb for maturation would also be an important step forward. He said that the broad use of this technology would be limited by donor availability, and he asked whether there were alternative strategies to engineer whole limbs. Dr. Dealy said that other research groups are working on alternative approaches to limb engineering. She said that although it might be challenging to engineer whole limbs, creating a scaffold to engineer small sections of a limb would be an improvement on existing technology and could help improve patient satisfaction and quality of life. Dr. Larkin said that current technology does not support the replacement of whole muscles. She said that small improvements to muscle

regeneration technology would be an important achievement. Dr. Garza said that using ectopic or rescued organogenesis as a model for regeneration might also help advance the field. Daniel Garry, M.D., Ph.D., said that some progress has been made in rescuing musculogenesis defects in embryonic pigs by introducing musculogenesis-competent cells from another embryo or induced pluripotent stem cells (iPSCs), and this might be a promising strategy to replace muscle.

Dr. Lehoczky asked attendees to consider what amount of regeneration would be clinically significant and useful to patients. She asked whether regenerating additional bone or muscle tissue would be enough or whether it is critical to regenerate a whole limb. Dr. Marasco said that the ability to regenerate individual tissue types, such as muscle or neurons, or to improve the skin interface, would be useful to people living with amputations. It would also be useful to regenerate some distal tissue or extend an amputation stump without regenerating an entire limb. Dr. Lehoczky said that because re-establishing the pattern of digits during regeneration might be difficult, regenerating individual tissues might be a more attainable goal. Dr. Ito said that separating out the goals of muscle, skin, and bone regeneration might improve the chances of success. However, because limb regeneration involves coordinated responses among several cell types and tissues, it might be difficult to successfully regenerate a single tissue type independently. It is important for researchers to examine how different cell types communicate during regeneration, and this can be achieved through multidisciplinary collaborations. David Morgenroth, M.D., said that it can be very difficult for people with very proximal amputations to use a prosthesis; techniques to lengthen short residual stumps could improve such individuals' quality of life. Dr. Dealy said that although blastemas in amphibians consist of only regenerative cells, single-cell RNA-Seq experiments show that the mammalian blastema consists of a mix of repair and regenerative cells, and these cells could be harnessed to extend stump length.

Dr. Sammarco asked attendees about the current level of knowledge about the mechanisms that can accelerate regeneration. She asked whether researchers could combine rapid cell proliferation, like that seen in cancer cells, with patterning to speed up regeneration. Dr. Garza said that there might be an active restraint of regeneration and wound healing. Dr. Lehoczky said that during deer antler regeneration, regenerating bone cells have significant metabolic differences from other bone cells, and further studies of these differences might help identify factors that can speed up bone regeneration. Dr. Crespi said that endocrine factors could accelerate regeneration. For example, in tadpoles, food restriction slows down regeneration, and hormones such as leptin accelerate regeneration. Dr. Sammarco said that some treatments for osteoporosis rely on hormones. Dr. McCusker said that the speed of regeneration depends on the size of the limb being regrown. Larger limbs take more time to regenerate. Dr. Dealy said that because human limbs are large, the calorie demands of regenerating an entire limb should be considered when regeneration models are being debated. Researchers and clinicians might have to use supplementary exogenous cells to facilitate limb regeneration in humans. Dr. Ito

said that to understand how to form a blastema, it is important to study the heterogeneity of fibroblasts and bone cells in the blastema.

Dr. Whited said that in past experiments, researchers have found that when they inserted an amputated newt limb into the animal's body cavity, the wound site obtained proximal–distal polarity cues and positional information that later influenced regeneration. It is therefore possible that decellularized limb grafts might obtain positional information from the body during regeneration. Dr. Crespi said that in the emergency room, doctors sometimes insert amputation injuries into a patient's abdomen to encourage healing and regeneration. Dr. Garza said that it would be interesting to identify the youngest successful arm or hand transplanted and whether the growth rate changed in the host.

Dr. Garza asked whether it is feasible to have a cocktail that induces limb bud formation and outgrowth. Cliff Tabin, Ph.D., said that there are transcription factors that, when misexpressed in early mouse or human fibroblasts, will turn them into early functional limb bud cells, leading to limb bud formation instead of blastema formation after an injury. Because limb bud cells are self-organizing, this leads to the formation of an embryonic limb and not a regenerated full-sized limb. Dr. Garza asked what the latest timepoint in development is at which a transplanted limb bud can form a full limb. Dr. Tabin said that this has not been tested experimentally. Dr. Seifert said that researchers in the past have transplanted limb buds into adult frogs.

Dr. Morgenroth said that most amputations in humans are due to vascular impairment caused by diabetes. He asked whether such vascular impairment would affect the possibility of limb regeneration in humans. Dr. Crespi agreed that it might be more difficult to have successful regeneration in suboptimal situations, such as in patients who have serious health conditions. Dr. Garza said that researchers might have to adopt a graded process of first perfecting regeneration technology in patients without other serious health conditions, then moving to people who have amputations as a result of traumatic injuries, and then to people who have serious health conditions that might affect how well regeneration works. Dr. Crespi said that researchers have conducted studies on how to recapitulate proper wound healing. Dr. Ito said that wound healing is essential for epimorphic regeneration, and this might have implications on the success of regeneration in patients with compromised wound healing. Dr. Dealy said that certain mesenchymal cells have anti-inflammatory properties, and introducing such cells into the regenerative environment might help control inflammation locally in cases where wound healing is impaired.

Dr. Mukhopadhyay thanked the speakers, panelists, and attendees for their insightful presentations, questions, and discussions.

Day 2: February 18, 2021

Welcome to Day 2

Joe Bonner, Ph.D., NICHD

Dr. Bonner briefly reviewed the Day 1 discussions, which began with a historical perspective of regeneration research, from research in lower vertebrates and mice to clinical case studies and finger regeneration in humans. Models of limb regeneration were presented, and participants discussed the role of and need for various cells, tissues, and stem cell niches. The day concluded with discussions on volumetric muscle loss and advanced prosthetics. The Day 2 agenda would shift to translational and clinical research on limb loss, both its present state and its future potential.

Dr. Bonner introduced the keynote speaker, Cato Laurencin, M.D., Ph.D., from the University of Connecticut. Dr. Laurencin is the Albert and Wilda Van Dusen Distinguished Endowed Professor of Orthopedic Surgery, a professor of biomedical engineering, and the president of the Regenerative Engineering Society. He has been elected to the National Academy of Engineering (NAE) and the National Academy of Medicine. Dr. Laurencin was the recipient of the 2019 NAE Simon Ramo Founders Award. President Barack Obama presented Dr. Laurencin with the National Medal of Technology and Innovation, the nation's highest honor for technological achievement, at the White House in 2016.

Keynote

Limb Regeneration Using a Regenerative Engineering Convergence Approach

Cato T. Laurencin, M.D., Ph.D., University of Connecticut

Dr. Laurencin put the topic of limb regeneration into a personal context, noting that limb loss is a devastating event that affects real people. The science is important, but it is really all about the patients. He said he had been contacted recently by the parents of a girl who lost a finger and by a wounded warrior who had lost a leg, both families hoping to learn about the latest treatments and the prospects for the future. Scientists must have a sense of urgency about solving these problems.

Dr. Laurencin said his interest in limb regeneration began in 2007 at the U.S. Committee on Biomechanics, when he first suggested exploring limb regeneration. It solidified in 2010 during the Society for Biomaterials annual meeting, when he served on the Biomaterials Grand Challenge panel and announced that the grand challenge was limb regeneration.

Tissue engineering will be required to accomplish limb regeneration. Dr. Laurencin defined tissue engineering as "the application of biological, chemical, and engineering principles toward

the repair, restoration, or regeneration of living tissues using biomaterials, cells, and factors alone or in combination." The area of tissue engineering started with Y.C. Fung, Ph.D., at a National Science Foundation workshop in 1987. The science has moved to a revolutionary phase of convergence, bringing together the life sciences, the physical sciences, and the field of engineering to apply insights and approaches from these very distinct fields to address grand challenges in a way that was unimaginable even just 15 years ago. In a 2012 article in *Science Translational Medicine,* Dr. Laurencin predicted that the future was "regenerative engineering," which he defined as "the convergence of advanced materials sciences, stem cell science, physics, development biology, and clinical translation for the regeneration of complex tissues and organ systems."

Dr. Laurencin described his work with soft tissue regeneration in rotator cuff injuries, which affect more than 2 million people in the United States each year; about 300,000 require surgery. Dr. Laurencin focuses on rotator cuff tendon repair and regeneration, addressing the weakest link: the insertion of the rotator cuff. He and his staff designed and engineered first-generation systems using nanofiber matrices, reinforced repairs to facilitate the natural regeneration and repair of the rotator cuff, and created biomimetic textile systems that recapitulate the rotator cuff tendon insertion. Dr. Laurencin said his lab also collaborated to develop systems that deliver biologic growth factor using polylactic acid-caprolactone (PLA-CL) matrices for regeneration of the rotator cuff at the insertion point and created stem cell niche systems that deliver biological factors directly to the rotator cuff for regeneration. To address muscle loss, a consequence of rotator cuff tendon tears, they designed systems to regenerate the muscle by examining insulin-like growth factor 1 (IGF-1) to create biologic delivery systems for regeneration of rotator cuff enthesis. His lab also created electroconductive matrices for reversing muscle atrophy in chronic degenerative tears and regenerating the muscle in the rotator cuff, because without muscle regeneration the risk of re-tear is very high.

Dr. Laurencin elaborated on using stem cells in combination with his lab's matrices to influence tendon regeneration. To accomplish this, all of the materials must be able to interact with cells to create the required functional characteristics. He described combining the beneficial characteristics of polycaprolactone (PCL) and poly[(ethyl alanato)$_1$(p-methyl phenoxy)$_1$] phosphazene (PNEA-mPh) to enable rotator cuff repair augmentation. His lab created polymer blends for the rotator cuff, prioritizing the cultivation of stem cells that would remain as stem cells. Studies were conducted on repaired and augmented rat shoulders, by introducing matrices either with or without stem cells. The stem cell–seeded matrices accelerated the tendon body remodeling and resulted in increased tendon biomechanics. This mechanism is likely paracrine (trophic) effects from the stem cells, coupled with an immunomodulatory effect. These matrices were developed to enable the stem cells to be dedifferentiated within the matrix over the area of repair, allowing the immunomodulatory effect to take place and create a stem cell niche for regeneration.

Dr. Laurencin described his work using engineered ligament regeneration on the anterior cruciate ligament (ACL), the major intra-articulator of the knee. He took a hierarchical approach by creating a system that would mimic that of the natural ACL. Because the ligament connects bone to bone, engineering two types of tissue was required, with bone on either end and the soft tissue ligament in between. The result was a degradable three-dimensional, braided, poly-L-lactic acid (PLLA) scaffold with bony articulated ends for the regeneration. Dr. Laurencin said years of work went into this project, starting with a model for ACL regeneration in rabbits, a success that was noted in 2012 in *National Geographic,* which ranked this discovery 30th in its article on 100 scientific discoveries that changed the world. The ACL work was then scaled up to sheep, where regular dense collagen tissue and full regeneration of the ACL were achieved. This led to soft tissue being implanted in humans, and now ACL ligament regeneration has been used in humans for 7 years. Dr. Laurencin won the 2021 Kappa Delta Award from the American Association of Orthopedic Surgeons for his 30 years of work in bone regenerative engineering.

Dr. Laurencin said that convergence tools enable his lab to engineer nearly every tissue in the lower extremity, including not only bone and ligament but also skin, cartilage, fascia, nerve, tendon, and vascular tissue. The goal of regenerative engineering is to bring all these technologies together for limb regeneration. Dr. Laurencin described the overall regenerative engineering goal as "regenerative consilience," the linking of principles from different disciplines to form a comprehensive theory. For example, it is important to harness what has been learned about organisms that can actually regenerate, such as axolotls and newts. Dr. Laurencin said that part of his work has been to identify the regeneration-enabling signals from axolotls to inform the improvement of human repair mechanisms. Learning from axolotls could enhance complex tissue regeneration in mammals. Humans have limited regenerative ability in many areas (e.g., heart, hair, blood, liver), while axolotls have extensive regenerative ability throughout their lifespan. Axolotls exhibit key signaling during limb regeneration in wound healing models, sending signals from nerves to influence blastema formations. Growth and pattern information is also contained during the regeneration process.

Dr. Laurencin said he believes that limb regeneration is mediated by interactions between cells. Pattern-forming cells provide positional information, and pattern-following cells remake the missing parts according to positional information (e.g., progenitor cells, adult stem cells). Understanding these signaling mechanisms is critical to moving forward.

Dr. Laurencin described his collaboration with David Gardner, Ph.D., from the University of California, Irvine, who created a "limb from scratch" by using purified growth factors combined with purified heparan sulfate proteoglycan (HSPG) from a mammal. The researchers believe that heparan sulfate (HS) may hold a key to controlling pattern formation, since various HS-related genes are upregulated during the limb regeneration process. They were able to identify HS-rich cells with multiple branching cell processes in the axolotl dermis. They described these positional information cells, which were arranged in a grid with multiple overlapping cellular

processes, as groups that are regenerative, interspersed, and dendritic (GRID) cells. GRID cells stained heavily for HSPG, were localized within the connective tissues in the limb and associated with dermis, nerves, muscle, and blood vessels. GRID cells were not evident at the early stage of blastema formation distal to the amputation plane, but they reappeared at a later stage of blastema formation distal to the amputation plane, although not the distal tip. One of the most exciting findings was an abundance of mammalian GRID cells in neonatal mouse skin and mouse limb skin. Dr. Laurencin summarized the work with GRID cells, saying this unique cell population was identified in both axolotls and mice. The cells have stellate morphology with long projection and show network in axolotls and ontogenetical decrease after birth in mice. GRID cells are candidates for pattern-forming cells and have the potential to provide positional information to enhance regeneration in mammals.

Dr. Laurencin discussed his work on osteoarthritis (OA) using amnion (AM) as an advanced biologic for tissue regeneration. He developed an AM-based hydrogel delivery system for adipose-derived stem cells (ADSCs) for treating OA. The amnion hydrogel (AH) supported adequate ADSC adherence, viability, and proliferation and maintained the ADSCs' "stemness." The anti-inflammatory and chondroprotective effects of ADSCs and AM were confirmed. Improvement was seen after intra-articular injection of ADSCs in AH in an OA rat model. Dr. Laurencin summarized this work saying that AM can serve as a delivery system for hydrogel ADSCs. ADSCs and AH synergistically exert anti-inflammatory and chondroprotective effects in an OA environment. This injectable AH system could be used in both basic research and clinical applications, leading to a major breakthrough in stem cell therapeutics for knee joint regeneration and OA treatment.

Dr. Laurencin concluded by again noting that the regeneration field is about people first, and there must be urgency to addressing the crucial issues of limb loss. He announced the Hartford Engineering A Limb project, which has a goal of regenerating a human limb by 2030 and redefining the clinical treatment of musculoskeletal disease.

Dr. Laurencin called the attendees' attention to his textbook, *Regenerative Engineering;* his new journal, *Regenerative Engineering and Translational Medicine;* his new society, the Regenerative Engineering Society, which is the first society for everyone, including nonscience members; and the upcoming International Summit on Limb Regeneration, sponsored by the Regenerative Engineering Society, in December 2021.

Discussion

- An attendee asked whether the joint or the long bones were the biggest challenges for regenerative medicine. Dr. Laurencin said regeneration of the joint is critical for limb regeneration, and many programs are focused solely on the joint. He noted that successful regeneration of a joint would result in new treatments in the short term, but

regenerating a limb will require regenerating a joint. A convergence approach is needed to change the internal environment of the joint to allow for regeneration.

- An attendee asked whether Dr. Laurencin had tried seeding with the scaffold he described for the ACL. Dr. Laurencin said he had not. He wants to get a better understanding of the GRID cells. In the spirit of consilience, the next phase will be to explore whether these cells can be used in other regenerative systems.
- An attendee asked about the challenges of working with smaller organisms, particularly whether they are biologically capable of sustaining the replacement, given the mechanical demands. Dr. Laurencin noted that in moving from rats to rabbits to sheep to humans, at every point the designs and implants for the functional demands worked better as they were scaled up. The ACL function work was more successful in humans than in rabbits for two reasons: The materials used in scaling up provide a better functional result, and the environment is more controllable. Dr. Laurencin noted that the rabbits ran around constantly, which was not the case with humans.

Session 3: Supporting Function in the Proximal Limb for Amputees

How Does Pre-Amputation Clinical Decision-Making Affect Functional Outcomes?

Christopher Attinger, M.D., MedStar Georgetown University Hospital

Dr. Attinger, a plastic surgeon who works only with limb amputation, discussed amputation and diabetes in the United States, the importance of a multidisciplinary approach, function as the primary outcome of all surgeries, and the changing role of amputations with the advent of targeted muscle reinnervation (TMR).

There are 185,000 lower-limb amputations in the United States each year; 54% of those are due to complications of diabetes and peripheral vascular disease. The southeast part of the country has the heaviest concentration of people with diabetes, the highest morbidity among adults with self-reported obesity, and the highest percentage of physical inactivity among people with diabetes.

The mortality rate after amputation is high. The 5-year mortality rate for a foot ulcer is 31%; for comparison, the pooled mortality rate for all cancers is 31%. The 5-year mortality rate for a major amputation is 57%. A retrospective review of all nontraumatic lower extremity amputations (LEAs) showed a 64% mortality rate at 5 years. When the mortality rate was broken down by type of amputation, the above-knee amputation (AKA) rate was close to 70% in 5 years; the below-knee amputation (BKA) rate was 57%. In comparing the relative risk of mortality in people with type 1 and Type 2 diabetes mellitus (DM) with the LEA population without DM, the rates were similar after 1 year, but at 5 years the mortality rate in the LEA population without DM was 47.2%, compared with 63.2% for the LEA population with DM.

The high mortality associated with amputation has shifted the emphasis to limb salvage. The literature on diabetic foot ulcers focuses on wound healing. Dr. Attinger said the physician's goal should be to create a functional limb, either by amputation or by salvage. The patient's goal is to maintain independence, which is dependent on maintaining ambulation, by limb salvage or use of a prosthesis. Ambulation is a particularly important goal because physical activity plays a significant role in increasing life expectancy. Dr. Attinger described a patient who spent 5 years trying to salvage his leg after an accident. During that time, he was unable to walk. He finally underwent a knee disarticulation and was walking with a prosthesis 6 weeks later. Many service members with AKAs have been able to engage in incredible physical activity. Dr. Attinger said patients with conditions such as diabetic foot ulcers often get sent to various physicians without ever getting the proper treatment. It is preferable for patients to go to one location and consult with a multidisciplinary group of physician experts.

Dr. Attinger discussed the surgical algorithm approach, which is driven by quality of life considerations. It requires:

- Finding functional solutions for the long term to avoid recidivism
- Angiosome-based revascularization
- Infection control
- A functionally based reconstruction, whether that is limb salvage or amputation

There is a bias toward AKAs with major amputations, because they heal more easily. However, there is a two- to three-fold lower chance of achieving ambulation with AKAs. Although BKAs do not heal as well as AKAs, there is always the option of moving to an AKA if healing remains poor. The real benefit of BKAs is ambulation using a prosthesis, which has an ambulation success rate ranging from 16% to 78%. Patients with BKAs at risk of being unable to wear a prosthesis are those who were nonambulatory before surgery, are on dialysis, or have DM.

People do not ambulate after major LEAs because of pain (e.g., residual limb pain, phantom limb pain, effects of taking narcotics for pain) or a neuroma, which forms from cut nerves and makes the prosthesis too painful to wear. Dr. Attinger said that burying a cut nerve in muscle, capping it, or connecting it to a similar nerve will not prevent a neuroma. However, taking a sensory nerve and cutting a small motor branch to a muscle to connect the two, causing the axonal sprouting to travel down the motor branch and dissipate in the muscle, can prevent neuroma formation.

Dr. Attinger observed that a BKA could be the start of a new life and a new leg for the patient, and surgeons should not approach this type of amputation as a failure. The most senior surgeon should do the surgery, and physical therapy should be involved from the start. Problems include leaving the amputation too short and not reattaching the muscles, which leads to muscle wasting and bone prominence. Dr. Attinger recommended using a posterior

flap design to reattach all the muscles and create a good cover for the distal end of the amputation. It should be between 12 and 18 centimeters from the tibial tubercle so there is enough length to allow for revision, if necessary, without having connective tissue that is too short. The current protocol after leg amputation is to make sure all the nerves are dissected out for future TMR or traction neurectomy.

Dr. Attinger said a primary BKA TMR has minimal added risks. The TMR is done with the superficial peroneal nerve and the tibial nerve. The traction neurectomy is done with the deep peroneal nerve, the saphenous nerve, and the sural nerve. The common peroneal nerve is also released just below the proximal fibular head. The myodesis is done by attaching the peroneal muscles to the lateral tibia and then attaching the anterior tibial and posterior tibial muscles to it. The soleus muscle is then attached to the anterior tibia, and the Achilles tendon is attached to the anterior tibial fascia.

For the athletic patient with a BKA, the Ertl procedure (named after Janos Ertl, M.D.) is done by fusing the tibia and fibula using a vascularized bone graft, by either screwing it in or tying it in with wires. This prevents a rotation of the fibula past the tibia when the person twists and turns and allows more accurate translation of that movement to the artificial ankle.

Dr. Attinger said 2014 data from his group indicated a 4:1 BKA/AKA ratio, with a 23% operative revision rate after BKA and a 2% BKA-to-AKA revision rate. Patients who did not undergo the Ertl procedure (i.e., no bone bridging) had a 78% ambulatory rate; patients with bridging had a 100% ambulatory rate. A comparison of TMR with non-TMR groups at 3 months showed the TMR group with a 92% ambulatory rate and the non-TMR group with a 71% ambulatory rate. The TMR group also experienced significantly less pain, with severe to moderate pain decreasing from 45% to 8%, and narcotic pain medication use decreasing fourfold. The 5-year survival rate for both major (n = 195) and minor (n = 224) amputations was 69%, with a 72% survival rate for minor amputations and a 65% survival rate for major amputations. The 5-year survival rate was highest for toe and transmetatarsal amputations and BKAs. These data help inform decisions between limb salvage and amputation. The functional algorithm for choosing between salvage and amputation showed no significant difference between the two, and both are routinely considered for every patient, particularly since longevity is not severely affected.

Dr. Attinger offered these conclusions:

- Chronic wound care and limb salvage is complex.
- A multidisciplinary approach is the standard of care.
- A surgical algorithm that focuses on function ensures increased function and longevity.
- Ambulation, whether via limb salvage or amputation, is the *only* goal.
- BKA with myodesis and TMR is a viable alternative to limb salvage in the appropriate patient.

- Always consider biomechanics and function.

Do Amputation Strategies Affect Functional Potential in the Proximal Limb?

Chris Dearth, Ph.D., Department of Defense (DoD)–Department of Veterans Affairs Extremity Trauma and Amputation Center of Excellence

Dr. Dearth said his talk would focus on the unique features of the DoD population and highlight the opportunities for limb regeneration to make meaningful improvements in the outcomes for these patients. He agreed with Dr. Attinger that function should be the primary outcome.

To understand the limb loss population within the DoD, it is necessary to understand combat-related traumatic extremity injuries. Extremity injuries (compared with head and neck, abdomen, and thorax injuries) account for the majority of inpatient admissions (63%) and the greatest utilization of medical resources (65%). These injuries have a profound negative impact on force readiness; service members with extremity injuries are most often found unfit for duty and discharged from active duty.

Dr. Dearth noted that patients with combat-related extremity injuries that result in severe trauma experience primary and secondary amputations or attempt limb salvage. This often results in an acutely threatened or functionally impaired limb. The DoD limb loss population is a severely injured, complex, trauma patient population and often must also deal with multiple comorbidities caused by blast injury (e.g., muscle loss, nerve injury, poor wound closure, brain injury, facial trauma). However, unlike the civilian population, the DoD limb loss population is young, with 80% of patients under 30. All of these factors have implications for the opportunities of regenerative biology and limb regeneration in this population. Over the past two decades, advances in the care of service members with traumatic extremity injuries have enabled the restoration of an unprecedented level of function. However, many of these advances were lost in the years between wars, only to have to be relearned at the start of a new conflict.

Dr. Dearth reviewed the DoD surgeons' list of factors to be considered in amputation surgery, including level selection, length preservation, delayed closure, stabilization of proximal fractures and deep soft tissues, and nerve management. He noted that the limb regeneration field may be able to intervene and improve on some of these factors for better regenerative outcomes, particularly by using a multidiscipline, convergence approach.

Dr. Dearth said some of the newer developmental strategies for the limb loss population that might present opportunities for collaboration within the limb regeneration field include osseointegration, regenerative peripheral nerve interface, agonist–antagonist myoneural interface, and vascularized composite allotransplantation, an important strategy from a functional outcome perspective.

DoD strategic drivers that have implications for the limb regeneration field include aligning strategic priorities to ensure that the ultimate goal of any technology supports operational readiness and return to duty, aligning clinical gap areas with the overall DoD health program, and focusing on the near-term translation of research that will have a profound impact on the outcomes of patients.

Dr. Dearth said that facilitating improved clinical outcomes for patients with limb loss in the near term will require:

- Considering a regenerative rehabilitation framework, including development and evaluation of complementary technologies
- Promoting transdisciplinary and multisector collaborations to foster federal, academic, and industry partnerships
- Examining the impact of confounding factors, including comorbid injuries and acute and chronic injuries associated with limb loss
- Expanding and refining preclinical models to achieve harmonization and standardization of clinically relevant outcome measures
- Finding new funding opportunities, such as a consortium model and the T.E.A.M. approach (Together Everyone Achieves More)
- Fostering education and training opportunities to raise awareness and train the next generation of scientists and clinicians
- Generating higher levels of evidence by validating findings with clinically relevant animal models and facilitating clinical translation where possible

Discussion

- An attendee asked to what extent heterotopic ossification (HO) is an issue for complex limb trauma in service members. If HO is a stem cell differentiation problem, it may be an area where developmental biology would be relevant. Dr. Dearth said much has been written about HO in the complex blast injury patient population, and it is important not only from a cosmetic appearance standpoint but also from the standpoint of driving impairments, function, and prosthetic fit. It is certainly one of the confounding factors.

Limb Function and Amputee Health Issues: Challenges in Prosthetic Restoration

Linda Resnik, Ph.D., PT, Brown University

Dr. Resnik said her remarks would focus largely on the area of upper limb amputation. She noted that the functions of the upper and lower limb are very different. The upper limb has a high degree-of-freedom system and is involved in both gross and fine motor activities, many of which are complex (e.g., self-care, self-expression, interaction with others). The proximal upper limb stabilizes the rest of the limb, attaches it to the body, and allows movement of the hand

into a range of positions known as “the functional envelope.” The shoulder joint is a high degree-of-freedom joint, which allows the terminal end of the upper limb to be placed in many different positions and also allows the movement of the body over the distal limb when it is fixed, such as in crawling.

By contrast, the function of the lower limb is primarily maintenance and the achievement of an upright stance to promote locomotion (e.g., walking, running, hopping). The proximal lower limb stabilizes and supports the body weight, positions the foot on the ground, and moves the body over the foot in the gait cycle.

Dr. Resnik discussed the etiology of limb loss, noting that vascular disease accounts for more than 80% of all amputations. However, there are significant differences in the etiology of upper and lower limb amputations. Vascular disease accounts for 90% of lower limb loss; trauma accounts for 80% of all upper limb loss. For service members, combat is the primary etiology for limb loss (36%), and it is often combined with other associated injuries (e.g., hearing loss, burns, abdominal trauma). Combat veterans with both upper and lower amputations routinely have secondary health conditions, including depression, post-traumatic stress disorder, traumatic brain injury, arthritis, back pain, and phantom pain. Of these, arthritis has been found to worsen as the veterans age, a substantial issue to consider in their treatment. Dr. Resnik’s Department of Veterans Affairs survey of persistent, moderate-to-severe pain conditions experienced by people with upper limb amputations revealed that more than 70% have back, contralateral, and phantom pain, and more than 60% have neck and residual limb pain.

Dr. Resnik reviewed the clinical implications of the various challenges of limb loss:

- Dysvascular etiology and ongoing disease is associated with tissue healing problems, further amputation, and shortened life expectancy.
- Traumatic or combat etiology is associated with other major injuries (e.g., impaired vision, impaired cognition), which makes using a prosthesis more difficult.
- Residual limb health issues (e.g., HO, neuroma, swelling, skin breakdown) are associated with limb pain, problems with prosthetic fit, and inconsistency with surface myoelectric control.
- Arthritis and other painful conditions are associated with less tolerance for prosthetic use and fear of falling in people with lower limb amputations.
- Soft tissue damage and lack of use is associated with contracture and weakness, making it difficult to use the prosthetic limb. This can limit range of motion and lead to harmful compensatory motion.

Dr. Resnik said prosthetic restoration varies greatly depending on the amputation level and the number of joints that have been lost. Interscapular-thoracic amputation presents a far greater challenge than rehabilitation of the hand, because of the need to restore all the function of the

shoulder, elbow, hand, and wrist. There are clear differences in dexterity and activity performance between people with distal limb loss and those with proximal limb loss, and in all cases, people with transradial amputations are more functional and have better dexterity than those who have undergone transhumeral and shoulder amputations. However, the level of impairment is very substantial even for those who have undergone transradial amputation.

Dr. Resnik compared prosthesis use (i.e., whether one is used) and engagement (i.e., how it is used) in lower limb and upper limb amputations. Among individuals with lower limb amputations, only 5.8% to 15.8% do *not* use a prosthesis. However, even among people who do use a prosthesis, about 25% use it only indoors and about 4% use it only for transfer. In the upper limb amputation group, 24% to 29% of people with unilateral amputations do *not* use a prosthesis; only 5% of those with bilateral amputations do not use one. Even among people who do use a prosthesis, only 26% to 42% use it for their daily tasks. Dr. Resnik said her study of home use of a prosthesis found that people with unilateral amputations use the prosthesis in only about 19% of all manipulations. Of those manipulations, 79% are nonprehensile in nature, meaning they do not involve much grasping but rather are activities such as pushing, pulling, and stabilizing objects, functions of the upper limb prosthesis that are very important. Dr. Resnik noted a resource she developed, the TULIP Manipulation Taxonomy, which describes the variety of functions of the upper limb, aside from grasping activities.

Falling is a significant challenge for people with lower limb amputations. Half of people in this group have fallen in the past 12 months, and many (49%) report a fear of falling; even more (65%) report low confidence in their balance. These concerns affect people's willingness to participate in physical activity, which has been shown to positively affect life expectancy. Falls are more likely with AKAs and among people who have back pain, joint pain, and multiple residual limb and prosthesis problems.

Dr. Resnik provided a summary of the challenges in prosthetic restoration, which include:

- Suspension
    - Short or irregularly shaped residual limbs
    - Changes in limb volume causing positioning and skin breakdown, interference with myocontrol, and poor suspension
- Lack of sensation
    - Requires visual attention, and loss of proprioception affects balance
- Painful conditions
    - Residual limb health problems
    - Phantom and other musculoskeletal pain
- Hardware
    - Issues related to durability and weight
- Difficulty controlling multiple degrees of freedom

  - Makes activities slower and usually requires sequential movements
- Consistency of function in a variety of environmental conditions
  - Difficulty with wet environments, temperature extremes, and dust and dirt

Dr. Resnik concluded with a report on a survey she conducted in conjunction with the Food and Drug Administration (FDA) on patients' willingness to consider surgery that might improve the use of upper limb prostheses in the areas of restoration of sensation, improved control, and osseointegration. The strongest interest in this surgery was among service veterans, perhaps because they face such great challenges.

Discussion

- An attendee asked whether the most impactful advance in limb regeneration would be improving fit, improving function, or a combination of those advances. Dr. Resnik said that improving residual limb length is extremely important, particularly because irregularly shaped residual limbs create a problem for prosthetic socket fit. She noted that some of the discussions about muscle regeneration to improve the shape of the residual limb could be very important for prosthetic fit.
- Dr. Bonner asked about the advantages of using some of the advanced upper limb prostheses. Dr. Resnik said work is ongoing for sensory restoration and direct interfaces with the nervous system. This work is not about regenerative medicine but about improving the interface between the person and the prosthesis.
- An attendee asked whether there are any professional or clinical barriers to providing the most appropriate prosthetics and opportunities for subsequent modifications. Dr. Resnik said there are many barriers, including insurance reimbursement issues and lack of skilled surgical, therapy, and prosthetic providers. Better training for providers and better insurance coverage are needed. Dr. Bonner recalled Dr. Attinger's comment that senior surgeons should be performing these surgeries, especially in the private sector, and functional outcomes should be the main goal.

Barriers to Realizing the Potential of Prosthetic Devices

Joan Sanders, Ph.D., University of Washington

Dr. Sanders described the process of fitting a BKA residual limb into a prosthesis. First, a sock-like thermoplastic elastomer (TPE) prosthetic liner with gel on the inside, is fitted onto the residual limb. The bottom of the liner has a suspension system designed to accept the prosthetic socket. The socket is custom made for the person; the rest of the prosthetic is off-the-shelf.

Dr. Sanders outlined the problems that can occur after a prosthetic fitting. Because the residual limb is not static, limb maturation or fluctuations in volume, shape, and size that occur over the course of the day can affect the socket fit. Some prosthesis users have weak physiological

adaptation and simply cannot adapt well to the prosthetic socket, which limits their activity and could lead to injury. The emotional and mental burden of the need to continually monitor the prosthesis is very high. Failure to adjust the socket when necessary may lead to pain, limited activity, and injury.

Limb volume is one of the major factors affecting socket fit. Even a 1% change in limb volume can cause a clinically detectable change in socket fit. Dr. Sanders monitors changes in limb fluid volume using bioimpedence analysis, injecting a small electrical current through the residual limb and measuring the voltage in different regions. Her research has shown that residual limb volume decreases over the course of the day, resulting in socket fit deterioration. The most common solution to this problem is to add a sock to counteract the decrease in limb volume and maintain an acceptable fit. However, many people find this inconvenient, and as their pain increases over the day, they become less active. Dr. Sanders noted this is unfortunate, because her research has shown that most people actually gain limb fluid volume when they walk; standing is what contributes to limb volume loss. Walking is an opportunity to better stabilize limb fluid volume over the course of the day and maintain acceptable socket fit.

Dr. Sanders showed some of the latest “adaptable” prostheses designed to help users adjust their socket fits more quickly and easily. The manual socket adjustments range from a dial-like adjustment, similar to those used in ski boots, to very complex adjustable socket systems. Dr. Sanders noted that the problem with these technologies is that the user must implement them. She and her team have developed an auto-adjusting system using sensors implanted in the wall of the socket. A microcontroller reads the data from the sensors and automatically adjusts the panels to maintain fit, obviating a great part of the personal burden of socket management. The researchers were able to make this technology work successfully in such a short time because of two advances: the variety of chips, microcontrollers, and powerful batteries that are now available and the additive fabrication ability for three-dimensional printing. She noted there are other technologies for socket fit, including an elevated vacuum device that applies continuous negative pressure to help reduce residual limb fluid volume. Dr. Sanders said this device works reasonably well, but its “finicky” technology can frustrate users, especially those who are not tech-savvy. There are other auto-adjusting technologies that use microcontrollers and onboard computers, particularly for ankles and knees.

Dr. Sanders suggested that there is a great opportunity for harmony between a regenerative biological approach and a microcontroller-driven prosthetic device. The prosthesis offers a unique platform to support bioengineered technologies, perhaps in the development of a biological hand. Dr. Sanders emphasized that an entire limb is not automatically needed to start the process. For example, there is a great need for a controlled way to drive the recovery of limb volume. One tissue regeneration strategy might be to grow a muscle-substitute tissue within the residual limb that is capable of changing size under activation. This would enhance

suspension, reduce the need for a tight socket, and support residual limb volume recovery during activity.

Dr. Sanders discussed direct skeletal attachment (DSA). She noted that people are advised not to run when they have DSA, because the force applied at the interface of the implant and the bone is driving a huge bending moment. Dr. Sanders suggested an alternative strategy of fastening a tendon-like structure and a microcontroller to the posterior aspect of the tibia and attaching that to the heel of the prosthesis to manage tether length and counter the bending moment on the implant. Bypassing the passage of the tendon through the skin and fastening the tendon substitute proximally at the bone could accomplish this. Distally, the tendon is an "eyelet" shape positioned within a skin flap. The "post" going through the tendon substitute is tethered to a microcontroller mechanism at the foot to continuously manage its length. Dr. Sanders suggested that the DSA implant might serve as a scaffold for regeneration of the tibia and offer a way to avoid immediate loading of the implant. A microcontroller device could manage a spring-and-roller assembly at the distal end to control how much load to deliver to that implant interface as the limb regenerates. Dr. Sanders said these were simply some ideas to move the discussion forward.

Dr. Sanders summarized her main points:

- Limb regeneration strategies can create innovative solutions to current challenges for people using lower-limb prosthetic devices.
- The prosthesis offers a unique platform to support a combined regenerative biological and microcontrolled prosthesis approach.
- These solutions may be the initial steps toward a completely regenerated lower limb.

Discussion

- An attendee asked whether insurance covers the more sophisticated prosthetics. Dr. Sanders said payment is done on a case-by-case basis, but there can be problems with coding. Insurance companies seem to be receptive if there is evidence that these technologies improve outcomes, which is why it is so important for studies to evaluate outcomes. Dr. Resnik added that it can take years for a new technology to get a new code. Dr. Bonner said that the question of getting a code for reimbursement is a separate issue.
- Dr. Sanders observed that the tissues that Dr. Laurencin can engineer (e.g., tendons, ligaments) and the biomaterials and scaffolds that Buddy D. Ratner, Ph.D., works on present a great opportunity to solve many immediate problems that people with amputations grapple with, and it would be a tremendous service to address some of those issues on the way to full limb regeneration.

Panel Discussion: Maximizing Function for Amputees Today

Chair: *Sarah Greising, Ph.D., University of Minnesota*

Panelists: *David L. Butler, Ph.D., University of Cincinnati; Alan R. Davis, Ph.D., Baylor College of Medicine; Yong Li, M.D., Ph.D., Western Michigan University*

Dr. Butler posed two questions for discussion. First, how is function defined? Is it bringing patients to some level that improves their quality of life, or would scientists and engineers have a mechanical, biological, or clinical definition of function? Second, what constitutes success? How does it vary for different investigators, disciplines, and patients?

Dr. Li said he was experimenting with a repurposed drug for functional fibrosis after surgery and how that affected the need for revision. Success at this point is finding that the number of revisions is down.

Dr. Laurencin said success depends on the clinical situation, with different levels of success for different disease states. One measure of success is certainly a measurable improvement in function. Dr. Laurencin noted that having engineered a real foot with skin and bone was a success, even though it is not functional. Success in orthopedic surgery always involves good outcomes. Success in regeneration will involve defined functional outcomes. Dr. Butler said that given the complexity of the limb and all the tissues in it, the demands that are placed on those tissues to be able to function seem to put a limit on what needs to be done for such things as muscle or connective tissue regeneration. Dr. Laurencin said the functional demands of individual tissue levels in the limb have already been defined. Multisystem regeneration systems are being designed that combine muscle, tendon, and bone into a functional limb.

Dr. Dearth was asked what defines success with traumatic injuries. He said that there are so many clinical manifestations of traumatic injuries in service members that there is no single definition of success. From a DoD perspective, the overarching goal would be force readiness and return to duty, which would be considered success because of the high-level activity required for such a recovery. There has been an evolution of the definition of success, from the initial success of the surgical procedure, to success with DSA procedures and what that means to the person's quality of life. He agreed that researchers should provide metrics of overall success. Success is not a static, preordained outcome. In considering functional fibrosis, even without a long-term robust regenerative outcome, success could be mechanical fixes of the proximal ends that facilitate improved outcomes in the short term.

Dr. Greising noted that, in thinking about functional fibrosis in muscle loss, if muscle regeneration is the goal, that has not been achieved yet. However, preserving the ability to allow force transmission can occur with fibrosis and can be important for the individual in improving function. Limitations occur when fibrosis becomes pathologic to the point that it tips

the scales and drives the loss of range of motion or other disabilities beyond what is protective to the contractile aspects.

Dr. Li said his first publication on skeletal muscle fibrosis was in 2001. He suggested that fibrosis is not good or bad; it is a balance. Tissue regeneration with fibrosis formation at a certain time will produce a good result and improved function. It is also true that fibrous scars can be a problem. Fibrosis plays an active role not only in regeneration but also in tissue pain and aging. Dr. Li said his lab is working on limb muscle regeneration using muscle stem cells. He noted his work regenerating the soft tissue (but not the bone) of an amputated mouse digit and suggested that new regeneration models are needed that bring together more comprehensive ranges of disciplines. He wondered whether a combined amphibian–mammal model might lead to new discoveries.

Dr. Greising said the question of the perfect model is complex, and success will depend on stages of translation. She observed that it is difficult to bridge the gap between her studies with rodents and large animals. She asked whether a better model is needed to fully answer questions on limb regeneration.

Dr. Davis said he has found the cells in neuroma to be much the same as in HO and has discovered compounds that inhibit both HO and neuroma. Neuromas, which involve nerves, afflict nearly all people with amputations. Dr. Davis said he is referring to HO totally away from any skeletal bone. Although people experience problems with the HO that is attached to the skeletal bone, the mechanism is different for HO that is away from the skeletal bone and appears to be more neurological. His studies using single-cell RNA-seq show that osteoblasts and macrophages can come from these nerves. This finding is not signaling; it is the actual cells coming from the nerves. One reason for HO, especially in the military—where more than 60% of patients with blast injuries have heterotopic ossification—is that the shock of the blast causes a degradation in both the blood–nerve and blood–brain barrier, allowing the cells to more easily escape from the nerve. He suggested that certain systems require understanding the component parts in addition to the entire system.

Dr. Muneoka said he agreed with Dr. Li's balanced view of fibroblasts, suggesting that in regeneration models, the focus should be on harnessing these cells to understand and control their role in development, not viewing them as the enemy; they are likely to be very important for making functional connections between tissues. Dr. Wellik agreed with Dr. Li and Dr. Muneoka, adding there are numerous fibroblast subsets, many of which are not well understood. An understanding of which subsets are helpful, harmful, or critical is needed.

Dr. Butler asked whether the biologists have a definition of success and a definition of function. Dr. Monaghan said in terms of success, making a functional product that can increase function in a patient is often not considered in limb regeneration. He noted that studying whether

salamanders recover from a large loss of muscle is not done, because more attention is paid to the salamander's ability to regenerate an entire limb. Dr. Monaghan suggested a good transition would be to study what occurs in volumetric muscle loss in humans, look at whether that also occurs in the salamander and what tissues they use for organization, and bridge that to a mouse model for translation of what has been learned from the salamander about specific tissue regeneration strategies aimed at function. In attempting to develop a functional muscle, a lot can be learned from the lower vertebrate about muscle regeneration.

Dr. Li noted that histology has long been used to identify new regeneration myofibers in collagen. He referred to Dr. Larkin's illustration of a three-dimensional tissue reconstruction for muscle repair that showed clear massing of collagen deposits in the tissues. He asked whether such a physiological examination would now be used for confirming muscle regeneration or whether histological evidence will continue to be the standard. Dr. Greising suggested that as studies move to larger animals and humans, there is less sampling of the muscle, so the physiologic outcomes will have to supersede the histology, which is a very small sample of what the muscle looks like.

Dr. Dealy agreed with Dr. Resnik's comment that success depends on the stage of research translation. Even in the clinical realm, every patient is an experiment, because each has unique and specific problems, so every operation is research translation. Identifying progenitor stem cells and the transcriptional profile of the blastema are also research translations. It really does depend on the stage of the research translation, because that relates to achieving goals that can be leveraged into developing a strategic plan.

Dr. Davis said it is also important to concentrate on detailed basic science. For example, the basic science research on the cystic fibrosis gene is changing the course of an entire disease. It is a mistake to base everything on translation; basic science is critical as well.

Prayag Murawala, Ph.D., emphasized the importance of fibroblasts in tissue regeneration, because these are the cells that produce scars. Before producing a whole new limb, the first goal should be to reduce scarring. Fibroblasts differentiate during axolotl limb regeneration. The key will be to manipulate cells in mammals to reduce scarring and promote differentiation toward blastema or mesenchymal identity.

Dr. Garza said that when he was ready to move his cell therapy research from healthy volunteers to volunteers with limb loss, FDA told him that it would not define the metric of success; he would have to ask his participants. He noted that everyone with an amputation has a different story and a different perspective. The experience made him realize that to understand what success is, ask the patient.

Dr. Ratner said he works with fibroblasts and microphages. When a biocompatible biomaterial is implanted in the body, first the macrophages invade it and then a fibrotic capsule forms around it. The order is important: First come the macrophages, then the fibroblasts are called to do the "dirty work" of fibrosis. There are now biomaterials that do not fibrose, because the macrophages switch to a proregenerative polarization. The complexity of limb regeneration is very difficult to comprehend, because there are so many different proteins and factors involved. But it may be that the trigger is the macrophage, which can call in other cells. Macrophages can be used with some biomaterials to produce skin or with other biomaterials to produce scar tissues. Dr. Ratner suggested more exploration is needed about the clues that suggest that the microphages control the fibroblasts. Dr. Davis agreed with the importance of the macrophage, noting its role in bone formation.

Dr. Li noted the differing ratios of collagen deposits in tendons and muscle tissue. He suggested that collagen deposition plays a role, but its effect on function is challenging to quantify. Different models may be needed.

Dr. Muneoka echoed Dr. Monaghan's point about salamanders, noting that the blastema is the autonomous structure that forms the limb in salamanders. He recalled a study where investigators created a very large fracture of the axolotl leg, inserted the blastema into the fracture, and showed that the cells mended the fracture. The blastema has not only the potential to become the entire limb but also the plasticity to involve itself in a localized repair mechanism. More basic science research is needed on the blastema and what allows it to be regenerative in so many different ways.

Dr. Dearth agreed with the patient-centered approach, which is critical for clinical translation. Work is ongoing to understand the best use of patient-reported outcomes and develop a system specifically for patients with limb loss. This might necessitate development of new metrics or new tools to provide a comprehensive picture of the patient beyond just one outcome measure. Dr. Greising agreed, observing that amputation patients are very different from one another, with different etiologies, age ranges, and survival rates. She wondered about leveraging the physiology across these differences to identify areas that diverge or match up.

Dr. Butler asked whether, given the great differences among patients, there are envelopes of demands put on tissues within the functional limb that is ultimately to be created. These demands on the muscles, tendons, and ligaments might be used by biologists to come closer to developing functional tissues that will lead to true limb regeneration, as opposed to having patients depend on prosthetics to provide some quality of life.

Dr. Morgenroth suggested that certain elements are common across all people with limb loss. Translating what is happening in the basic sciences in limb regeneration research to functional success in the future will benefit all people with these common elements. There will also be

concepts that are categorical because they are very specific to the individual due to differences in etiology, age, and activity level. Another important area to consider is the concept of personalized rehabilitation to determine what will be useful to individual patients for their particular lifestyles. Basic scientists do not necessarily think about patient definitions of success, because that is so far along the translational pathway. But it is important to find a middle ground among the basic scientists, the clinician scientists, and the rehabilitation researchers, so they can speak a common language that furthers the discussion of success on both ends.

Dr. Dealy said having more feedback from the clinical community will inform basic research. Another consideration is the feasibility of getting approvals for new tools. For example, obtaining approval from FDA for the use of iPSCs would be a substantial barrier. Perhaps the scientific community should work on approaches to generating critical cell populations that are more FDA-compliant. She emphasized that she was not advocating for a specific approach, simply noting that there are other factors that determine where science will go. The risk is that scientists should not be so translation-targeted that they do not conduct the critical basic science that leads to serendipitous discoveries.

Dr. Davis noted that at one university, every faculty member must work with a clinician. This policy ensures that teams of clinicians include basic scientists and may prevent basic scientists from working on projects that have no practical applicability. Dr. Butler said the concept of starting the research with the end in mind does not mean that basic scientists have to adhere to every clinical problem, but it puts some focus of activity on tissues that could ultimately be useful. He noted that it is not only basic scientists working with clinicians but also multidisciplinary teams that will be important. Dr. Wellik agreed, saying work among disparate fields will enhance progress from both ends.

Dr. Laurencin said that it is necessary to redefine the field of regenerative engineering to bring together all different disciplines and to think of them not as extremes at two ends but rather as a circle in which their work is constantly integrated. He suggested that someone studying the axolotl should be aware of what is happening in stem cell science and clinical translation. There are great centers of regenerative engineering across the country to move this work along. Dr. Laurencin said his biggest concern is that 10 years from now everyone will still be asking the same questions. There are grand challenges ahead and no easy shortcuts, but it starts with goal-oriented requests for applications that bring teams together to make limb regeneration a reality.

Dr. Greising said these comments highlight how important the multidisciplinary approach will be. She asked Dr. Dearth to comment on translation into clinical use for the trauma population, especially how to conduct clinical trials for some applications.

Dr. Dearth said that understanding the clinical problem and the strategy to solve the problem is the real premise for research funding. The DoD intramural research program, which embeds researchers in points of care across the country to bring researchers and clinicians together, could be leveraged to support the translational bench-to-bedside activity. However, large-scale clinical trials for the amputation population are challenging because of study fatigue and practical considerations related to the number of participants needed for large-scale trials when there are so many different clinical presentations in this population.

Session 4: Future Directions

Moderated Discussion: What Can Be Achieved in the Near Term Through Collaborations Between Basic Scientists and Clinicians That Could Profoundly Accelerate the Pace of Progress?

Co-chairs: *Thomas Rando, M.D., Ph.D., Stanford University; Cliff Tabin, Ph.D., Harvard University*

Panelists: *Lea Goentoro, Ph.D., California Institute of Technology; Virginia Byers Kraus, M.D., Ph.D., Duke University; David Morgenroth, M.D., University of Washington; Ken Muneoka, Ph.D., Texas A&M University; Buddy D. Ratner, Ph.D., University of Washington*

Dr. Nitkin said this panel discussion was an opportunity to bring together all the valuable insights from this meeting and identify what research and collaborations are needed to advance the limb regeneration field and how NIH can help.

Dr. Rando said he is a clinical neurologist and a stem cell biologist studying muscle regeneration. He is also interested in muscle, bone, and skin injuries caused by trauma and in regenerative rehabilitation. Dr. Rando introduced the panelists, briefly summarized their work, and asked each member to make an opening statement about where the field is now and how it can advance to clinical applications.

Dr. Muneoka is interested in limb generation animal models, with a focus on mammalian limb regeneration. He noted that the blastema is a developmental structure that exists on an adult organism. He suggested that there must be a way to prevent cells from being attacked by systemic influences that are part of the adult physiology. The blastema tends to be avascular, and enhancing vascularization completely shuts down the regenerative response. The blastema actively proliferates; when proliferation is prevented, differentiation occurs. In the absence of proliferation, many types of differentiation can be induced at a wound site. Dr. Muneoka said this concept of the blastema being able to proliferate in the adult body is unique. The blastema is also hypoxic, and the oxygen levels in the blastema act as switches to transition the blastema from a growth phase to a differentiation phase. It is worth considering how this developmental structure is forming in an adult environment and is not completely isolated. There is an opportunity with the mouse to introduce human cells, to try and understand the cell types that

are important for regeneration and cell types that antagonize regeneration and tease apart those components as a first step.

Dr. Goentoro uses a systems biology approach to scale from molecules to organisms, with a focus on signaling and developmental programs. She emphasized the need for more collaborations between basic scientists and clinicians. She said there is also a need for more physiology. It has been said that regeneration is necessarily *ad hoc*, so the control might not be fine-tuned. An overarching issue would be determining the upper-level controller. Looking at this from a physiological perspective could unify the many themes that have been presented, such as macrophages in relation to the immune system, endocrine hormonal signaling, and key product metabolism. Bioelectric signaling may be another mechanism for global control that is not well studied. There has also been developmental biology work showing that biomechanics influences not only a passive scaffold but also the shape of things by signaling pathways that lead to gene induction. This is another area where there is much more to learn.

Dr. Kraus is a professor of medicine and a leading expert in osteoarthritis, having done pioneering work on the pathogenesis and treatment of OA. She observed that one easily available option would be to compile all the incremental information about limb regeneration that could be applied to repair and regeneration in general, independent of the limb. She noted that the ability of some organisms, including mammals, to regenerate digital tips wanes or is lost with age, and she suggested looking at things in reverse by studying what is lost that once was there. Are there active repressors that cause the loss? Considering the natural repair ability that is present in infancy and childhood could augment this type of study. Dr. Kraus said this suggestion arises from her perspective of working with ankle, knee, and hip joints and finding that the blastema messenger RNA (mRNA) that control limb regenerations are present in abundance in the ankle, in intermediate amounts in the knee, and in very low amounts in the hip, corresponding to the regenerative capacity of proteins in the cartilage. Obviously, some of the mechanisms are present, but perhaps some of the drivers are missing. Dr. Kraus suggested that there would be a great benefit in exploring “negative spaces,” such as the loss of an ability that once was present.

Dr. Morgenroth is a professor of rehabilitative medicine with a research focus on the principles of biomechanics and prosthetic engineering to improve quality of life. He said he hoped this meeting would lead to more collaboration and fewer silos. As a clinician who works with patients with limb loss, Dr. Morgenroth said it is tempting to think of limb loss as involving only an anatomical structure to be restored through regeneration. He urged the participants to focus not only on the absent limb but also on the person with the limb loss and the challenges they face. A patient-centered approach, even at the basic science level, would aim current regenerative techniques toward affecting key clinical targets along the road to full regeneration. He enumerated specific clinical targets, including residual limb length and wound healing. A majority of amputations in the United States are due to complications from diabetes

and vascular disease, often related to plantar ulceration. Enhanced wound healing could prevent many of these amputations. People who have experienced limb loss due to diabetic or vascular etiology are at great risk for contralateral foot ulceration and residual limb alteration, so wound healing advances would have an impact on prevention. Another problem is the issue of bony protuberances affecting socket fit. Any regenerative advances to modify the soft tissues or skin characteristics would be a beneficial short-term target. Dr. Morgenroth said that most patients with limb loss endure various types of pain. A great advance would be to use regenerative neural approaches to obviate phantom limb and neuroma pain. HO is another cause of socket fit issues and pain. There may be bone regeneration or bone-signaling pathways that could help mitigate HO. Finally, functional limb loss (e.g., shock arthroscopy or peripheral neuropathy that cause relative limited functional use of a limb) should be considered as another target for regenerative pathways.

Dr. Ratner is a professor of chemical engineering and bioengineering with a research focus on biomaterials in the context of tissue engineering, particularly organ reconstruction tissue regeneration. He said that although he is an engineer, he does a lot of work with cell cultures. The number of signaling factors, gene regulations, and system interconnections involved in limb regeneration is almost overwhelming. He emphasized the importance of the macrophage. The blastema is rich in macrophages. Macrophages can have a polarization that leads to fibrosis, but when comparing the blastema of the amphibian with the scar or fibrosis in mammalian healing, it is clear that the macrophage can go either way. The macrophage makes nearly every important factor. Dr. Ratner suggested that, instead of trying to tease out every detail, start at the beginning of the process that leads to a new leg in the amphibian. He also noted that there are a number of biomaterials that, instead of leading to fibrosis, lead to a vascularized reconstruction. It may be possible to make a pseudo-blastema using some of these materials. One unique porous material has been able to harness the macrophage, which phenotypic studies show moving in a proregenerative fashion. Dr. Ratner noted that sclera in human implantations, which tends to fibrose, heals in a vascularized reconstructive manner with this type of material. Dr. Ratner offered two ideas: Consider the blastema versus fibrosis and elucidate the biology of how the macrophage “knows” what occurs in this process; and, since amphibian limb regeneration occurs underwater, consider the need to develop a strategy to keep the future human regenerated limb under water during development and healing.

Discussion

- An attendee asked about the comparisons between the blastema and the regenerated portion of the mouse. What are the right ways to think about correlates between those two systems? What can be learned to translate one from the other in a meaningful way? Dr. Muneoka said regulating fibroblasts is important for regeneration and fibrosis, and those functions are not necessarily antagonistic. It depends on regulating how these cells respond and how many types of fibroblasts there are. It would be important to

know whether there are defined types of fibroblasts and to categorize them as pro- and antiregenerative.

- Dr. Monaghan said that when an entire salamander arm is irradiated so nothing divides, and a fibroblast cuff is grafted on, amputating through that area leads to regeneration of an entire arm, so fibroblasts' importance is clear. Epigenetics could inform positional information, such as how cells know where they are and what they need to become. After injury, a salamander's cells can access developmental programs; in mice, except in the limited digital tip, cells cannot access these programs after injury. In amphibian regeneration, the epigenetic state of these developmental programs is a complete black box. There has not been much discussion about epigenetics, but the competency of cells is very important. Macrophages are important, and they can be inducers, but having cells become competent is critical. Epigenetic engineering and reprogramming will be exciting areas to move the field forward. Dr. Rando agreed that epigenetics is needed to understand where positional memory is encoded, but in a broader sense, this relates to what the key regulators are for the epigenetic state.
- Dr. Kraus said the whole process of limb generation is so complex that it stands to reason that there must be an upstream master regulator. Epigenetics seems to fit that bill, not only for chromatin remodeling but also for the vast number of microRNA (miRNA), each of which controls numerous downstream mRNAs. She noted her research has shown a correlation of blastema miRNA in human cartilage with the regenerative potential of the cartilage. The question is whether there is specific miRNA that humans do not make but that limb-regenerating organisms do express. Conducting RNA-seq on human limb tissues has shown that some things are missing—or perhaps there are other factors that wane with aging or are blocking the process. Is there a magic missing miRNA? It is important to have more collaborations to ask common questions across species all the way to humans, and epigenetics has great potential to clarify the master regulatory process.
- Dr. Poss agreed that the goal of finding a master regulator, if there is one, is important. The question is how to deliver such a master regulator if it is found and whether there is some powerful factor that can reprogram chromatin to enable blastema formation in a human residual limb. He said he is also curious about gene therapy and viral vectors to infect stump tissue in a safe way, and he asked for the group's thoughts about the use of gene therapy if these nonmammalian vertebrates do confine those factors. Gene therapy could be used in combination with hydrogels and different types of prosthetic devices. Dr. Rando noted that the aging field is interested in taking advantage of the Yamanaka programming factors, not to reprogram cells for potency but to reprogram them from old to young. The idea that there is a partial reprogramming that can change the epigenome from old to young and enhance the function of cells relates to the kinds of differentiation reprogramming discussed in this workshop and that could be done by delivery of subsets of Yamanaka factors by viral vectors. There may be ways of thinking

about the delivery of reprogramming vectors that could span the spectrum from fundamental organisms to humans in the area of regeneration.

- Dr. Tabin said he is submitting a paper reporting having factors that can push adult limb cells back to being embryonic limb cells. He emphasized the importance of defining the epigenome of different cells types *and* understanding the interactions between the cells, calling this a systems biology question. Dr. Tabin said his experiment is essentially the Yamanaka process, except for using the limb at the end state rather than iPSCs.
- Dr. Goentoro said she is optimistic that there is a master regulator of limb regeneration. Dr. Tabin noted that certainly during development the mammal knew how to make a limb, and it would be interesting to get back to that point on an embryonic scale. To do this on an adult scale and recapitulate what is happening in amphibians is a different issue entirely. He suggested that there are likely to be regulators, but it is unlikely that there is a master regulator.
- Dr. Rando, noting the great interest in biomaterials for any kind of scaffold needed for regrowth of a tissue or limb, asked Dr. Ratner whether biomaterials alone would be sufficient. Dr. Ratner said there is one particular class of materials that steers the macrophage into a proregenerative phase, and the biomaterials field is learning how to reverse scarring and provide a very prohealing environment. These biomaterials are being used clinically because they do not scar. He noted that the biomaterial that he uses becomes heavily infused with cells almost immediately, and whatever tissue is put in (e.g., bone, skin) is reproduced. This is very important for cell therapy, because, rather than needing thousands of patients to donate cells, the biomaterials approach harnesses the macrophage to make the cells. He noted he had just published a paper on skin healing with materials seeded with mesenchymal skin stem cells. This holds great promise for regeneration studies.
- Dr. Laurencin agreed that biomaterials that interact with stem cells are important, but he suggested that the key is consilience, the combining of developmental biology with biomaterial science and other disciplines to create new theories. The clinical translation is also critical; regeneration happens in the operating room every day. He noted that his newly created Regenerative Engineering Society will foster this interdisciplinary collaboration.
- Dr. Poss introduced his colleagues to another new society, the International Society for Regenerative Biology, for which he serves as president. It will bring together a large group of people interested in all areas of regeneration (e.g., limb, spinal cord, heart) to share technologies and perspectives.
- Dr. Li noted a hypoxia application for the stimulation of blastema. His team has experimented with memo muscle cells and hypoxia, which promotes Yamanaka factor expression or cell reprogramming. He suggested this is a unique factor that involves regulation, indicating that hypoxia plays a role in initiating cell reprogramming.

- Dr. Rando noted the relatively new field of regenerative rehabilitation. There is great interest in clinical outcomes, and it is important to understand at what point and to what extent after surgery to introduce physical therapy. Dr. Morgenroth said this is a very important collaborative space between those who study regeneration and those who study biomechanics and rehabilitation in the clinical setting. Dr. Goentoro noted the importance of basic scientists being exposed to clinical settings to promote discoveries and improvements that are important *and* practical.
- Dr. Muneoka said that introducing human cells into mouse models may help identify cell types that are antiregenerative or provide other cell-type data to inform clinical decisions. Dr. Rando said he has transplanted human muscle cells into an immunocompromised mouse, but these are laborious experiments, and a higher-throughput system would be welcome. Dr. Muneoka said his lab is moving its regeneration response work *in vitro.* Having the ability to replicate the *in vivo* response *in vitro* enables him to determine the response at the cell level. Moving the regeneration response *in vitro* provides the opportunity to do more high-throughput cultures with human cells. Dr. Tabin observed that some questions might be best answered with a humanized mouse, others with an *in vitro* culture. If the questions are defined correctly, *in vitro* systems can be very powerful.

Wrap-Up

Theresa Cruz, Ph.D., Director, National Center for Medical Rehabilitation Research, NICHD

Dr. Cruz thanked the speakers, panelists, and attendees for the rich discussions, emphasizing the importance of fostering conversations between basic scientists and clinicians. She reviewed some of the highlights of the past 2 days, including the two superb keynote addresses that provided such great insight and optimism, the Day 1 presentations that so well described the questions that remain and the real possibilities for answering them, and the Day 2 presentations that focused on the clinical and translational work from a surgical, research, and rehabilitative standpoint. Dr. Cruz said the 2 days have been incredibly inspiring and that the success of this meeting will be measured in the new perspectives that were put forward, the transdisciplinary collaborations that will occur, and the advances to come. She urged all of the investigators to contact NIH about investigator-initiated applications.

Dr. Cruz concluded by saying it was impossible to name all the people who had put this meeting together, including the NICHD staff, the contractors and science writers, and the technical support team, but their efforts were greatly appreciated. She expressed particular thanks to Dr. Bonner, Dr. Mukhopadhyay, and Dr. Nitkin.

Dr. Bonner and Dr. Mukhopadhyay added their thanks to all the participants.