BioPharmaceutical Technology Center
I N S T I T U T E

10th Annual Wisconsin Stem Cell Symposium

Engineering Limb Regeneration: Recapitulating Normal Development and Regeneration?

SPEAKER ABSTRACTS

Mechanism and Evolution in Vertebrate Limb Regeneration

Jeremy P. Brockes, Ph.D.

Our understanding of limb regeneration as an evolutionary variable may have implications for our long term strategy to promote limb regeneration in mammals. Salamanders are the only adult tetrapod vertebrates able to regenerate their limbs. Limb regeneration could either be an ancestral vertebrate property that is lost by other tetrapods, or could depend on some evolutionary novelty in salamanders. From an evo-devo perspective, limb development in larval salamanders is different from other tetrapods, particularly in relation to the property referred to as pre-axial dominance (PAD). It has been suggested that the origins of PAD, which are reflected in the fossil record of the Paleozoic amphibian Apateon, may be connected to the origin of limb regeneration (1), and I will describe our mechanistic evidence in support of this viewpoint. It is unclear how they might be connected, and whether PAD operates in limb regeneration in extant species.

We have described the salamander-specific three finger protein called Prod 1, which was identified and analysed for its role in newt limb regeneration, both in relation to nerve dependence and to positional identity (2,3). Prod 1, which is readily identified as a unique TFP structure by its twelve residue alpha-helical stretch, is not found in other vertebrate genomes such as zebrafish or Xenopus. We have analysed the expression and functional role of Prod 1 in larval forelimb development in the newt, using TALEN-mediated gene disruption. We also find that PAD is a feature of larval limb regeneration, in particular the early appearance of digits 1 and 2, but this changes after metamorphosis in the eft form, and in the adult newt. Our work supports the idea that an evolutionary novelty that impacted on the development of the larval salamander limb, also impacted on its ability to regenerate this structure (1).

1. Frobisch, N.B. & Shubin, N.H. Dev. Dyn. 240, 1087-99 (2011)
2. Kumar, A., Godwin, J.W., Gates, P.B., Garza-Garcia, A.A. & Brockes, J.P. Science 318, 772-77 (2007)
3. Garza-Garcia, A.A., Driscoll, P.C. & Brockes, J.P. Integr. Comp. Biol. 50, 528-35 (2010)

Bridging Developmental Biology, Stem Cell Tissue Engineering and Clinical Translation after Musculoskeletal Tissue Injury

David L. Butler, Ph.D.

Given the frequency of musculoskeletal injuries and the lack of consistency in surgical outcomes, it is not surprising that researchers have turned to novel therapies like tissue engineering to

regenerate rather than just repair these damaged structures. While some have focused their research on incremental improvements in adult tissue healing, others have employed the paradigm of mimicking aspects of normal tissue development to improve the result. In this talk, I will first examine current and future challenges in using stem cells to generate functional ligament, tendon, bone, cartilage, and muscle in a timely and effective manner. These include the need to control cell phenotype and protein deposition in space and time as well as the challenges in creating constructs with inherent stiffness sufficient to tolerate the very large in vivo forces expected after surgery. Using the murine tendon model midsubstance and enthesis into bone, I will also discuss the challenges of mimicking developmental signaling events in an adult setting plus issues of homology across species when engineering functional tissues in the adult. I will then conclude with a discussion regarding how recent advances in developmental and regenerative biology might help overcome these challenges. It is hoped that this talk will further stimulate the dialog regarding the merger of developmental biology and human regeneration in engineering truly functional musculoskeletal tissues after injury.

The Role of Stem Cell Exhaustion in Aging and Disease
Johnny Huard, Ph.D.

Aging is characterized by the progressive erosion of tissue homeostasis and functional reserve in all organ systems. Although controversy remains as to the molecular mechanism(s) underlying the process of aging, accumulated cellular damage, including DNA damage, appears to be a major determinant of lifespan as well as age-related pathologies. Moreover, there is evidence that the accumulation of damage in stem cells renders them defective for self-renewing and regenerating damaged tissues. We have demonstrated that a population of muscle progenitor cells(MPCs) isolated from the ERCC1-deficient mouse model of accelerated aging, are defective in their proliferation abilities, differentiation capacity and resistance to oxidative stress. We have observed that intraperitoneal (IP) injections of wild-type (WT)-MPCs into Ercc1 knockout (Ercc1-/-) mice resulted in an improvement in age related pathologies. Although the mechanisms by which the transplantation of WT-MPCs extend the lifespan of these progeria mice is still under investigation, we have obtained evidence that the beneficial effect imparted by the injected cells occur through a paracrine effect that involve angiogenesis. In an attempt to determine whether the defect observed in ERCC deficient MPCs was not exclusive to this progeria model, we have isolated and characterized MPCs from another progeroid mouse model, the zinc metalloproteinase (*Zmpste24*) knock-out mouse, an animal model of the *Hutchinson-Gilford progeria syndrome* (HGPS). Similar to ERCC deficient MPCs, we have observed that *Zmpste24*-/- MPCs have proliferation and differentiation defects, characteristics also observed in MPCs isolated from naturally aged mice. These results suggest that the defect in MPCs is not specific to a particular model of progeria and can also be observed in naturally aged animals. Finally, we have investigated whether a defect in MPCs can also be observed in skeletal muscle disease such as Duchenne muscular dystrophy (DMD), which is a degenerative muscle disorder characterized by the lack of dystrophin expression at the sarcolemma of muscle fibers. Interestingly, DMD patients lack dystrophin from the time of birth; however, the onset of muscle weakness only becomes apparent at 4-7 years of age, which happens to coincide with the exhaustion of the MPC pool. There are several lines of evidence that support this concept including the gradual impairment of the myogenic potential of MPCs isolated from DMD patients during aging, which results in a reduction of muscle regeneration in older DMD patients. In addition to muscle weakness, DMD patients acquire osteopenia, fragility fractures, and scoliosis indicating that DMD may represent a model of ***premature musculoskeletal aging***

with a potential dysfunction in MPCs. Here, we report that dystrophin–utrophin double knockout (dKO) mice, an animal model of DMD, exhibit a spectrum of degenerative changes in various musculoskeletal tissues including skeletal muscle, bone, articular cartilage, and intervertebral discs. In contrast to that observed with MPCs isolated from the mdx mice (dystrophin deficient and mild phenotype), we have recently shown a defect in the MPCs isolated from dKO mouse. We have observed that the MPC defect from the dKO mouse model appears to be age dependent and not specific to MPC since other stem cell populations also appear to be affected. ***These results taken together support the concept that stem cell exhaustion plays a role in aging and disease.***

Epithelial-mesenchymal Interaction Underlying Digit Tip Regeneration
Mayumi Ito, Ph.D.

Mammalian digit-tips can regenerate upon amputation, like amphibian limbs. Previous studies including clinical observations have shown that digit regeneration occurs only when the nail can regrow. However, it is unknown why this capacity is limited to the area associated with the nail. The involvement of the nail epithelium in digit regeneration is currently elusive, because analysis of the lineage, growth, and differentiation of nail epithelium has been lacking. In this study, we investigated how nail epithelium differentiates under homeostasis and upon amputation and how these mechanisms are associated with digit regeneration. First, using genetic lineage analysis and colony forming assays, we identified nail epithelial stem cells (NESCs) niche in the proximal nail matrix that undergo Wnt-dependent differentiation into the nail plate. Upon digit amputation, early NESC progeny de-differentiate into a NESC-like state with concomitant downregulation of Wnt signaling and rapidly cover the wound site. By depleting Wnt signaling using genetic mouse models, we found that Wnt activation in regenerating nail epithelium subsequent to reepithelialization is required for directing its differentiation into nail. At the same time, this Wnt activation is required for attracting nerves that promote mesenchymal blastema growth, leading to regeneration of the entire digit. Amputations proximal to the Wnt-active nail progenitors result in failure to regenerate nail/digit. Nevertheless, forced Wnt activation in NSCs, but not skin epithelium, induced their regeneration. These results establish a link between NSC differentiation (nail regrowth) and digit regeneration, suggesting a utility of NSCs in developing novel treatments for amputees.

A Tissue Engineering Approach to Restore Function, Mechanics and Structure after Ligament/Tendon Injury
Lisa Larkin, Ph.D.

End-stage organ failure or tissue loss is one of the most devastating and costly problems in medicine. Limitations associated with tissue donation such as tissue availability, donor site morbidity, and immune rejection have led investigators to develop strategies to engineer tissue for replacement. The creation of engineered musculoskeletal tissues will not only restore the function of complex tissues such as muscle, tendon, ligament, bone and nerve following traumatic injury, but can also be used as a model for studying developmental muscle biology and muscle pharmacology. Dr. Larkin directs the Skeletal Tissue Engineering Laboratory (STEL) at the University of Michigan that has developed a scaffold-less method to engineer three-dimensional (3D) muscle, nerve, tendon, bone and ligament constructs from primary, bone marrow stromal cells (BMSCs) and adipose stem cells (ASCs). The research aims of STEL are to fabricate 3D musculoskeletal tissues, interface the tissues and evaluate the structural and histological

characteristics, implant the tissues in vivo to expose them to the actual mechanical and biochemical environments of a hindlimb, evaluate alterations in the structural, functional and histological characteristics of the tissues in response to strain-shielded and unshielded mechanical environments, and utilize the engineered tissues for tissue repair and replacement.

Chondrogenic Differentiation of Stem Cells Regulated by a Network of Signaling Pathways
Wan-Ju Li, Ph.D.

Cartilage regeneration by stem cells derived from either adult tissue or embryo is appealing to the field of orthopedic medicine given that cartilage is unlikely to repair itself if injured or degenerated and the current therapy using autologous chondrocytes fails to achieve desired outcomes.

With decades of development, stem cell-based cartilage regeneration has achieved several milestones that suggest a promising future of treating joint diseases using a tissue regeneration approach. However, a number of challenges affecting the success of stem cell-based applications for cartilage still remain. One of them is associated with the appropriate stem cell type for cartilage formation and another with precise control of chondrocyte differentiation. My group is interested in solving these two problems by working on the projects that aim to study the chondrogenic potential of mesenchymal stem cells (MSCs) from human bone marrow (BM) or embryo origin and to develop a viable approach to induce hyaline chondrocyte differentiation without hypertrophy.

We have found that MSCs derived from embryonic stem cells (ESCs) represent a cell source that holds promise for the future of regenerative medicine. Although ESC-MSCs are less responsive to induction for mesenchymal differentiation by current protocols compared to BM-MSCs, we have shown that the modified differentiation protocols can enhance ESC-MSC differentiation. Our key finding demonstrates that chondrogenesis of H1-MSCs can be greatly increased by BMP7 and TGFB1 compared to that by the gold standard TGFB1.

In addition, our data have demonstrated that differentiating MSCs' responsiveness to growth factors varies throughout the time course of differentiation and showed that MSC chondrogenesis can be enhanced over the current differentiation conditions using the sequential administration of multiple growth factors. Further, we have found that endogenously-produced IHH is both necessary and sufficient for the successful execution of MSC chondrogenesis, as knockdown of *IHH* almost completely blocked the induction of chondrogenesis by TGFB1, and overexpression of IHH was able to induce MSC chondrogenesis even when TGFB and BMP signaling pathways were blocked. Overall, our study demonstrates that the cooperative activities of the IHH, TGFB, and BMP signaling pathways together induce the most pronounced differentiation into the chondrogenic lineage.

Customized Scaffolding for Skeletal Tissue Regeneration
William L. Murphy, Ph.D.

Control over the presence of biologics (e.g. stem cells, growth factors) is a common theme in natural tissue formation, and also an emerging theme in functional tissue engineering strategies. However, a persistent challenge in tissue engineering approaches has been to effectively combine biologics with devices, such as "scaffolds", while maintaining optimal physical and chemical properties of the device. In particular, there is often a design trade-off between effective biologic delivery and optimal scaffold physical/chemical properties. This talk will

present a series of coating strategies we have used to deliver genes, growth factors, and stem cells from tissue engineering scaffolds. Fundamental mechanisms of affinity binding are used to incorporate biologics and enable uniquely high stability and biological activity. Controllable nucleation and growth of coatings also allow for spatial and temporal control over biologic delivery. In addition, coatings can be formed on a variety of devices, ranging from 3-D printed scaffolds to injectable microparticles. Coatings can be independently optimized for intended biologic delivery without influencing bulk properties of the underlying device. This "modular" approach results in scaffolds that have optimized properties from the macroscopic scale to the molecular scale. Our recent studies also demonstrate that array-based strategies can select coating chemistries for specific biological or biomedical goals. Examples include coatings that optimize long-term protein stabilization, autologous cell capture, stem cell differentiation and gene delivery.

Building a Blastema: A Historical Perspective
David L. Stocum, Ph.D.

Spallanzani was the first to describe external views of the urodele limb regeneration blastema in the late 18th century. He speculated that the blastema was the product of "expanding germs". The development of histological technology in the late 19th century enabled the study of biological processes at the tissue level. Using these technologies, Fritsch (1911) was the first to understand that the blastema was a collection of undifferentiated cells. A logical question then was, what is the origin of the blastema cells? Experiments by R. Hertwig in the 1920s showed that the blastema was limb-derived as opposed to the blood, and irradiation experiments by Butler in the 1940s proved that the tissue contributions were local to the amputation. Histological, electron microscopic, and labeling studies from the late 1930s to the 1980s suggested that the blastema cells arose by a process of histolysis and dedifferentiation. The question of origin received little more attention until the laboratory of Elly Tanaka created axolotls transgenic for GFP (Sobkow et al (2006)). This breakthrough has enabled a much more accurate way to trace the lineages of blastema cells. Some earlier conclusions have been verified, but there have been some surprises as well, and with increasing refinement of the transgenic technology, there may be more surprises ahead on which I will speculate.

Anuran Limb Regeneration: An Intermediary Model between Amphibians and Mammals
Koji Tamura, Ph.D.

Limb regeneration in urodele amphibians (newts and salamanders) is perfect with a correct number of digits, whereas adult limb regeneration in *Xenopus* (an anuran amphibian) results in a simple and unpatterned structure, including a spike-like shaft of cartilage that has no bifurcation/branching. Regardless of its patternless regeneration, we suggest that limb regeneration in the adult *Xenopus* is an epimorphic regeneration with blastema formation mediated by nerve-dependent cellular/molecular programs common to amphibian limb regeneration. Once the blastema is made after limb amputation, the urodele blastema reconstructs the limb structure by a re-patterning process that is incomplete in *Xenopus*. Our findings indicated that the incomplete patterning in *Xenopus* limb regeneration is closely associated with deficient re-expression of a key gene for the antero-posterior pattern formation, *shh*, and we suggest that epigenetic gene regulation plays an important role in re-expression of *shh*.

We also demonstrate correlations between the blastemal formation and skin wound healing by elucidating the molecular framework in the early phase of those events. A key for this elucidation is a limb-specific enhancer of the *prx1* gene, which is activated both in the limb blastema and wound-healing mesenchyme, but the enhancer is not activated in the mouse wound healing process. Wounded adult amphibian skin can repair perfectly without scarring, whereas mammalian adult skin repairs by the formation of scar tissue. Thus, it is possible that the perfect wound healing (skin regeneration) and limb regeneration in amphibians have the same molecular basis, including *prx1* re-activation, and that molecular mechanism of skin wound healing in mammals is different from that in amphibians. Accumulation of molecular information and techniques in *Xenopus* enables us to address many unsolved issues regarding limb regeneration and wound healing. Our goal is successful limb regeneration in mammals through bridging mammalian wound healing to amphibian skin regeneration, as is closely associated with limb regeneration in amphibians.

Regeneration in Newts: Lessons from Transcriptomic Analysis
Panagiotis Tsonis, Ph.D.

Among vertebrates, the regenerative abilities of newts have no parallel. No other vertebrate comes close in regenerating their limbs, heart, brain, spinal cord and eye tissues. Naturally the field was in much need to delineate patterns of gene expression that correlate with regeneration. In the past few years work from our lab and from others has succeeded in sequencing whole transcriptomes from regenerating tissues. The patterns that emerge provide very important insights into the mechanism of regeneration. I will be discussing common and unique expression profiles from different regenerating tissues.