BRS Liverpool 29 June – 1 July 2016

Invited speaker abstracts

IS1

Identifying and tracking early cells of the osteoblast lineage in vivo

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Most studies of putative osteoblast precursors involve analyzing the properties of cells grown in culture or in non-physiologic conditions, such as after subcutaneous implantation. With the advent of lineage tracing strategies, it has become possible, for the first time, to identify cells in bone that can be shown to be precursors of osteoblasts in normal homeostatic conditions. These strategies use transgenic promoters to drive the expression of cre recombinase, often with cre covalently linked to a mutant form of the ligand-binding domain of the estrogen receptor that can only be active in the presence of tamoxifen (cre-ERt). We chose to use promoters (those from the genes encoding collagen II or SOX9) active in mesenchymal condensations, because we hoped that these promoters might mark early bone precursors not only in embryonic life but perhaps also in adult mice.

We found that coll II-creERt, combined with a tdTomato reporter (fluorescent only after the action of cre recombinase) marked perichondrial cells in condensations that, over time, became both collagen I-expressing perichondrial cells as well as cells that streamed into the developing bone, along with blood vessels, to form the precursors of trabecular bone. When one dose of tamoxifen was given three days after birth to mice bearing either the coll II-creERt or the SOX9-creERt transgenes, marrow stromal cells and new osteoblasts continued to be generated for as long as 1.5 years. These same cells became CXCX12-expressing stromal cells, osteoblasts, osteocytes, and adipocytes over time. When mice were given parathyroid hormone 1-34 (PTH) in an "anabolic" protocol, the number of tdTomato osteoblast precursors and osteoblasts substantially increased in number after 3-7 days. After several weeks of administration of PTH, followed by cessation of PTH administration for several more weeks, the amount of fat in the proximal metaphysis of the tibia increased dramatically. Many of those adipocytes were descended from cells marked with tamoxifen administration many weeks earlier (when the metaphysis had no discernible fat.) Thus, we have identified growth-associated stem cells that, over time become chondrocytes, osteoblasts, and adipocytes. We can now study the regulation of the fates of these cells.

Disclosure: Amgen: sponsored research and consultation. Novartis: consultation. Chugai: sponsored research and consultation

IS2

Discovery and clinical development of abaloparatide for the treatment of postmenopausal osteoporosis

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Abaloparatide is a novel 34 amino acid peptide selected to be a potent and selective activator of the parathyroid hormone receptor 1 (PTHR1) signaling pathway with 41% homology to PTH(1-34) and 76% homology to PTHrP(1-34). Abaloparatide is differentiated from PTH and PTHrP ligands based on its high affinity and >1,000-fold greater selectivity for the RG (vs R0) conformation of PTHR1 which favors bone anabolism with a limited effect on bone resorption. In nonclinical models of ovariectomy-induced osteoporosis in rats and monkeys, abaloparatide treatment restored BMD through increased bone formation, without a significant effect on bone resorption, consistent with a predominantly bone anabolic mechanism of action. These findings correlated with increased biomechanical bone strength at vertebral and nonvertebral sites. ACTIVE was a double-blind, active comparator Phase 3 clinical trial with treatment with abaloparatide-SC, placebo or teriparatide in 2463 postmenopausal women with osteoporosis for 18 months. Abaloparatide significantly increased BMD and significantly reduced new vertebral fractures by 86%, nonvertebral and all clinical fractures by 43% and major osteoporotic fractures by 70% compared to placebo. Kaplan-Meier curves indicated early separation between abaloparatide and placebo for nonvertebral, clinical and major osteoporotic fractures. Compared to teriparatide, abaloparatide treatment resulted in significantly greater gains in BMD at the femoral neck and total hip, and reduced major

osteoporotic fractures. Subjects who completed 18 months of treatment with either abaloparatide or placebo in the ACTIVE trial, were eligible for up to 24 additional months of treatment with alendronate (70 mg/wk). A total of 1139 (91%) of eligible subjects from ACTIVE were enrolled in the ACTIVExtend trial. Over the 25 months of treatment, including the first 6 months of ACTIVExtend, there was an 87% relative risk reduction of new vertebral fracture, a 52% reduction in nonvertebral fracture, and a 58% reduction in major osteoporotic fracture risk in the abaloparatide/alendronate group, compared to placebo/alendronate group. The overall results suggest that abaloparatide may provide an effective treatment option in the management of postmenopausal osteoporosis.

Disclosure: Employee and shareholder - Radius Health

IS3

Heavy mice and lighter things: using solid-state NMR spectroscopy to probe the molecular structures in bone

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The detailed molecular structures of the extracellular molecules in bone determines how those molecules self-assemble into e.g. the fibrillar structures that provide mechanical strength in the tissue, whilst ligand protein binding sites within the extracellular matrix are essential for cell communication, adhesion and mobility. NMR spectroscopy information on local molecular geometries of the major components of the matrix, the accessibility of ligand protein binding sites, the molecular mechanical properties that ultimately are responsible for the bulk material properties and not only the atomic structure of the mineral components in bone, but what binds them to the organic matrix.

An NMR spectrum for a given NMR-active nucleus, i.e. ^{13}C, consists of a signal from each chemically-distinct ^{13}C nucleus in the sample at characteristic frequencies for the chemical structure and local molecular geometry, allowing basic composition and structural information to be immediately obtained. Considerably more structural information is available in two-dimensional NMR spectra in which the NMR signals of two (for instance, ^{13}C) nuclei are correlated, showing which ^{13}C nuclei are close in space. At the most basic level, even without any interpretations of the correlation signals, a 2D ^{13}C-^{13}C correlation NMR spectrum gives a "fingerprint" of the molecular structures in the sample and their conformations – which means easily accessible comparisons of molecular structures between tissues. Spectral editing techniques using e.g. paramagnetic labels, allows further refinement of the spectral information content, and information on, e.g. the accessibility of ligand binding sites.

Key to the success of NMR methods here is to be able to introduce NMR-active nuclei, ^{13}C and ^{15}N, that have low natural abundance, into the tissue. This talk will describe how we have done this and what it has achieved in terms of new information on the organic matrix structure in bone.

A combination of ^{31}P, ^{13}C and ^{1}H NMR experiments in conjunction with high-level molecular modelling has informed not only on the mineral structure in bone, but on its interface with the surrounding organic matrix. I will describe our work here and hypothesise about the importance of small organic acids in bone mineral.

Disclosure: None declared

IS4

Tidal dynamics in the structure of the bone cartilage interface

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Background: Normal joint function is dependent on the maintenance of interfaces between hyaline articular cartilage (HAC) and articular calcified cartilage (ACC) and ACC and bone. Regulation of the ACC mineralising front and processes that influence ACC resorption and replacement by bone are not well understood. Mechanical overload is a risk factor for osteoarthritis (OA) although mechanisms linking trauma and OA are poorly characterised.

Material: the osteochondral junction in man, mouse, rat, rabbit, elephant and horse.

Tissue processing: embedding in PMMA to maintain integrity of hard and soft tissue junctions: avoidance of demineralisation and microtomy: micromilling or polishing of block surfaces: removal of mineralised tissue to leave a plastic cast of the soft tissue/cell space (Figure 1): or maceration of unembedded tissue to remove all cells and non-mineralised matrices (Figure 2).

Microscopy: 2D, 2.5D or 3D backscattered electron scanning electron microscopy: confocal scanning light microscopy: embed in iodinated resin: stain with triiodide solution: dry staining with elemental iodine vapour: x-ray microradiography: x-ray microtomography: nano-indentation.

Findings: Equine exercise/training experiments establish that the regulation of the rate and density of calcification of ACC occurs at the mineralising front and is influenced by exercise and position in the joint, indicating that loading conditions affect ACC parameters. Exercise inhibits vascular invasion of ACC.

Extension of cutting cones through ACC decreases the attachment of HAC to bone and is a major feature in human ageing.

Responses of bone to overload exercise in large mammals involves a repair mechanism whereby microcracks are sealed by the intrusion of very dense, acellular, mineralised matrix: this may extend into HAC from the ACC mineralising front. The resulting high density mineralised protrusions have been shown in several human and equine joints, and may even occupy full thickness HAC. This hard, dense material may fragment, migrate within the HAC and eventually enter the joint space as an abrasive.

Difficulties in producing good histological imaging of HAC, ACC and the marrow space compartment of the subchondral bone are diminished using undemineralised tissue, studying the block face, SEM, and iodine staining methods, all approaches highly suited to the investigation of normal and diseased joints.

Figure 1. Dynamic resorptive and formative activity of cutting cones in the subchondral region can be inferred from study of resin casts of soft tissue space. PMMA cast of palmar condyle of 2 year old Thoroughbred racehorse.

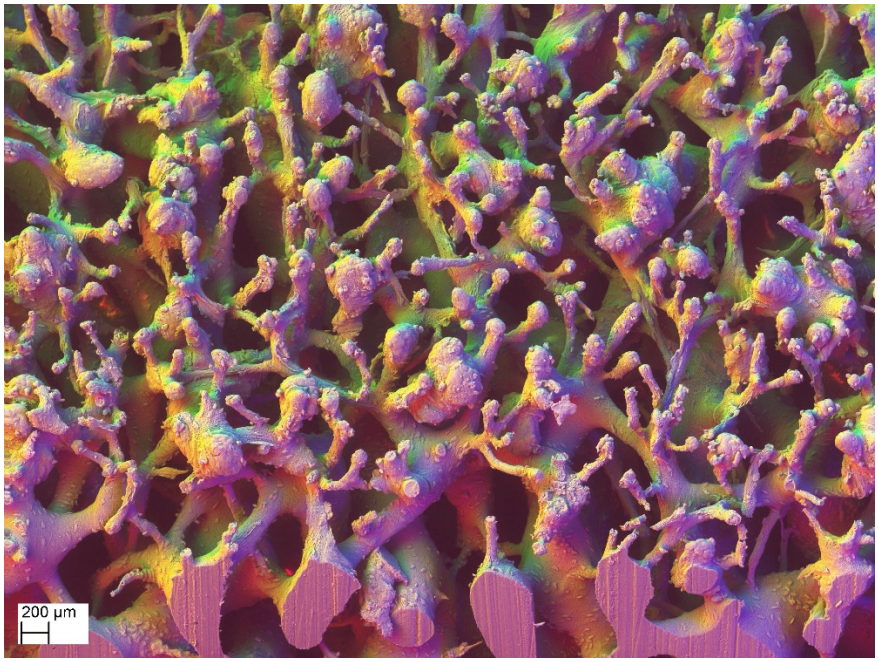
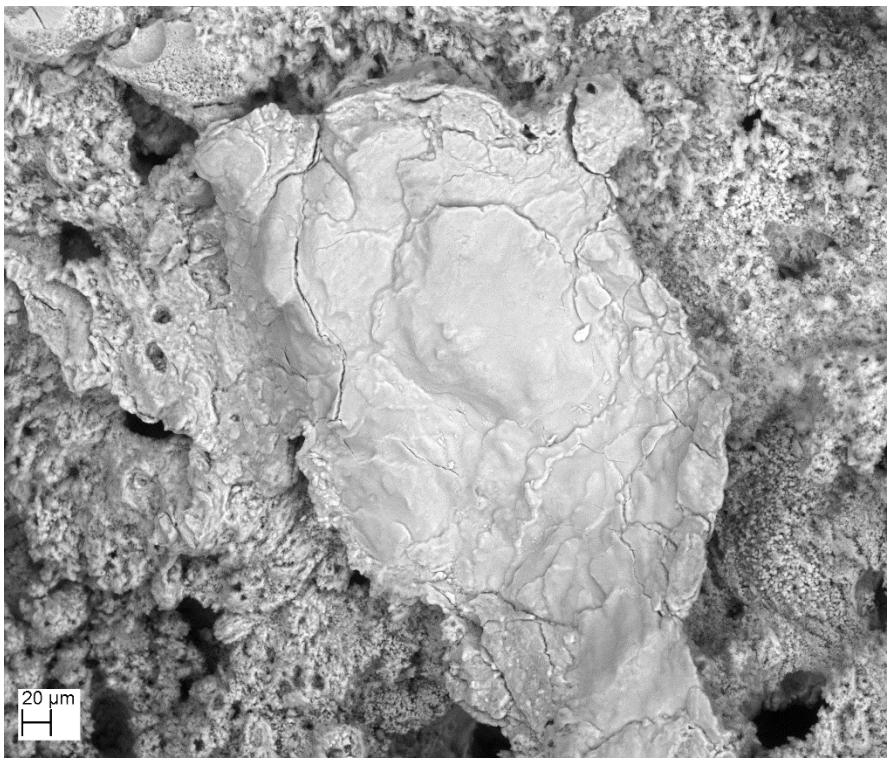


Figure 2. Sodium hypochlorite bleach macerated human osteoarthritic patella showing a high density mineralised protrusion from the ACC mineralising front.



Disclosure: None declared

IS5
Cartilage and bone biomarkers in osteoarthritis
Virginia Byers Kraus, Duke University School of Medicine, Durham, USA

Osteoarthritis (OA) is disorder of the whole joint organ. The cartilage and bone play reciprocal and vital roles in OA pathogenesis, progression and symptomatology. This is well illustrated by gene expression analyses of cartilage and site-matched bone revealing that 27 (of 61) candidate genes were coordinately up- or down-regulated in both tissues in a new human model of OA progression (Chou 2013a). Based on a subsequent 44 k microarray (Agilent Technologies), many of the most significantly regulated genes identified in bone have documented roles in the pathogenesis of OA, arthritis or bone formation including POSTN, ASPN, COL6A3, COL3A1, OGN, DIO2, TNFSF11 (upregulated) LEP, APOB (downregulated) (Chou 2013b). Urinary alpha-CTX, reflecting turnover of newly synthesized bone, is localized to high turnover areas of OA subchondral bone, is associated with dynamic bone turnover of knees as signified by scintigraphy, and is associated with progression of both osteophytes and radiographic joint space narrowing (JSN) (Huebner 2014). Moreover, the progression of the combination of pain and knee JSN is also predicted by bone biomarkers, including by alpha-CTX (Kraus 2016, in press). Based on recent data, serum TRAP5b activity that likely reflects osteoclast activation during bone resorption, is also associated with knee OA-related pain, subchondral sclerosis and pain worsening (Nwosu 2016). Taken together, these and other data support the involvement of bone turnover in the generation of pain and structural change in OA that is modifiable by bone acting agents.

Disclosure: None declared

IS6

Challenges of imaging muscle and bone – an evolutionary and biomechanical perspective

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Investigating the musculoskeletal system on an evolutionary timescale poses many fascinating conceptual and technical challenges that necessitate innovative solutions of broader application and significance. Not least of these challenges is how, in the absence of genetic data and with limited soft-tissue evidence, to demarcate the boundaries between intraspecific phenotypic plasticity and interspecific variations and thus infer the taxonomic affinities and functional capabilities of extinct species. The present paper explores the approach of investigating extant wildtypes and experimental models with novel imaging and computational methods to discover and map the relative capacity for morphological and functional plasticity. These data can then form a framework for interpreting extinct, fossil, forms. Approaches considered range from integrated MRI and CT and contrast-enhanced microCT through to geometric morphometric shape analysis and finite element analysis.

Disclosure: None declared

IS7

Bone muscle interactions and vitamin D

Paul Lips, Endocrine Section, Department of Internal Medicine, VU University Medical Center, Amsterdam, the Netherlands.

Bone and muscle are highly interrelated. Muscle atrophy due to neuromuscular diseases or nerve trauma leads to bone loss and osteoporosis. On the other side, exercise can increase muscle mass and strength, leading to bone growth and increased bone mineral density. The bone-muscle interrelationship uses humoral mechanisms for communication either way. These mechanisms are partially controlled by vitamin D. The skeleton serves as calcium reservoir, and adequate control of serum calcium level is crucial for neuromuscular function.

The active vitamin D metabolite 1,25–dihydroxyvitamin D (1,25(OH)2D) stimulates calcium absorption from the gut. Bone mineralization itself is a passive process, that can proceed without 1,25(OH)2D. The 1,25(OH)2D stimulates bone resorption by osteoclasts. It also stimulates osteoblasts. The vitamin D receptor (VDR) is present in bone cells. When the VDR is mutated in the mouse, the VDR null mouse, this leads to rickets, decreased longitudinal growth and muscle atrophy. Overexpression of the VDR increases bone formation.

Vitamin D is related to physical performance. In case of severe vitamin D deficiency, poor bone mineralization is coupled to muscle weakness leading to falls and fractures. This can be reversed by vitamin D supplementation.
Muscle tissue secretes myostatin, which inhibits muscle hypertrophy. Vitamin D decreases myostatin and this decrease is associated with higher bone mass. Vitamin D stimulates vascular endothelial growth factor (VEGF) and IGF-1 synthesis in muscle, and these growth factors can play an anabolic role in bone. The cytokine interleukin 6 (IL-6) is secreted by muscle following exercise. It may stimulate bone resprption, but it is decreased by vitamin D.
Osteocytes also produce VEGF and IGF-1 which can stimulate muscle growth. Osteoblasts produce osteocalcin, important in the final phase of bone formation and mineralization. It may also have a role in carbohydrate metabolism and mitochondrial function in muscle. Osteocalcin is regulated by vitamin D. Osteocytes produce sclerostin in the last phase of osteon formation, where it suppresses further bone growth. When absent as in sclerosteosis, bone formation continues with very high bone mass and neurological complications as a consequence. Sclerostin decreases after muscle loading and increases after vitamin D treatment.
These examples confirm the tight interaction between bone and muscle. Knowledge of this interrelationship is important for therapeutic interventions in bone and muscle diseases and during aging when osteoporosis and sarcopenia occur.

Disclosure: Advice to Friesland-Campina (dairy industry)

IS8

Novel roles for stem cells in skeletal muscle adaptation and aging

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Satellite cells are the primary stem cell in skeletal muscle, required for postnatal muscle growth and adult muscle regeneration. Satellite cells are activated to proliferate and normally contribute nuclei to growing myofibers in response to hypertrophic stimuli. We utilized the discrete expression of Pax7 in satellite cells to develop the Pax7-DTA mouse, whereby the use of Cre-lox technology allows for the specific and inducible depletion of satellite cells following tamoxifen-induced expression of diphtheria toxin. A detailed analysis of multiple muscles in sedentary mice revealed that, despite reduced regenerative capacity, the life-long reduction of satellite cells did not accelerate nor exacerbate sarcopenia. However, life-long satellite cell depletion was associated with increased extracellular matrix accumulation in muscle. To examine the role of satellite cells in muscle adaptation, synergist ablation surgery, where removal of synergist muscles places functional overload on the plantaris, was used to stimulate robust hypertrophy. Myofibers hypertrophied in the absence of satellite cell fusion, although growth was eventually blunted, associated with fibrosis and diminished force production. Myofiber hypertrophy was impaired in aged mice regardless of satellite cell content. Myonuclear accretion occurred in response to functional overload, which was prevented by satellite cell depletion, demonstrating that myonuclear addition is insufficient to drive myofiber hypertrophy. These data argue against satellite cell contribution to the maintenance of muscle size or fiber type composition during aging; however, satellite cells regulate the muscle environment, and their loss during aging may contribute to fibrosis, particularly during periods of remodeling.

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Disclosure: None declared

IS9

Breast cancer bone metastasis: Biomarkers and beyond

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Our studies within breast cancer bone metastasis (BCBM) have two aims: (1) to discover biomarkers which can identify patients at greatest risk of developing bone metastases who would benefit most from adjuvant bone-targeted drug therapy and, (2) the elucidation of key regulatory molecules within the mechanism of BCBM that may have potential as novel drug targets.
This talk will present highlights of our research programme aimed at tackling the challenges of biomarker discovery and identification of key BCBM regulators. We have applied proteomic techniques to analyse human BCBM cell models leading to the identification of a number of potential biomarkers, three of which have been clinically validated. We have also identified potential regulators of BCBM that have promise as novel drug-able targets and biomarkers.
Using breast tumour tissues collected from patients recruited to the phase III AZURE trial (BIG01/04-ISRCTN79831382), we have correlated expression of three of these proteins with bone metastatic outcomes. In one study, high expression of the proteins CAPG and GIPC1 in patients who did not receive zoledronic acid was found to be significantly associated with first recurrence of cancer in bone (hazard ratio [HR] = 4.5, 95% confidence interval [CI] = 2.1 to 9.8, $P < .001$). Similarly, in a second study, we found that high expression of an additional protein was prognostic for skeletal recurrence (HR 2.13, 95%CI 1.06-4.30, p=0.034). We are currently assessing other proteins for biomarker potential with the aim to develop a definitive panel of biomarkers for clinical application.
Bioinformatic analysis of protein expression levels in our cell models has led to the identification of many functionally-interconnected proteins specifically associated with the bone-homed cell-phenotype. Within these a core group of 11 proteins demonstrate promise as potential regulators of bone-homing in our model owing to their established cellular functions including cell cycle control, signal transduction and tumour suppression. These proteins have potential as drug-able targets for which inhibitors are currently available, and nine also have potential to act as biomarkers of BCBM. We are currently pursuing further work to extend these studies and test the potential of these proteins to impact upon clinical practice.

Disclosure: None declared

IS10

Role of LOX in bone metastasis

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Bone is the primary site for many cancer types and can account for up to 80% of cancer-related deaths in certain tumours. The progression from a discrete solid primary tumour to devastating and painful bone metastases is a complex process involving multiple cell types and steps. There is increasing evidence that modulation of the extracellular matrix (ECM) plays an important role in this process. However, it is not know which occurs first, abnormal ECM, which supports secondary tumour formation, or the arrival of cancer cells into the bone that create abnormal ECM. Recently it has been shown that tumour-derived factors circulate the body and exert effects on ECM remodelling within distant organs, creating so-called pre-metastatic niches. One such factor is Lysyl oxidase (LOX), which is highly expressed by invasive/metastatic cancer cells, enhances tumour progression and is high in patients with lower metastasis-free survival. LOX is critical for pre-metastatic niche formation in soft-tissue (lungs, liver and brain) enhancing bone marrow-derived cell invasion and thereby enabling colonisation of metastasising tumour cells. Recently we have begun to unravel the role of LOX in the bone pre-metastatic niche, in particular the early events governing osteolytic lesion formation. Using multiple *in vitro* and *in vivo* models, and a large clinical cohort, we show that LOX gene expression is significantly correlated with osteotropism and bone

relapse. We show that high expression of LOX in primary breast tumours or systemic delivery of LOX *in vivo* leads to osteolytic lesion formation, and that silencing or inhibition of LOX activity abrogates this. The enzymatic activity of tumour-secreted LOX affects both osteoclasts and osteoblasts, disrupting normal bone homeostasis leading to bone lesion formation. These changes and lesions occur prior to tumour cell arrival in the bone and act to provide the initial foothold for circulating tumour cells to colonise the niche and form bone metastases. Mechanistically, we identify tumour-secreted LOX as a novel regulator of osteoclastogenesis through NFATc1 transcription factor translocation. In summary, we have uncovered a novel step in bone metastasis and mechanism of bone homeostatic regulation, opening up new opportunities for therapeutic intervention with important clinical implications.

Disclosure: None declared

IS11

Modeling the skeletal maladies in Neurofibromatosis type 1

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Forty percent of patients with Neurofibromatosis type 1 (NF1) present with skeletal manifestations, which include dystrophic scoliosis and tibia bowing and non-union after fracture (pseudarthrosis or TPA). The natural history and pathogenesis of these skeletal abnormalities are poorly understood. Therapeutic options are limited and mainly involve bracing strategies and invasive surgeries when fracture occurs. Attempts to stimulate bone union via local BMP treatment have been made but clinical outcomes are still poor.

Diseased bony tissues from these cases are rare and difficult to obtain, hence we have developed over the years a number of mouse models with the goal of identifying the cell of origin leading to NF1 skeletal dysplasia and pseudarthrosis. These studies revealed that these skeletal defects are likely to result from primary osseous abnormalities of endochondral bone formation, caused by loss of *Nf1* function in osteochondroprogenitor cells. They uncovered a number of potential new therapeutic targets by identifying the functional and molecular abnormalities of osteoprogenitors lacking *Nf1*, and provided valuable pre-clinical mouse models as well, which are currently used to assess the efficacy of targeted drugs in improving bone healing and union in NF1.

Disclosure: research grants from NIH, DOD

IS12

Cell therapies and regenerative medicine - the dawn of a new age or more hype than hope?

Anthony Hollander (Liverpool, UK)

Anthony Hollander (University of Liverpool), Martin Birchall (University College London), Ashley Blom (University of Bristol), Michael Whitehouse (University of Bristol), Jonathan Eldridge (North Bristol NHS Trust).

Methods for deriving chondrocytes from mesenchymal stem cells (MSCs) have been developed in a number of laboratories, but turning these methods into clinical therapies has been particularly challenging. We have been able to develop an optimised protocol for cartilage tissue engineering from MSCs. Whilst the original purpose of our work was to develop treatments for cartilage degradation in osteoarthritis, we were able to adapt our method in order to engineer a functional airway that was successfully implanted into the left bronchus of a patient with bronchomalacia). We have also developed a stem cell therapy for torn meniscal cartilage that has been tested in 5 patients. Turning these ad hoc therapies into routine clinical practice will however take a lot more work to help us develop treatments that are safe, effective and low-cost.

Disclosure: Azellon Limited: Co-founder and Director