MEETING REPORT FROM THE IBMS HERBERT FLEISCH WORKSHOP

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The first Herbert Fleisch Workshop was held in Brugge, Belgium on March 16 to 18, 2014. The aim of this initiative of the IBMS Board was to attract involvement of predominantly young investigators (including graduate students, postdocs, and young faculty), brought together with peers and a few senior scientists, with the main objectives of having them discuss science in progress, to have them network with other scientists with similar interests and complementary expertise, and to showcase unpublished data to obtain constructive feedback and comments.

The only invitees were six senior investigators, each of whom presented a state-of-the-art lecture on a topic related to their work. Most importantly, all were available throughout the meeting as mentors, to comment, critique and advise regarding the work of the young investigators. All 80 submitted abstracts were presented as posters throughout the meeting, and 30 of these gave oral presentations. With total participants a little over 100, the meeting achieved throughout the few days a level of formal and informal discussion that was rewarding for all.

The naming of this event as the Herbert Fleisch Workshop reflected its genesis as a support for young scientists in the field of bone and cartilage research, and the legacy of the late Herbert Fleisch: the Davos meetings that he organised over several decades until 2006 were notably supportive and encouraging of young scientists in the field. As a site for this small meeting, Brugge was an admirable choice, lacking the snow attraction of Davos, but presenting the beauty and history of a significant part of Europe. In honouring the memory of Herbert Fleisch in this way, we were very pleased that his wife, Maria Pia, and two of his daughters, Marie Gabrielle and Isabelle,

attended the opening of the Brugge meeting. In addition the meeting was attended by Segvi Rodan and by 5 (past) presidents of IBMS.

The plenary lectures of each of the senior scientists highlighted what was the latest in the fields covered by their respective expertise, from basic science at the cellular, genetic and molecular level over translational research and clinical applications of new therapeutics. They presented the findings in a context that was optimal as a teaching exercise, and that set the scene for discussion of the oral and poster presentations.

Henry Kronenberg (Massachusetts General Hospital, Boston, MA, USA) focused his presentation on the work of his group on the cells of the osteoblast lineage, providing insights into their functions and flexibility, and the need to identify the ultimate precursor cell *in vivo* – the skeletal stromal cell. He indicated the role of osteolineage cells in providing support for hematopoiesis, particularly focusing on B lymphocyte development (1), and ways in which parathyroid hormone (PTH) can use the osteoblast lineage to produce its anabolic effect, including "waking up" the lining cells to resume an anabolic activity (2). Particularly instructive was the use of lineage tracing models with different gene promoters labeling subsets of cells and different reporter readouts marking the fate of the labeled cells at later time points, a strategy used to explore the relationship between fetal life and adult homeostasis and repair settings. In one such setup, inducible mutagenesis was used to obtain postnatal ablation of β-catenin in osterix-producing cells, which was shown to lead to predominant adipocyte formation from osterix-expressing cells. This supports the conclusion that once the cell starts differentiating into the osteoblast lineage, continued β-catenin

signaling is needed for it to stay on that pathway (3). Recent ongoing work includes the use of other gene promoters, including the nestin promoter (4), to mark earlier cells and follow their programming in the lineage, in search of the skeletal progenitors that could be key to modulating the provision of osteoblasts for bone formation and repair.

Among submitted abstracts, Deepak Balani (Boston) elaborated on this theme. Lineage tracing was used to show that the collagen-II promoter, driving tamoxifen-inducible CreERt mice, labels (among cells with other fates) osteoblast progenitors that differentiate into osteoblasts. as evidenced by the double labeling of Rosa26-Tdtomato reporter-identified cells with Collagen type I-GFP or osteocalcin-GFP. Moreover, treatment with PTH increased the abundance of such double-labeled cells. Garyfallia Papaioannou (Boston) further showed how Ras signaling activation in osteoprogenitor cells marked by either collagen-II- or osterix-driven CreERt expression dramatically increased the amount of bone and the number of stromal cells.

A matter of increasing interest in the field is the relationship between fat and bone, the subject discussed by **Clifford Rosen (Maine Medical Center Research Institute, Scarborough, ME, USA)**. The various types of fat tissue – including the white adipose tissue of the visceral fat deposits, brown inter-scapular fat, marrow fat in the skeleton – are each characterized by specific properties related to the mitochondrial en energy pathways they use, and they stand in varying correlations to bone. The relationship of marrow fat to bone is also context-specific, for example if mice are severely calorie-restricted there is an increase in marrow fat and they lose bone, as happens in patients with anorexia nervosa. Ageing as well correlates with loss of bone volume and

increased marrow fat. Another example was discussed later in the session by Anneke Greetje Veldhuis-Vlug (Amsterdam), who showed the results of a prospective study in 10 women revealing quite dynamic changes in bone marrow adiposity during the menstrual cycle.

Cliff Rosen further made the point that we need to understand the metabolic pathways providing osteoblasts with the energy required for their work, hence what fuel and energy pathways the osteoblast uses to differentiate and lay down collagen. Using Seahorse technology to measure glycolysis and mitochondrial function (5), energy metabolism was quantified in the context of osteoblastic differentiation in MC3T3 cells. Interestingly, cells late in osteoblast differentiation *in vitro* shift from oxidative phosphorylation of glucose to anaerobic glycolysis, at times when collagen synthesis is increased. Notably, PTH treatment increased glycolysis in differentiating osteoblasts, indicating that it was favouring this pathway of glucose utilization for the work it is stimulating. This recalls the similar observations of W.F. Neuman in the 1970's and 1980's, showing that PTH promoted lactate accumulation in media of bone organ cultures (6, 7).

A number of submitted papers were addressed to this area. Tara Brennan-Speranza (Sydney) summarized recent work showing that osteoblasts mediate the insulin resistance of glucocorticoid treatment, which could be mitigated by heterotopic expression of osteocalcin (8). She also presented data showing that increasing circulating osteocalcin levels by *in vivo* gene therapy is associated with bone loss and increased bone turnover in mice, irrespective of whether osteocalcin was used or a

mutant osteocalcin that could not be carboxylated. Karla Jade Oldknow (Edinburgh) presented work on the role of Phospho1, a bone-specific phosphatase, in the regulation of whole body lipid and energy metabolism. Using knockout mice and primary osteoblasts derived thereof, Phospho1 was found to regulate the expression of Esp, although this was not associated with altered serum levels of osteocalcin (uncarboxylated or undercarboxylated). These data thus suggested that Phospho1 deficiency improved the metabolic profile of the mice and conferred resistance to obesity and diabetes through an alternative mechanism, possibly related to the levels of circulating ceramide. Streptozotocin-induced diabetes in mice was used by Sergio Portal-Nunez (Madrid) to show that diabetes negatively affects bone structure in aged mice by a mechanism independent of oxidative stress.

The subject of bone formation control by Wnt signaling and osteocyte-derived sclerostin was discussed by **Michaela Kneissel (Novartis Institutes for Biomedical Research, Basel, Switzerland)**, and particularly how this pathway might be exploited therapeutically. The powerful effect of neutralizing, monoclonal anti-sclerostin antibodies was summarised, and it was pointed out that attempts to find small molecule drugs that could favourably influence protein-protein interactions in Wnt signaling have been so far unsuccessful. The anabolic effect of anti-sclerostin is reversible, with bone lost after cessation of treatment, at about the same rate as it had been gained. This emphasizes the continuing need for safe, effective anti-resorptive drugs in treatment of osteoporosis, even with such a powerful anabolic. Some intriguing questions are raised with anti-sclerostin treatment. First, the increase in bone formation markers after anti-sclerostin treatment is only transitory (9), whereas it is maintained with PTH treatment

(10). Second, it is notable that there is a significant, sustained reduction in resorption markers with anti-sclerostin treatment (9), perhaps due to an increase in production of osteoprotegerin associated with increased Wnt signaling (11). Based on the decreased bone matrix mineralization seen in *sost* knockout mice, the possibility was raised that anti-sclerostin might be useful in the hyper-mineralisation of osteogenesis imperfecta ('brittle bone disease', mostly caused by mutations in collagen type I, and often associated with elevated matrix mineral content). Other applications of anti-sclerostin therapy that were discussed include adult-onset hypophosphatasia ('soft bone disease', caused by mutations in the alkaline phosphatase gene ALPL), fracture repair, osseo-integration of dental implants, and periodontal regeneration. Thus, this lecture covered a range of new biology and medicine that provided exciting perspectives for the participants, illustrating the rapid transit from basic discovery to applied drug that is sometimes possible.

The session proceeded with presentations work related to osteocyte biology and quantification,. In support of the notion that the influence of osteocytes extends well beyond the skeleton, Mari Sato (Sapporo) found that reducing the number of osteocytes affected remote organs and functions in mice. A diphtheria toxin (DT) mediated approach was used to render adult DMP1 promoter-driven DT receptor transgenic mice "osteocyte-less". Interestingly, the mice suffered from lymphopenia and severe thymic atrophy, as well as lipodystrophy with a progressive loss of white adipose tissues. These defects were not rescued in a parabiosis model, indicating that humoral factors were unlikely to be the cause; the question whether osteocytes may elicit effects on the thymus and peripheral fat via the central nervous system is further explored. Christina

Vrahnas (Melbourne) showed her work on the effects of ephrinB2 in osteocytes (using DMP1-promoter mediated conditional knockout mice) compared with cells earlier in the osteoblast lineage (osterix-promoter), stressing how bone remodeling and the maintenance of optimal bone mechanical properties are to a significant extent determined by delicately regulated, stage-specific signaling within the osteogenic cell lineage.

The osteocyte and sclerostin were also central to the discussion by **Jo Price (University of Bristol, Bristol, UK)**, who uses a number of valuable experimental models of bone loading to investigate the response of the skeleton to changing loads. It is well established now that loading of bone results in a rapid decrease in osteocyte sclerostin (12) and increase in bone formation, and that the reverse results from unloading of bone. The osteoblast proliferation that results from strain is blocked by sclerostin, and study of this response in *β-catenin*-deficient mice revealed an impaired proliferation response to strain. Evidence was presented that the estrogen receptor (ERα) mediates the osteogenic response to loading, with *ERα -/-* mice showing a reduced response (13-15). The great interest in this area lies in dissecting the roles of Wnt signaling components in controlling the cellular responses to skeletal strain. The questions that need to be addressed here are important ones related to bone growth and repair.

There were several presentations related to bone growth, repair and regeneration were next presented, including the following. Rana Abou-Khalil (Paris) used a non-stabilized mouse fracture model to address the role of muscle stem cells during bone regeneration, showung that physically inhibiting the contact between muscle and bone resulted in

failed repair, while surgical implantation of muscle at the fracture site enhanced bone regeneration. Using transgenic reporter readouts, cells from the muscle graft were found to contribute to the fracture callus. Bone regeneration was severely delayed in mice lacking Pax7 and in mice in which Pax7+ satellite cells (also known as muscle stem cells) had been selectively ablated by a DT approach, with delays in both cartilage and bone deposition. Altogether, the presented data made a strong case suggesting that muscle can contribute to bone regeneration through either satellite cells themselves, or via osteoanabolic growth factor products of satellite cells. An exciting submitted presentation by Saravana Ramasamy (Muenster) described a novel line of crosstalk between vascular endothelial cells of bone and osteoblasts active in the formation of bone. Two papers on this work were published in Nature the weekend of the Brugge meeting (16, 17). Notch signaling promotes endothelial cell proliferation and vessel growth in bone, whereas it does the opposite in other organs. When notch signaling was disrupted in endothelial cells (through VE-cadherin-CreERt mediated induced mutagenesis), blood vessel growth in bone was impaired, which was associated with a disturbed growth plate morphology and reduced bone formation, with loss of trabeculae and bone mass. The bone effects were rescued by treatment with noggin, which is an endothelial product of notch signaling. Induced deletion of notch signaling in osteoblasts (Collagen I-CreERt mediated) had little or no effect on bone, and the conclusion is that a secreted factor from notch signaling in the endothelium provides a stimulus to the osteoblast lineage to form bone (17). These findings greatly add to our understanding of the coupling between angiogenesis and osteogenesis, which remains a challenging but very promising route towards the improvement of bone repair and regeneration and the prevention of bone loss. Linda Vi (Toronto) approached the

problem of failing repair from the perspective of ageing. Given that the capacity to repair upon fracture diminishes with age, she explored which factors could help rejuvenate the repair process. A parabiosis model indeed indicated that fracture healing could be improved by sharing the circulation of a young mouse and an old mouse. Accelerated healing could also be achieved by transplanting bone marrow of a young mouse into an old mouse. Co-culturing bone marrow stromal cells (BMSC) derived from old mice with non-adherent marrow cells taken from young mice, improved the osteoblastic differentiation of the old cells. Further experiments indicated that F4/80+ macrophages are the prime responsible mediators of the rejuvenating effects, by secreting a soluble 'youth factor' whose identification is eagerly looked-for.

Brendan Lee (College of Medicine, Houston, USA) addressed the area of human skeletal dysplasias, and how they come about from gene defects and errors in signals from cells and from the extracellular matrix., with a number of the candidate genes for skeletal dysplasias found among transcription factors, matrix proteins and morphogenic signals. A particularly instructive example that he gave was the increased TGFβ signaling in some forms of osteogenesis imperfecta (OI). In a model of a recessive form of OI, the Crtap -/- mouse, increased TGFβ signaling was evident, and the phenotype could be rescued by treatment with a neutralizing antibody against TGFβ. Evidence along the same lines suggested that increased TGFβ signaling might also be involved in dominant OI, with the G610C model (mice expressing a mutant type I collagen) showing increased expression of TGFβ target genes. The possible mechanism explaining how alterations in collagen that cause increased TGFβ signaling may involve a proteoglycan such as decorin, which binds to collagen and can also bind TGFβ. If the

collagen mutation disrupts this proteoglycan binding, free TGFβ can be released. The possibility of increased TGFβ as a common mechanism in OI is an intriguing one. The relevance to this question of the increased bone turnover in mice transgenically overexpressing TGFβ (18) was discussed. The dysplasias are of course a collection of disorders of differing pathogenesis, each of which is uncommon, but there are useful approaches to treatment that are being developed, based on improved understanding of pathogenesis. Among those discussed were the bisphosphonates in pediatric OI, PTH (Teriparatide) in adult OI Type I, anti-sclerostin in a model of OI caused by Wnt1 mutations (19), and anti-TGFβ, possibly in combination with anti-sclerostin, in adult OI Types III and IV. The lecture also covered insights in the relevance of mutations in Notch signaling genes in skeletal disorders, osteosarcoma and bone metastasis, as well as progress in understanding the chondro-protective role of Prg4, the gene encoding the secreted glycoprotein lubricin that causes joint failure when mutated (20, 21), and its relevance to osteoarthritis (22).

Among a number of young investigator presentations, one dealt with an especially intractable and devastating disorder, fibrodysplasia ossificans progressiva (FOP). Sarah Hatsell (Tarrytown, USA) presented an inducible mouse model of this disorder, in which global expression of the mutated gene (ACVR1, also known as ALK2, encoding a BMP signaling receptor) was activated in adult mice upon administration of tamoxifen (23-25). The mice displayed spontaneous, dense heterotopic ossification of the axial skeleton, long bones and thorax, resembling the progressive heterotopic ossification seen in FOP. This is an early step towards understanding the molecular and cellular processes that result in FOP.

Although skeletal aspects of cancer were not strongly represented among the submitted abstracts, a number of presentations comprised a session on multiple myeloma. Julia Paton (Sheffield) discussed a new approach to growing human myeloma cells in NOD/SCID/GAMMA mice, and Michaela Reagan (Boston) talked about growing myeloma cells *in vitro* and *in vivo* in silk scaffolds that allow assessment of spatial and temporal growth of myeloma in a bone-like environment.

The impact upon the field of the discovery of regulation of Wnt signaling in bone has been enormous, so not surprisingly, another of the major presentations was on this subject, with **Roland Baron (Harvard School of Dental Medicine, Boston, MA, USA)** providing some intriguing new data that illustrates how much more we still need to learn about this pathway. An example is his account of the R-spondins (RSPO1-4), which are co-activators of Wnt signaling in the presence of Wnt ligands, by binding to the receptors together with the Wnt ligand. RSPO3 appears to be the most important R-spondin in the skeleton and in osteoblasts. However, heterozygous loss of RSPO3 (in RSPO3+/- mice) results in increased bone formation and a higher bone volume; *in vitro* experiments using knockout cells indicate that Wnt pathway signaling through b-catenin is increased in the absence of RSPO3. The latter might thus be another inhibitory target to promote bone formation. Some data was summarized on contributions of the non-canonical Wnt signaling pathway. Wnt5a as an osteoblast product promotes RANK production in osteoclast precursors through non-canonical signaling, and thereby promotes RANKL-induced osteoclast formation (26), whereas it acts also within the osteoblast lineage to promote bone formation (27).

Most of the matters discussed by Roland Baron were directly pertinent to mechanisms of bone remodeling, which were the subject of much discussion in submitted abstracts. Pia Rosgaard Jensen (Vejle, Denmark) showed data on the canopy, a structure considered as a source of osteoprogenitors that has been shown to cover bone multicellular units (BMUs) in human and rabbit bone (28, 29). The canopies were studied in sections of lumbar vertebrae of rabbits treated with either alendronate or the cathepsin K inhibitor, odanacatib. Alendronate, which inhibits bone formation as well as resorption, reduced the extent of canopy coverage over osteoclasts, whereas odanacatib, which inhibits resorption without killing osteoclasts, did not reduce canopy coverage. This was interpreted to suggest that coupling of bone formation to resorption is better maintained with cathepsin K inhibition than with a bisphosphonate. Among other interesting local events in bone, Isabel Orriss (London) showed that activation of the P2Y2 receptor in osteoclasts led to increased ATP release from the cells. P2Y2 -/- mice had increased bone, with the suggestion that local ATP release is a necessary stimulus to osteoclast activation, and its ablation impairs activity. Kazuki Inoue (Matsuyama, Japan) explained the DNase-sequencing approach he took to discover novel RANKL-induced transcription factors that play roles in osteoclast differentiation, and showed how in vitro knockdown of a number of these factors drastically reduced osteoclastogenesis (30).

The poster sessions allowed extensive presentation of all abstracts (whether orally presented or not) and generated lively discussions among the young participants and in their interactions with the senior scientists. There were many particularly pleasing

aspects of the Brugge meeting. First, it was attended by a most enthusiastic group of around 100 young scientists from many countries, including several European countries, the USA, Japan, Australia and New Zealand. Second, they responded enthusiastically to the opportunity to interact with each other, and particularly, to get the close attention of the senior scientists as mentors and discussants. Third, the senior scientists fulfilled their mentoring role gracefully and devotedly – they gave inspiring lectures, and vigorously interacted with the young participants during the oral and poster sessions as well as informally over coffee breaks and meals. This interaction was highly appreciated by the attendees as revealed by a post-meeting questionnaire. Finally, Brugge was a wonderful place to hold such a meeting – a most beautiful, ancient European city, small and attractive, and with a venue that made it easy to keep the Workshop participants together as a communal group. Herbert Fleisch would be the first to agree that it substitutes well for Davos, of course without the snow.

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