

## **Cartilage Metabolism and Cell Based Therapies for Tissue Regeneration**

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The Autumn Meeting was held at The School of Biosciences, Cardiff University on the 8<sup>th</sup> and 9<sup>th</sup> of September, 2008. The meeting entitled “**Cartilage Metabolism and Cell Based Therapies for Tissue Regeneration**” was held to honour the scientific achievements of Professor Tim Hardingham (Wellcome Trust Centre for Cell: Matrix Research, Manchester University) upon his retirement. The meeting focused on Tim’s key interests as defined by his research career including matrix metabolism and tissue engineering strategies for cartilage repair. The meeting was organised and hosted by Dr Emma Blain and Dr Clare Hughes, and attracted 193 delegates (one of the largest BSMB meetings ever) and financial support was gratefully received from: Smith and Nephew, Arthritis Research Campaign, The Company of Biologists, The Matrix Biology Institute, MD Biosciences, Stryker, Seikagaku and Flexcell™ International. Trade exhibits were displayed by Jencons and Millipore.

Tim’s contribution to the field of matrix biology was not only celebrated through the scientific programme but by a dinner dance held at the Millennium Stadium (home to the current Six Nations Champions!). A Welsh theme interweaved itself through the menu of the four-course dinner culminating in the much anticipated speeches. The BSMB Chairman Professor Bruce Caterson started off the speeches, and as we have become accustomed to, had everyone up and down in their chairs continually “raising a glass” – of the customary port! Further speeches by past and current co-workers of Tim including Professor Martin Humphries (see the ditty penned by the Manchester crew on the coach to Cardiff on the BSMB website) were eventually curtailed before Tim gave a humorous and warmly received series of acknowledgements and anecdotes from his career. With everyone sufficiently relaxed from the wine and port (!), the evening’s entertainment was provided by a ceilidh band called Cats Claws. A special mention to the Manchester trio of Professor Martin Humphries, Dr Dave Thornton and Dr Alan Murdoch for their most impressive commitment to lasting the longest on the dancefloor! And equally to the younger BSMB members for their enthusiasm in embracing the “do-si-do” so energetically!

### **Session One – Cartilage Metabolism**

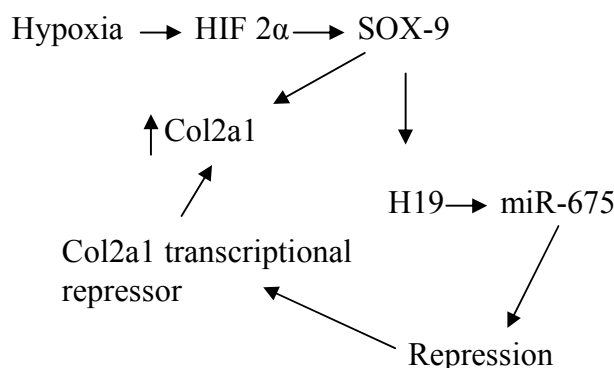
Co-chaired by Professor Vic Duance (Cardiff University) and Dr Liz Canty-Laird (Manchester University), the first session was opened by **Professor David Eyre** (University of Washington,

USA) with a presentation entitled 'Cartilage Collagen: Evolution of a Complex Polymer'. He began by briefly describing how all different types of cartilage share variations on a distinctive "network" fibrillar organization. He then focused on adult articular cartilage which consists largely of type II collagen fibrils which are formed as a heteropolymer where collagen IX molecules are covalently linked to the surface and collagen XI acts as a template driving the assembly of the collagen fibers. Both collagen IX and collagen XI are essential for collagen II fibril formation since mutations in the genes corresponding to these proteins cause chondroplasia syndromes and early onset of Osteoarthritis. Professor Eyre then talked about how research into the site specific interactions between different molecular types of collagen and into the evolution of the collagen gene families can reveal how individual collagen molecules and their post-translational features have contributed to fibril development. He then described how collagen type XI is a very important molecule in the process of collagen II heteropolymer formation and how differences in the chain composition of collagen XI vary with tissue type. Professor Eyre and his colleagues used techniques such as ion exchange chromatography and ion trap mass spectrometry to assess the chain compositions of collagen XI and V molecules within type II collagen heteropolymers in different tissues and found that adult articular cartilage has a chain composition of  $[\alpha 1(XI) \alpha 1(XI) \alpha 3(XI)]$  yet epiphyseal cartilage has a chain composition of  $[\alpha 1(XI) \alpha 2(XI) \alpha 3(XI)]$ . More fibrous tissues such as bones and tendons have a chain composition of  $[\alpha 1(V)_2 \alpha 2(V)]$  and the nucleus pulposus contain collagen XI and V chains together. These data suggest that the different compositions of the collagen II template result in diversity of the fibrillar forms in different tissue types. Collagen IX can covalently cross-link to Collagen II and V/XI fibrils and different linkages between these molecules may affect the shape and aggrecan trapping properties of the network in different tissues. Professor Eyre went on to explain that post-translational modifications including lysine hydroxylation, glycosylation and potentially prolyl-3-hydroxylation are important for regulating the tissue specific quality of the cross linking chemistry. Defects in prolyl-3-hydroxylation cause recessive osteogenesis imperfecta and under-hydroxylation of prolyl and lysyl residues from a defect in zinc transport causes Ehlers-Danlos syndrome (skeletal dysplasia phenotype). He concluded that common themes and molecular diversities in the structural biology of cartilagenous tissues, and insights from genetic defects in these processes can help in understanding evolutionary mechanisms in collagen biology in general.

The second talk of the session was presented by **Dr Gareth Hyde** (Manchester University). Gareth presented the results from his recent work investigating the cellular origins of the constituent tissues within the knee joint, in a talk entitled 'Col2a1 lineage tracing reveals that the meniscus of the knee joint develops from a number of cells with different origins'. In order to study the movement of cells within the prospective joint, Gareth utilised a Col2a1-Cre/R2R murine model

which expresses  $\beta$ -galactosidase under the Col2a1 promoter. Using this murine model, Gareth discovered that the interzone forms when cells within the Col2a1 expressing anlagen stop expressing Col2a1 and not through cellular invasion of the anlagen. Those cells which ceased Col2a1 expression were found to form the cruciate ligament and inner medial meniscus of the knee. After interzone formation at E14.5, cells which had never expressed Col2a1 appeared in the joint and formed the lateral and outer medial meniscus. No Col2a1 expression was noted in the meniscal cells between E13.5 and birth, although 1 week after birth Col2a1 expression was observed. These findings indicate that the knee joint develops from cells with different origins and Gareth thinks this may explain the heterogeneity of tissues found within the meniscus. The data presented here may provide a new model for knee development which will have implications in regenerative tissue engineering of the meniscus.

The final talk of this session was given by **Dr Chris Murphy** (Kennedy Institute of Rheumatology). His presentation entitled 'The role of Hypoxia inducible H19-derived miR-675 in promoting chondrocyte function' outlined his work on the H19 – derived primary micro RNA miR-675 identified through gene-array work on human articular chondrocytes (HACs) undergoing hypoxia-induced differentiation. MicroRNAs such as miR-675 function as endogenous post-transcriptional regulators of gene expression and Chris wanted to investigate the function of miR-675 during hypoxia-induced differentiation. Chris used real-time PCR assays to measure levels of expression of the primary transcript H19 and the subsequently derived miR-675 in isolated non-diseased primary HACS in both 20% oxygen and physiological levels of 1% oxygen tension. Through this work it was found that H19 transcript expression was comparable to that of the most abundant matrix genes in articular cartilage, namely Col2a1 and Aggrecan. Chris then used siRNA and antisense oligonucleotides to inhibit the expression of two genes whose products are known to be involved in hypoxia induced differentiation, HIF2 $\alpha$  and HIF1 $\alpha$  to investigate the pathways through which H19 was induced. From this experiment it was found that similar to other cartilage matrix genes, H19 was hypoxia inducible through HIF2 $\alpha$ . In addition, following siRNA knockdown of SOX9, hypoxic induction of H19 was abolished. Through similar experiments, mature miR675 derived from H19 was also found to be hypoxia inducible and SOX-9 dependent in HACs. Finally, depletion of miR675 was found to significantly reduce hypoxic induction of the cartilage matrix gene Col2a1. Chris believes that these results potentially represent a new mechanism for regulating cartilage matrix expression, shown in the diagram below.



## **Session 2 - Hyaluronan Metabolism.**

Co-chaired by Professor Martin Humphries (Manchester University) and Dr Wa'el Kafienah (Bristol University), the second session was opened by **Professor Vince Hascall** (Biomedical Engineering, Cleveland, USA) who focused on Hyaluronan metabolism. He spoke about how hyaluronan modulates inflammation. His work concerned mesangial cells from kidney glomeruli of streptozocin-treated rats, which synthesise an abnormal hyaluronan extracellular matrix within one week, after the induction of hyperglycaemia. His current research showed that cyclin D3, a regulator of cell cycle progression, is up-regulated in the mesangial cells, concurrent with abnormal matrix synthesis. Furthermore, silencing cyclin D3 RNA inhibits the synthesis of the abnormal matrix. Confocal microscopy demonstrated coalesced cyclin D3 with an adipocyte marker, C/EBPa, associated with this matrix. Rat tibias isolated four weeks after streptozocin-treatment showed 17% loss of bone mineral. Demineralised sections stained for cyclin D3 or C/EBPa showed embedded adipocytes in an extensive hyaluronan matrix in bone marrow. An adipogenic cell line stimulated to divide in hyperglycaemic media (25mM glucose), synthesised a massive hyaluronan matrix that is adhesive for U937 cells. This extensive hyaluronan matrix with cyclin D structures was similar to those in diabetic bone sections. Thus, it was proposed that bone marrow precursors divert to the adipogenic pathway in response to hyperglycaemia. This accounts for the accumulation of adipocytes in the bone marrow, and they initiate the synthesis of a hyaluronan matrix, which is an energetically efficient way to remove glucose. This synthetic pathway is related to that observed in other cell types in response to stress. It was hypothesised that the abnormal hyaluronan matrix produced by adipocytes in bone marrow in response to hyperglycaemia, recruits inflammatory cells and establishes a chronic inflammatory response that is critical for the demineralisation of bone in diabetic bone pathology.

The second talk of the session was given by **Mr Jason Webber** (Cardiff University), who discussed the role of hyaluronan in the myofibroblast phenotype, the cells which arise from the TGF- $\beta$  induced transdifferentiation of endogenous tissue fibroblasts. Fibroblasts were incubated with TGF- $\beta$  and myofibroblastic differentiation was assessed by the expression of the marker alpha-smooth muscle actin ( $\alpha$ -SMA). After the induction of differentiation, hyaluronan was measured. Data demonstrated that myofibroblasts produce endogenous TGF- $\beta$  and that TGF- $\beta$  signalling has a role in maintaining the myofibroblast phenotype. It appeared that there was a direct relationship between TGF- $\beta$  signalling and hyaluronan coat assembly, because inhibition of TGF- $\beta$  signalling lead to decreased expression of TSG-6, which is important in HA coat assembly, suggesting that hyaluronan plays a role in the maintenance of the myofibroblastic phenotype.

**Ms Helen Fielder** (Oxford University) continued the session with an analysis of complexes formed between polymeric hyaluronan and the G1 domain from human versican. It had been previously proposed that the G1 domain (VG1) interacts with hyaluronan (HA) every HA10, and that the interaction displays all or nothing co-operatively. However, interestingly data gained using size exclusion chromatography multi angle laser light scattering (SEC-MALLS), demonstrated that VG1 interacts with hyaluronan every 18-22HA, a length of polymeric hyaluronan approximately twice as long as previously thought. Furthermore, the VG1/HA interaction was not all or nothing co-operatively and instead it was suggested the interaction may be negative co-operatively. The binding of one VG1 molecule could stabilise an adjacent hyaluronan conformation, which is unfavourable for more VG1 to interact or alternatively, the binding of one VG1 molecule may sterically hinder the binding of an adjacent VG1 molecule. Continued SEC-MALLS investigations are underway to elucidate the co-operativity of this reaction.

**Professor Bryan Toole** (Medical University of South Carolina, USA) presented his work, which hypothesised that the interactions of hyaluronan with its cell surface receptor, CD44, are crucial to the behaviour of highly malignant sub-populations of cancer cells (stem-like or tumour initiating cells). Treatment of tumour cells, with hyaluronan oligosaccharides, which perturb constitutive hyaluronan interactions, induced radical changes at the cell surface, which resulted in the destabilisation of oncogenic signalling complexes. These HA-CD44-stabilised signalling complexes were enriched in highly malignant, therapy resistant, cancer-like stem cells, derived from patients' tumours or cancer cell lines. Additionally, hyaluronan oligosaccharides were shown to inhibit growth and invasion of stem-like tumour cells *in vivo*. Thus, these oligosaccharides may have important therapeutic use and the findings here also provide a paradigm for other disease processes, particularly those involving inflammation, where similar pathways are critical.

The session, and the first day of the meeting, was brought to a close by **Professor Dick Heinegard** (Lund University, Sweden), this year's winner of the prestigious BSMB Fell-Muir award. Early proteoglycan research was discussed, before the name aggrecan was coined, and specifically findings involving proteoglycan aggregation (Hascall and Sajdera) and the interaction of aggrecan with HA filaments (Hardingham and Muir). Aggrecan binds hyaluronan, via two link module motifs and also interacts with link protein, which in turn contains similar functional link module motifs. It has now been found that CILP (Cartilage immediate layer protein), found in the territorial matrix of cartilage efficiently binds to proteins containing the link module (it binds to link protein G1 in aggrecan). CILP is also a molecular marker of late Osteoarthritis. There is a second generation of molecular marker assays, based on the unique cleavage of neo-epitopes. Professor Heinegard has searched for proteins, which bind to aggrecan, as early aggrecan preparations tended to have high protein contents. Matrilin-1 was identified which binds tightly to aggrecan,

collagens and the leucine-rich proteoglycans decorin and biglycan to form a linker unit, consisting of either of the latter proteoglycans between beaded filaments of type VI collagen and collagen type II fibres or aggrecan. Other leucine-rich proteoglycans, fibromodulin, PRELP and chondroadherin (CHAD), bind tightly to fibrillar collagens, via the leucine-rich proteoglycan -repeat domain. In bone, CHAD was found to decrease expression of osteopontin, RANKL, IL-1 $\beta$ , IL-6 and IL-18 in osteoblasts and to decrease osteoclastogenesis. Furthermore, CHAD is down-regulated in Osteoporosis and a loss of CHAD was seen to occur in early Osteoarthritis. The tyrosine sulphate N-terminal domain of fibromodulin mimics heparin in interactions, such as those with cytokines and growth factors. This fibromodulin domain modulates collagen fibre formation, as in fibromodulin -/- knockouts, collagen fibres are thinner. CHAD and PRELP contain different heparin binding domains and both bind to the tyrosine sulphate domain of fibromodulin and to heparin sulphate. These molecules are able to network constituents of the matrix and bind back to the cells, by the combined interactions with collagens via their leucine-rich-domain and via their heparin binding motifs.

### **Session 3 – Matrix Degradation**

The first session of the second day, chaired by Professor Tim Cawston (Newcastle University) and Dr Sophie Gilbert (Cardiff University), was a Stryker sponsored presentation by **Dr. Mandy Fosang** (University of Melbourne, Australia), who presented her work on ‘the disturbed endochondral ossification in collagen II knockin mice resistant to collagenase cleavage’. Dr. Fosang’s group generated a knockin mouse with type II collagen which is resistant to degradation by collagenase at the primary cleavage site. Chondrocytes of this knockin mouse produce type II collagen which is resistant to collagenase cleavage as they have a mutation as G<sup>778</sup>-QRG<sup>779</sup> at the collagenase cleavage site which blocks the proteolytic effects of MMPs 2, -8, -13 and -14. Their aim was to compare the expression of growth plate differentiation markers in wildtype and knockin mice which they named “Bailey”. Bailey mice had elongated growth plates and delayed mineralisation. They showed that the cells in the hypertrophic zones (HZ) of wildtype cartilage stop proliferating while the cells in the HZ of collagen II knockin cartilage continue to proliferate suggesting disturbed chondrocyte hypertrophy and terminal differentiation. In knockin mice, collagen fibres were normal in the growth plate and fibrillogenesis was unaffected and similar to wildtype. However, at week 6 these mice had no marrow cavities and their growth plate closure was aberrant with intramembraneous ossification, instead of endochondral ossification. There was aberrant expression of collagen types II and X by Bailey chondrocytes. Chondrocytes in the HZ showed inappropriate expression of type II collagen, osteopontin, caspase-3, MMP-9 and upregulation of MMP-13. Morphologically, bones of the Bailey mice were 30% shorter and 10 %

wider compared to wildtype mice. Dr. Fosang concluded the first part of her talk by suggesting that collagenase cleavage is required for the production of mineralized collagen to build up for mineralized bone. Her results raised the interesting hypothesis that collagen II fragments were bioactive signalling molecules that influenced terminal differentiation in the growth plate. In the second part of her talk Dr. Fosang continued with their work on another knockin mouse “Jaffa” which is resistant to aggrecanase cleavage. The researchers challenged these mice with intraarticular BSA injection to create an antigen induced arthritis (AIA). Interestingly, following the challenge, at day 7 they observed loss of aggrecan in these mice. Dr. Fosang explained that this degradation resulted from the effects of neutrophil enzymes and free radicals present within the joint. At day 28, however, aggrecan loss was observed only in wild type mice, being reduced or absent in Jaffa mice. In the light of their findings Dr. Fosang suggested that the blocking of aggrecan cleavage might have a potential protective effect on cartilage repair. Repeating similar experiments with Bailey mice, Dr. Fosang’s group showed that blocking of collagen cleavage by 50 % did not diminish aggrecan loss at day 28. Dr. Fosang’s results supported the protective roles of aggrecan in cartilage degradation. In Jaffa mice there was a reappearance of aggrecan at the pericellular site after 7 days following AIA induction and this finding was especially significant at the femoral rather than the tibial sections of the joint. Much more interestingly, in knockin mice the phenotype was restricted to the knees only.

Dr. Fosang’s talk was followed by two short presentations by Dr. Yoshi Itoh and Dr. Liz Canty-Laird. The second talk of the session was by **Dr. Yoshi Itoh** (Kennedy Institute of Rheumatology), titled ‘MT1-MMP in human rheumatoid arthritis’. In rheumatoid arthritis (RA), pannus formation is an unique pathology to the disease. The invasion of synovial pannus into cartilage requires degradation of the collagen matrix. Dr. Itoh and his group were particularly interested in membrane-type 1 MMP (MT1-MMP) in synovial pannus invasiveness. They used 3D collagen invasion assays (in this methodology, the areas degraded by the enzyme is visualized as white whereas the non-degraded areas are black) and cartilage invasion assays to study the role of the enzyme. Dr. Itoh’s group investigated the effects of MT1-MMP in the presence and absence of TIMP-1, -2 and GM6001 (a pharmaceutical inhibitor of MT1-MMP). They looked at the effects of a dominant negative MT-1 MMP construct lacking the catalytic domain. This construct inhibited collagenolytic activity and proMMP2 activation by MT-1 MMP on the cell surface. Dr. Itoh described the high expression of MT-1 MMP at the pannus cartilage junction of RA joints and MT-1 MMP dependent invasion of synovial fibroblasts in collagen matrix. This invasion could be blocked by TIMP-2 and GM6001 but not TIMP-1. The over expression of the dominant negative MT-1 MMP construct also inhibited the invasion of the matrix by synoviocytes. Dr. Itoh concluded his talk by suggesting an essential role for MT1-MMP during pannus invasion in human RA highlighting the need for developing new therapeutics to target MT1-MMP for RA therapy.

The next speaker, **Dr. Liz Canty-Laird** (Manchester University) described her work on the role(s) of BMP1/tolloid metalloproteinases in developing ovarian follicle. She opened her talk with an overview of the BMP family. BMP1 is expressed by ovarian granulosa cells, as is cleaved chordin (BMP inhibitor) and C-procollagen. BMP1 has both autocrine and paracrine activities. Both procollagen C-proteinase and chordinase activity were detected in follicular fluids, but not in serum from the same species. BMP1-like activity was greatest in immature follicles. Small follicles had higher activity compared to the larger ones. Dr. Canty-Laird suggested that BMP1 was likely to be involved in the deposition of collagen fibrils in the interstitial tissue of the follicular wall and had additional role in follicular maturation by modulating BMP signalling during follicle formation.

**Professor Gill Murphy's** (Cambridge University) talk was titled "Controlling extracellular proteolysis: The ECM... and More". Professor Murphy first emphasized the key roles of metzincin super family of zinc dependent metalloproteinases, which included the matrix metalloproteinases (MMPs), the disintegrin metalloproteinases (ADAMs) and the disintegrin metalloproteinases with thrombospondins (ADAM-TSs) in extracellular modulation of the microenvironment. The key roles of these enzymes included: a) clipping (the proteolytic activation or inactivation of chemokines and other chemoattractants by the removal of short N- or C-terminal peptides), b) shedding: the proteolysis of the ectodomain of cell-surface proteins, including chemokines, cytokines, growth factors, receptors, as well as adhesion molecules, c) RIPping: Regulated intramembrane proteolysis and d) cutting (cleavage of extracellular matrix (ECM) proteins, remodelling of cryptic ECM ligands). Professor Murphy presented her group's work on pre-capillary sprout-like formation from endothelial cell spheroids seeded in type-I collagen gels and tubule formation on co-culture of endothelial cells with fibroblasts and tumour cells in an *in vitro* mini tumour model. Professor Murphy's group showed that MT1-MMP induced sprout formation in co-cultures. They found that blocking the hemopexin domain of MT1-MMP (using an antibody against it) inhibited outgrowth of endothelial cells and fibroblasts. The cytoplasmic domain of MT1-MMP was also very important in regulation of signalling, recycling, endocytosis and VEGF transcription. VEGF was upregulated in MT1-MMP expressing cells. One molecule among many that interacted with MT1-MMP in enhancement of VEGF expression was Scr which interacts with the intracellular domain of the enzyme. In the final part of her talk Professor Murphy pointed out that both the catalytic and intracellular domains were needed for the activity of MT1-MMP.

The last speaker of Session 3 was **Professor Hideake Nagase** (Kennedy Institute of Rheumatology) who concluded the morning session by delivering a presentation on 'aggrecan breakdown in cartilage induced by proteolytic fragments of fibronectin'. Professor Nagase spoke on the cryptic functions of extracellular matrix such as release of growth factors, cytokines and chemokines or induction of matrix metalloproteinases. The interests of Prof. Nagase's group is on

the role of fibronectin and its fragments (FNfs) in cartilage degradation, because of the molecule's increased turnover in OA cartilage. They generated FN fragments from porcine cartilage explants by MMP and proteinase digestion. They found that fragments derived from a trypsin digest caused highest glycosaminoglycan release similar to interleukin-1 stimulation. Following purification, they identified two key regions in FN located in type III repeats, III (8-10) and III (13-14) which induced cartilage aggrecan degradation. These fragments were able to induce aggrecanase expression, cartilage aggrecan degradation and to elevate ADAMTS-4 and -5. A synergistic effect between FN III (8-10) and TNF in aggrecan degradation was observed by using an aggrecanase cleavage site anti-neoepitope antibody. Their work with MyD88 (-/-) mice suggested that the catabolic effects of FN III (13-14) fragments might be mediated by Toll-like receptor-4 (TLR4). Professor Nagase commented that TLR 4 (-/-) mice did not respond to FN III (13-14) in aggrecan degradation. Questions arising from the presentation given by Prof. Nagase included consideration of: "which natural enzymes produced FN fragments similar to trypsin?" and "what were the levels of synergistic effects between FN fragments and cytokines such as interleukin and TNF- alpha?"

#### **Session Four – BSMB Open Session**

The second session of the day, chaired by Professor John Couchman (University of Copenhagen) and Dr Chris Murphy (Kennedy Institute of Rheumatology), and sponsored by Company of Biologists, was a BSMB open session in which selected posters were presented. The first talk of the session was by **Dr Jim Melrose** (University of Sydney, Australia) who presented his work on fibromodulin. The molecule (59kD) is the most abundant small leucine rich proteoglycan (SLRP) and regulates collagen fibrillogenesis and TGF bioavailability. Dr. Melrose's work focused on the question "which enzymes were responsible for fibromodulin degradation *in vivo* ?". Comparison of naturally occurring fibromodulin fragments in pathological human tissue with those generated by MMP-13, ADAMTS-4 and -5 were made by western blotting using N and C terminal specific antibodies to fibromodulin (TsYG11, PR-184) and ADAMTS-4, 5 (BC-13) and MMP (BC-14) neoepitope antibodies. Fragments generated by enzymes were different sizes suggesting enzyme and tissue specificity in fibromodulin degradation, as in naturally occurring fragments. Data obtained from their work implicated the roles of MMP-13 and ADAMTS-4 in proteolysis of fibromodulin in cartilage breakdown.

**Dr. Ko Hashimoto** (Southampton University) presented the second poster talk in this session. Dr. Hashimoto's group previously reported the role of de-methylation in abnormal synthesis of matrix-degrading enzymes in osteoarthritis. He presented their work on effects of inflammatory cytokines such as TNF- $\alpha$ , oncostatin M (OSM) and interleukin 1-beta (IL1 $\beta$ ) on DNA de-

methylation in healthy chondrocytes from human femoral head cartilage *in vitro*. Methylation status was determined by bisulfite modification. TNF and OSM diminished DNA methylation, whilst IL-1 caused an ~15% decrease. Most interestingly, IL1 $\beta$  induced its own expression in healthy chondrocytes triggering a positive feedback mechanism. Dr. Hashimoto's findings give an insight into the complex mechanism of osteoarthritis progression.

The BSMB open session was continued by **Brendan Thoms** (Kennedy Institute of Rheumatology), who discussed the promotion of the chondrocytic phenotype by the inhibition of HIF (hypoxia inducible factor)-specific prolyl-hydroxylase domain (PHD) enzymes. Under normoxic conditions, the HIF- $\alpha$  subunit is hydroxylated by the PHD enzymes. This work investigated the potential selectivity of chondrocytic PHD enzymes, for either isotope HIF-1 $\alpha$  or HIF-2 $\alpha$ . Human articular chondrocytes were cultured in monolayer with 20% oxygen and the PHD enzymes were broadly inhibited using DMOG/DFO or specifically inhibited using siRNA against PHD1, 2 and 3. Data indicated that HIF-1 $\alpha$  was stabilised with the specific knockdown of PHD2. In addition, real-time PCR suggested that knockdown of PHD1 and PHD2 may stabilise HIF-2 $\alpha$ , although this will be further investigated. It was concluded that the inhibition of specific PHDs, stabilises both HIF-1 $\alpha$  and HIF-2 $\alpha$  under normoxic conditions in chondrocytes, which may have therapeutic applications, since the inactivation of one or more PHD enzymes could selectively stabilise HIF-2 $\alpha$  and promote cartilage synthesis/repair, whilst leaving the activity of HIF-1 $\alpha$  unaltered.

**Carole Bougault** (University of Lyon, France) presented her data on the molecular response of agarose cultured chondrocytes to dynamic compression. The study aimed to characterise the signalling mechanisms in chondrocytes in response to mechanotransduction because such pathways play an important role in chondrocyte physiology. Mouse chondrocytes were cultured in agarose hydrogels and the constructs were submitted to loading with the Flexercell® compression plus system™. Data indicated that cyclic compression stimulated the early response genes *c-fos* and *c-jun*, triggered ERK1/2 and p38 phosphorylation and modulated type II collagen promoter activity, suggesting that chondrocytes were responsive to compression in this model. Exploration of the role of  $\beta$ 1 integrins in mechanotransduction pathways using this model continue.

The next talk, presented by **Chris Watson** (Manchester University) was entitled 'Identification of a mutation in the UFM-1 specific peptidase 2 (UFSP-2) gene which segregates with the Beukes Hip Dysplasia phenotype'. In his research Chris wanted to investigate the genetic mutational changes which give rise to a rare autosomal dominant disorder Beukes Dysplasia (BHD) which causes joint pain in mid childhood and crippling in adulthood leading to early onset of osteoarthritis. Previous studies had revealed that this disorder was linked to a 2.79 mb region on chromosome 4q 35 and

Chris genotyped single nucleotide polymorphisms in order to fine map the linked region. Several candidate genes were identified including UFSP-2. UFSP-2 showed a T-C sequence change in exon 8 of the gene in all affected individuals causing a tyrosine to histidine amino-acid substitution in the protein. This mutation was found to add a Nde-1 restriction site within the gene making it possible to genotype individuals using amplification of exon 8 and a cut with Nde-1. Control individuals lacking this mutation would show one band after electrophoresis yet affected individuals would show two. Chris genotyped 360 control individuals and found that all of them were negative for this mutation. Through previous investigations it is known that UFSP-2 activates its substrate UFM-1, a protein involved in ubiquitination. UFSP-2 is also known to be able to remove UFM-1 from its substrates. To find out the effect of the Y290H mutation, Chris expressed protein targets and found that due to this mutation the proteolytic domain of UFSP-2 was diminished and cleavage of its substrate was abolished. Further work into the action of UFSP-2 in ATOCS cells has shown, using RT-PCR assays, that UFSP-2 expression is linked to type II collagen in the cells during differentiation. Genetic and functional studies are underway to investigate whether the UFSP-2 Y290H mutation plays a role in the aetiology of BHD.

**Dr Christina Kamma-Lorger** (Cardiff University) presented her research on corneal stroma regeneration following Laser *in situ* Keratomileusis (LASIK). During LASIK, laser beams break molecular bonds within the corneal stroma tissue to form a hinged flap. The flap is then pulled back and the cornea is laser ablated to re-shape the tissue and produce a refractive change. The flap is then replaced and usually heals within 24 hours although even after years of the operation the flap can easily be pulled back. Christina created LASIK like flaps in bovine corneas and the corneas were placed in organ culture and allowed to heal for up to four weeks. Histological sections of the flap showed that the epithelium became thicker than normal following wound healing and there was a significant increase in the cell number above the incision compared to the number below the incision. These cells appeared to be myofibroblastic in nature and there was a significant increase in the number of myofibroblasts after 3 weeks in culture. Christina hypothesised that cytokines released by the epithelium did not reach the flap bed. To investigate this theory, cytokines such as TNF- $\alpha$ , IL1- $\alpha$ , Fas ligand and TGF- $\beta$ 1 were added to the cornea during wound healing to see if this would increase the strength of the flap. The mechanical strength of the flaps were measured using a tensiometer in a 'pull to break' test. In addition to mechanical strength, the transparency of the flap was also measured after cytokine treatment. Compared to untreated flaps Christina found that all four cytokines increased flap adherence with Fas having the least effect and TGF- $\beta$  having the greatest effect, however, this came at the expense of flap transparency. As an alternative to trying to stimulate wound healing at the interface, Christina also tried to artificially crosslink the flap using a combination of Riboflavin/Dextran solution and UVA light for 30 minutes. This technique directly

increased flap strength immediately after treatment yet upon follow-up the flap strength decreased. Christina is now trying to identify the most suitable approach to increase flap-bed strength and maintain corneal transparency.

**Dr John Whitelock** (University of New South Wales, Australia) closed the fourth session with his presentation ‘the role of heparan sulfate on chondrocyte perlecan, to proliferate or not?’ John’s research is based on perlecan and its influence on the fate of chondrocytes through its interactions with member of the fibroblast growth factor family. Previous work has identified that mice lacking perlecan have been shown to have severe chondroplasia due to defective endochondral ossification, similar to the phenotype of FGF18 and FGFR-3 knockout mice. John wondered if perlecan, FGF18 and FGFR-3 share signalling pathways and the aims of his research was to define the GAG structures associated with perlecan produced by chondrocytes and to investigate its ability to interact and promote the signalling of FGF-18 or FGF-2. John and his colleagues purified perlecan from primary cultures of fetal chondrocytes and compared it to that directly isolated from the tissue. He found that these two perlecans showed remarkable similarities in GAG composition and structure with the protein core being decorated with heparan sulfate (HS), chondroitin sulfate and keratan sulfate. Plates coated with endothelial perlecan formed ternary complexes with FGF2, and FGFR-1 or FGF-18 and FGFR3 (subtype iii) which were disrupted with the removal of HS suggesting that these interactions are HS dependent. In contrast, chondrocyte derived perlecan was unable to signal FGF2/FGFR1 or FGF18/FGFR3 and de-glycanation had no effect on the activity. This work demonstrated that the HS on perlecan mediated the formation of FGF-FGFR complexes and controlled the signalling of FGF2. Furthermore he hypothesises that chondrocyte perlecan may act as an inhibitor of FGF activity in cartilage development to prevent over signalling of FGFR3.

### **Fifth Session – Tissue Engineering**

The final session of the meeting was co-chaired by Professor Charlie Archer (Cardiff University) and Dr Simon Tew (Liverpool University), and opened by **Professor Brian Johnstone** (Department of Orthopaedics and Rehabilitation, Portland, Oregon, USA). Professor Johnstone discussed his work on mesenchymal stem cells (MSCs) in a talk entitled ‘Mesenchymal Stem Cells and Skeletal Tissue Regeneration’. He started by introducing the concept of using MSCs for the tissue engineering of cartilage where focal lesions are evident. MSCs are a heterogeneous population of multipotential cells, which have been shown to be able to differentiate down distinct lineages to become fat, bone and cartilage. It is thought that this cell population is heterogeneous in nature and is made up of more “plastic” stem cells which have the ability to differentiate into all three lineages and more restricted progenitors which can only form one or two different cell types.

Professor Johnstone demonstrated that MSCs could be cultured in photopolymerizable hydrogels in which they can start to secrete a cartilagenous matrix although he highlighted that we need to time biodegradation of a scaffold with the production of an ECM by the MSCs. One scaffold, which fits this criterion, is a hyaluronan gelatin scaffold however one problem with both types of scaffold is that only a small amount of tissue can be made. In order to scale up these techniques we need to be sure of the starting population of cells to be used. In order to contribute to our understanding of the biology of MSCs, Professor Johnstone and his colleagues are examining MSCs from patients to firstly assess if biochemical assays could predict the subsequent performance of the MSCs and secondly show the effects of age, sex and disease status. In order to do this, MSCs were harvested from the bone marrow and subjected to Colony Forming Unit (CFU) assays, proliferation assays, and tested for markers of osteogenesis and chondrogenesis. The outcomes of these tests were correlated with age, sex, smoking, disease and drugs. Professor Johnstone found that cell counts at P1, alkaline phosphatase or GAG content did not correlate with age. CFU counts did not have any correlation with osteogenesis although the number of CFUs did correlate with chondrogenic potential. Professor Johnstone found that there was sexual dimorphism in MSC CFU counts with males showing more CFUs than females in both humans and mice. His group purified murine MSCs for common biomarkers and found that male mice have more CD45-CD11b-Sca-1+cKit- cells. Interestingly, he also found that virgin female mice had significantly less CFUs than females which had been pregnant. Professor Johnstone also found that mothers that had given birth multiple times had increased CFUs compared to those mice that had only given birth once. He then discovered that injecting females with oestrogen at a level similar to that observed during pregnancy (2.5nM) increased the numbers of CFUs significantly. This interesting study shows the importance of studying the MSC population more carefully and that factors such as hormone levels may increase CFU production.

The tissue engineering session was continued by the first of three short presentations given by **Dr Claire Clarkin** (Royal Vet College, London). Claire has studied the phenotypic plasticity of superficial zone chondrocytes, using sulphated glycosaminoglycan (GAG) measurement and angiogenic factor release profiles from regions of neo-cartilage grafts. Superficial zone (SZ) and deep zone (DZ) bovine chondrocytes were grown in transwell cultures and media from both the inner and outer wells analysed. It was found that grafts produced by SZ but not DZ chondrocytes showed increased GAG secretion. Interestingly, SZ chondrocyte grafts exhibited pronounced plasticity with exclusive release of VEGF, IL-6, leptin, MCP-1, angiopoetin-2, angiostatin and endostatin. It was suggested that this marked increase in GAG release from the SZ grafts, together with their exclusive repertoire of secreted angiogenic factors, indicated greater plasticity of the SZ chondrocytes than the DZ. This finding may indicate that SZ chondrocytes contribute the most to

zonation vital for cartilage function and highlight their potential in *ex vivo* cell based cartilage tissue engineering.

Following on was **Miss Sandra Strassburg** (University of Manchester) who discussed the effect of conditioned media from nucleus pulposus (NP) cells of the intervertebral disc, on the differentiation of mesenchymal stem cells (MSCs) in monolayer co-cultures. Conditioned media was obtained from normal or degenerate NP cells or from normal or degenerate NP cells directly co-cultured with MSCs for 4 days. Quantitative real-time PCR suggested that only conditioned medium obtained from degenerate NP cells co-cultured with MSCs induced increased gene expression of NP cell markers, in MSCs after 7 days. Thus, these findings suggest that soluble factors in conditioned medium from degenerate NP cells co-cultured with MSCs are potent enough to direct MSC differentiation into an NP-like phenotype in monolayer culture. Importantly, data suggested that cell-cell contact between MSC and degenerate NP cells (but interestingly not normal NP cells), is a necessary event for the production of these soluble differentiation factors, findings that have relevance for MSC-based tissue engineering of the NP.

Next, **Dr Debbie Mason** (Cardiff University) described her research on the interactions between osteocytes and osteoblasts in a novel 3D co-culture system. Osteocytes (MLO-Y4 cell line) were embedded in type I collagen gels, with osteoblasts (MG63/SaOS2 cell line) layered on top of the gel. The osteocytes were seen to form a network of dendritic cells, with extensive processes connecting neighbouring osteocytes and extending towards osteoblasts on the gel surface. When the gels of co-cultures of osteocytes and osteoblasts were compared with control gels of osteoblasts cultured on empty gels, it was revealed that osteocytes inhibit both invasion into the gel and the expression of type I collagen by osteoblasts. The dendritic marker E11 is expressed by MLO-Y4 osteocytes and not normally expressed by MG63 osteoblasts. However, when the osteoblasts were incorporated within the gel they expressed E11. Furthermore, human primary osteoblasts embedded within collagen gels assume a dendritic morphology, down-regulate RUNX2 and up-regulate osteocalcin, indicative of osteocytic differentiation. Hence, it was concluded that osteocytes regulate osteoblast function, in this co-culture model, and that embedding osteoblasts within type I collagen induces differentiation to an osteocyte-like cell.

The conference was closed by **Professor Frank Barry** (University of Galway, Ireland) who spoke on 'Cellular Therapies in Arthritic Diseases'. The main focus of his talk was on the capacity of "mesenchymal stem cells" (MSC) to differentiate into cells of connective tissue lineages including bone, fat, cartilage and muscle. In a typical bone marrow aspirate, MSCs constitute approximately 0.001-0.01% of the mononuclear cell population. The pharmacologic importance of MSCs was related to four points: MSCs secrete biologically important molecules; they express specific

receptors; can be genetically manipulated; and are susceptible to molecules that modify their natural behavior. Professor Barry pointed out several issues that still need to be clarified despite the on-going research on MSCs. First of all, as MSCs are expanded in large-scale culture for human applications it is important to define culture techniques to ensure better reproducibility and enhanced safety. Secondly, further characterization of the cells is essential to purify the stem cells successfully. In addition to the property of plastic adherence elegant methods involving identification of specific surface markers for selection, detection and testing of MSC preparations needs to be developed. Finally, although early pre-clinical and clinical data demonstrate the safety and effectiveness of MSC therapy there were still questions to be answered in assessing the therapeutic potential of MSC in humans. The therapeutic efficacy of transplanted cells and the mechanism(s) of engraftment, homing and *in vivo* differentiation still needs evaluation. The need to carry out appropriately designed toxicology studies to demonstrate the long-term safety of these therapies was also discussed. In the second part of his talk Professor Barry gave some striking examples of the therapeutic use of MSCs in repair of tissues in the treatment of osteoarthritis, myocardial infarction and breast cancer xenografts. He addressed the role of trafficking between stem cells and injured tissues. He talked about an animal model of osteoarthritis in which following induction of injury (meniscectomy with/without anterior cruciate ligament injury), infused MSCs labelled with green fluorescent protein were retained at the site of engraftment. The second example provided by Professor Barry was on a myocardial infarct model. Researchers created ischaemia-reperfusion in myocardium by ligation of the coronary artery for 45 minutes followed by 24 hours of reperfusion. The ability of intravenously infused MSCs to migrate to the site of injury was determined by radioactive or fluorescent labelling of MSCs. To their surprise, after 7 days of injury, there were MSCs present at the injury site suggesting that differentiation happened in local myocardium cells but not in infused MSCs. In another animal model utilising MSCs therapeutically, Professor Barry spoke about the detection of IV-delivered MSCs, with PKH26 labelling in multiple sites in the lining of blood vessels in breast tumour tissues. Professor Barry concluded his presentation by summarising aspects that need to be considered in the implanted cell-host model to understand the mechanisms underlying stem cell therapies such as: (1) the host immune response to implanted cells, (2) the homing mechanisms that guide delivered cells to a site of injury, and (3) differentiation of implanted cells under the influence of local signals.

