

Plenary Speakers

Robert Mecham (Washington, USA) opened the first session describing the formation of elastic fibres within cells transfected with an expression construct containing tropoelastin in frame with a novel reporter, Timer. Timer is a derivative of Dsfluorescent protein, which changes from green to red over a period of 6-12 hours, thus allowing new protein to be distinguished from old protein. Dynamic imaging video microscopy was used to capture fibre formation and results suggest that fibre formation can be separated into two stages, microassembly and macroassembly. The first step in fibre formation is the organisation of small cell-surface-associated elastin globules (microassembly), which are then transferred to pre-existing elastic fibres to form larger structures within the extracellular matrix (macroassembly). Further antibody labelling studies indicate that elastic fibre proteins are involved in this process, with fibulin-5 associating with elastin globules during microassembly and fibrillin and MAGP1 forming components of the fibrous network involved in macroassembly. Finally Professor Mecham presented fantastic 3D images of the extracellular matrix (ECM) in tissues and cells produced from quick freeze, deep etch (QFDE) microscopy. This method does not require chemical fixation, dehydration or heavy metal staining and enables interactions between the ECM and cell surface matrix receptors to be visualised. The imaging also identified unique cellular structures that may be involved in the formation of the ECM.

Holger Gerhardt (London, UK) gave a comprehensive overview on the coordination of cell behaviour and functions during vascular patterning. The first point raised was the importance of a balance between the tip-cell migration and stalk-cell proliferation. This balance is highly regulated by a VEGF-A gradient which shapes the vascular patterning. Another crucial factor in regulating which cell will become a tip-cell or a stalk-cell is Dll4 signalling through Notch1. The speaker showed that using the two models of development of the retinal vasculature in mice and the intersegmental vasculature in zebrafish, inhibition of Notch signalling pathway resulted in extensive filopodia formation and increased number of tip-cells whereas activation of this pathway inhibited formation of filopodia and tip-cells. Therefore he concluded that the angiogenic sprouting is achieved by a well-balanced mechanism between these two principles.

Eckhard Lammert (Dresden, Germany) highlighted the importance of cell-cell interactions within pancreatic islets. For a long time these islets were considered to be only formed of β -cells aggregates. Communication through Eph receptors and ephrin ligands between these cells regulate insulin secretion. However, these pancreatic islets are highly vascularised and every β -cell is in contact with an endothelial cell. This interaction is critical for insulin production and β -cell proliferation. These two cell types communicate via their respective basement membrane. The speaker concluded his talk by highlighting the potential link between vasculature within pancreatic islets and the onset of type II diabetes.

Suneel Apte (Cleveland, USA) gave the first plenary talk of the Thursday afternoon (10-07-2008), and discussed the independent and cooperative roles of ADAMTS metalloproteinases. The mammalian ADAMTS metalloproteinases play an important role in ECM remodeling and according to the manner of their working together, three different models can be observed: working alone (such as ADAMTS-13), parallel functions (such as ADAMTS-2, 3 and 14) and cooperative functions (Such as proteoglycan-degradating ADAMTS). Functionally similar ADAMTS proteinases usually are co-expressed and work cooperatively by contributing to thresholds of proteolysis. Therefore, their biological roles reflect not just their substrate preference, but also their transcriptional regulation and co-regulation. ADAMTS-9 and ADAMTS-20 are the most conserved members of the family. The fact of ADAMTS-9 $-/-$ mouse embryos die by 9 days suggests that ADAMTS-9 play an essential role for survival of the mouse embryo beyond gastrulation. ADAMTS-20 is found to be required for colonization of trunk skin by neural-crest melanoblasts through promoting survival of melanoblasts in a white-spotted mouse named belted (bt) but with otherwise normal development. To further investigate developmental functions for which both ADAMTS-9 and ADAMTS-20 may be essential, a bt/bt, ADAMTS-9 $+/-$ mice were developed. These mice die at birth with a full-penetrant cleft secondary plate suggesting a defect in elevation and migration of palatal shelves towards the midline. However, fusion of palatal shelves is completed in vitro suggests a specific defect in the neural-crest derived palatal mesenchyme. Significantly, both bt and ADAMTS-9 $+/-$ palatal shelves were delayed

in migration to the midline, but both fused eventually, suggesting a cooperative role for ADAMTS-9 and ADAMTS-20 in palatal closure. Meanwhile, there is more extensive depigmentation in *bt/bt*, ADAMTS-9 +/- mice when compared with *bt/bt* mice, suggesting that ADAMTS-9 works with ADAMTS-20 in influencing the function of two neural-crest derived populations. ADAMTS-5 plays an important role in proteoglycan degradation in articular cartilage and usually expressed by perichondrium and colocalised with versican. It was found that versican colocalises with fibulin-1 in interdigital mesenchyme, and fibulin-1 enhances versican proteolysis by ADAMTS-5. ADAMTS-5 and 20 double knockout mice develop syndactyly. BMP-2 and 4 are required for normal development of the interdigital webspace but they are not affected by TS knockout mice. Further studies confirmed fibulin-1 and versican genetically interact with ADAMTS-5 releasing the BMPs, thereby regulating its proteolysis activities.

Florence Ruggiero (Lyon, France) began her talk on the use of zebrafish as a model system to understand collagen function by outlining the challenge of elucidating the specific functions of the 29 distinct collagens currently identified, adding that the considerable structural diversity of the collagen family and the modular nature of each collagen type contribute complexity to the puzzle. She discussed the use of human genetic diseases and targeted gene disruption in mice in providing insights into the role of some collagens in development and disease. She then introduced the zebrafish (*Danio rerio*) as a vertebrate model commonly used in developmental studies but not exploited in the elucidation of collagen function, with advantages such as rapid and external fertilisation and development, transparent and manipulatable embryos and straight-forward knock-down of protein expression achievable using morpholino oligonucleotides. Her laboratory has been able to produce specific hybridisation probes, morpholino sequences for knock-down, and recombinant proteins to generate specific antibodies for use in analyses. They have begun to screen collagen function in the zebrafish model, starting with collagens of known functions to validate the model. The fibrillar collagen type XI was shown to be expressed in the cartilage of zebrafish, with knock-down resulting in defects in the shaping of the head, in chondrocyte stacking, and in the diameter of collagen fibrils in the cartilage extracellular matrix. The FACIT collagens XII and XIV were also studied, with collagen XIV localised in the skin, notochord and gut, and collagen XII, located throughout the fascia and encasing all tissue of the zebrafish tail. Type XII collagen was shown to co-localise with laminin, labelling the epidermal-dermal basement membrane. She then went on to describe how her group have used the zebrafish model to study collagen XXII function, which was previously undetermined. They found it to be a marker of tissue junctions, particularly the myotendinous junction, and to co-localise with dystrophin. The COL22A1 transcript was expressed in muscle cells of the developing zebrafish, and morpholino knock-down resulted in impaired mobility and a hooked tail, both of which they demonstrated to be due to muscle attachment failure and cell retraction. In addition, the myoseptum, which is analogous to the tendon in human, contained disorganised collagen fibrils and numerous fibroblasts when compared to the wildtype. This work demonstrates that the zebrafish is a reliable vertebrate model to analyze collagen function in development and diseases.

Stefan Mundlos (Berlin, Germany) discussed the heritable disorders of the skeleton, introducing them as a wide range of heterogeneous conditions affecting many aspects from development to homeostasis. Taking a developmental approach, he grouped these disorders into Dystososes, conditions arising from a disorder of patterning, Dysostoplasias, conditions caused by disorder during organogenesis, and Dysplasias, those caused by disorders in the growth and maintenance of the skeleton. For the disorders of patterning, he gave examples of heritable disorders affecting each of the three dimensional axes involved in patterning. Ectrodactyly results from a disorder of patterning along the proximo-distal axis caused by a mutation in *p63*, polydactyly is one of the symptoms of Greig Syndrome and is caused by abnormal antero-posterior signalling, whilst a mutation in the gene encoding *Wnt7A* results in disordered dorso-ventral patterning, as occurs in Schinzel phocomelia syndrome. He then went on to discuss the disorder Hoxd13 Associated Synpolydactyly, caused by the insertion of seven alanine residues into the gene encoding Hoxd13. Work by his laboratory has shown that wild type Hoxd13 and Hoxa13 suppress chondrogenesis, preventing progenitors from differentiating, with the alanine insertion resulting in an induction of Sox9 expression and chondrogenesis. Next he discussed dysplasias as mostly involving abnormal growth plates which may arise from abnormal matrix production, abnormal signalling or gene activation, abnormal proliferation, or abnormal metabolism. A number of dysplasias are linked to the Ras-MAPK

pathway and his group produced a mouse knockout with constitutively activated Ras. Increased Ras signalling resulted in decreased proliferation in the growth plate, through down-regulation of Indian hedgehog signalling. Bending of the long bones was observed in knockout mice, a symptom similar to that of patients with Neurofibromatosis type I (NF1) who suffer from bowing of the tibia and frequent fracture, leading his group to investigate a potential role of NF1 in bone signalling. Bones of knockout mice showed an increased porosity due to an increase in vasculature, and also a decrease in stiffness due to decreased calcium content. In addition, osteoblasts from knockout mice showed decreased differentiation and increased proliferation. The effects of Ras activation on endothelial cells (increased vasculature and bone porosity) and osteoblasts (decreased calcium and bone stiffness) all result in mechanical instability. Finally, he discussed a disorder caused by defective protein glycosylation. In addition to abnormal skin and subcutaneous fat distribution, patients with Wrinkly Skin Syndrome have slender long bones and osteoporosis. They show abnormal N-glycosylation of serum proteins as a result of a loss of function mutation in vesicular H⁺ATPase which affects the Golgi apparatus. As protein modification regulates development, growth and maintenance, mutations affecting this may result in dysplasias.

Johanna Myllyharju (Oulu, Finland) has generated mutant knockout (KO) mouse lines for the vertebrate collagen prolyl 4-hydroxylase (C-P4H). There are three isoenzymes of the C-P4H. Type I KO mice are embryonic lethal at day 10.5, having reduced mesenchymal cell density, dilated ER, decreased collagen and decreased number of capillaries with basement membrane devoid of collagen IV. Type II KO mice have no overt phenotype. They generated double mutant mice that were heterozygous for type I and homozygous for type II. These mice were smaller with a disorganised growth plate indicating type II was not essential for survival but has a role on skeletogenesis. She also presented data on hypoxia-inducible transcription factors regulated by C-P4H in O₂-dependant manner. C-P4H regulates efficiency of RNA interference (RNAi). She showed argonaute 2 binds to RNA and cleaves in the RNAi system as part of the RISC complex. The X-pro-Gly in argonaute 2 is hydroxylated by C-P4H and that the lack of C-P4H reduces the efficiency of RNAi.

Marie-Madeleine Giraud-Guille (Paris, France) opened her talk on “Collagen crystal stabilisation gels: Relevance to matrix morphogenesis, 3D cell culture and tissue engineering”, with a description of tissue morphogenesis and a comparison of collagen structures between organisms and tissues. Histological sections of connective tissue in polarised light showed an ordered assembly of collagen fibres. Evidence was presented to show that cells have some control of 3D collagen networks by adjusting the contour of the membrane. Macromolecule organisation in 3D matrices shows morphology analogous to that of liquid crystalline phases. At high collagen concentrations (50mg/ml), spontaneous collagen ordering was seen, indicating self assembling properties of collagen. Macroscopic structure of the crystalline phase was analysed using polarised light microscopy and small angle x-ray scattering. Type I collagen dispersal in aqueous solution experiments showed similar patterns to those in cholesteric liquid crystals. *In vitro* experiments showed that the cholesteric order is present with a soluble precursor in tissue morphogenesis prior to fibrillogenesis. pH triggered liquid collagen forms a fibrillar gel similar to that of tissue networks. Cell matrix relationships were studied with dense collagen matrices and human dermis fibroblasts, mouse fibroblasts (integrin $\alpha 1 \beta 1$ deficient) and primary cell line / transformed osteoclasts. It was concluded that this information is valuable in 3D tissue culture and tissue engineering.

Mario Raspanti (Varese, Italy) presented data from *in vitro* studies showing the effect of the interaction of collagen fibrils with the small leucine-rich proteoglycans (SLRPs) decorin, biglycan and fibromodulin, and the associated glycosaminoglycan (GAG) side chains dermatan sulphate (DS), chondroitin 4-sulphate (C4S), chondroitin 6-sulphate (C6S) and keratan sulphate (KS). His data highlighted that both the protein core and GAG side chains of SLRPs were able to interact with collagen molecules (with the exception of C4S) and that many were influential in the aggregation of collagen molecules into fibrils and fibrils into fibres. Many of the collagen binding ligands tested were found to inhibit lateral fusion of collagen molecules during fibril growth and all (with the exception of biglycan) influenced collagen fibril diameter. Furthermore, decorin, biglycan, DS and C6S were all shown to form interfibrillar links believed to highlight functional roles for SLRPs and GAGs in the gathering of collagen fibrils into fibres. These *in vitro* studies led Mario to conclude that direct interactions between

collagen molecules and GAG chains may occur *in vivo* and it was hypothesised that these interactions might contribute to the mechanical strength of tensile tissues.

Yann Barrandon (Lausanne, Switzerland) described how skin, which is squamous stratified epithelia, contains multipotent stem cells of ectodermal origin. This was proven via long-term serial transplantation assays in mice. Professor Barrandon also described how clonogenic cells taken from other stratified epithelia, such as the vagina and cornea, could be transplanted into the skin to form *de novo* cycling hair follicles and sebaceous glands in response to skin and hair morphogenic signalling, regardless of their germ layer origin. Transplantation of thymic epithelial cells into skin resulted in the formation of skin hair follicles, demonstrating that these cells share phenotypic and functional characteristics of the multipotent stem cells found in the skin. After transplantation the thymic cells were placed back into thymus tissue and still retained the capacity to participate in the thymic microenvironment. Professor Barrandon concluded that the stem cells of the stratified epithelia and epithelial of the thymus may be closely related and could be interchangeable, which could be of therapeutic interest.

Tomoyuki Nakamura (Osaka, Japan) began his talk with an overview of elastic fibre properties and elastogenesis. Elastic properties in tissues such as the skin, arteries and lungs are mediated by elastic fibres, the deterioration of which can result in age-associated diseases. DANCE (Developing Arteries and Neural Crest EGF-like), also known as Fibulin-5, was introduced as an integrin ligand essential for elastic fibre assembly, as truncated forms are unable to modulate elastogenesis. DANCE deficient mice have disorganised elastic fibres, and phenotypical characteristics include stiff arteries, loose skin and emphysema. Human aging phenotypes were similar to those seen in DANCE/ Fibulin-5 deficient mice, including a reduction in the elastic modulus of the tissue. It was reported that DANCE organises tropoelastin (by coacervation and alignment) and other cross linking enzymes (LOXL 1, 2 and 4) into microfibrils, possibly mediating the assembly and maturation of elastic fibres. It was reported that there is a decrease in full length DANCE expression with age, with an increase in truncated forms via proteolytic cleavage of the amino-terminal domain of the full length protein. The truncated form was not fully functional in elastogenesis in cell culture experiments. A knock in mouse model was presented, which lacked the amino terminal domain, showing very close results to those of the truncated form.

Birgit Leitinger (London, UK) presented a research on the discoidin domain receptors, DDRs. Those receptors are involved in regulation of developmental processes, cell adhesion, migration, control of cell proliferation and extracellular matrix remodelling. Nevertheless the detailed mechanism of molecular recognising of collagen by DDRs is not yet fully understood. The talk focused on recently defined sequence motifs in collagen that are recognised by DDR2. It was found that like integrins, DDR2 recognize discrete amino acid motifs on triple-helical collagen. For the first time three DDR2 binding motifs on collagen II were revealed and one, with the highest affinity, was found to be also conserved in collagen I and III. The specific motif was distinct from that of integrins. Knowing motif peptide sequences, the investigation was directed to characterising the minimal collagen sequence required for DDR2 binding; this sequence was defined as GVMGFO. Using triple-helical peptides that bind to DDR2 it was found that DDR2 activation does not require the presence of higher order fibrillar collagens.

Chris Overall (Vancouver, Canada) talked about quantitative proteomic analysis of proteolytic moulding connective tissues *in vivo*. All proteomes are moulded by proteolysis. Therefore, it is crucial to identify which proteases cleave a particular substrate and which substrates individual proteases cleave. In order to understand the biological role of proteinases by globally integrating the identification of all the elements of the proteinases, a number of unique quantitative proteomic platform technologies named degradomic have been developed. Gel-based proteomic identification of substrates is a kind of technology to separate and compare differences of protein spots from protease-treated and control samples on two-dimensional (2D) PAGE gels, especially using fluorescent 2D difference gel electrophoresis (DIGE). Protease-treated and control proteomes are labelled with either Cy3 or Cy5, pooled and electrophoresed together. After merging the Cy3/Cy5 images, substrate and cleavage products can be identified as individually coloured spots. Then mass spectrometry is used to identify the proteins. The second kind of technology isotope-coded affinity tag (ICAT) labelling. Protein from protease-treated and control samples are labelled on Cys residues with ICAT tags, pooled and digested with trypsin. The

ICAT-tagged peptides are then affinity purified by the biotinylated tag. These are quantified by analysis of peak pairs in the MS spectrum and identified by MS/MS. ICAT ratios of extracellular protease-to-control peak areas that are less than 1 indicates protein degradation, ratios that are greater than 1 indicate shedding. Another choice for the second technology is that protease-treated or control samples are trypsinised and all amino groups are then iTRAQ labelled. The samples were then analysed by MS/MS. The third technology that can be used to identify substrates is Neo-N-terminal isotope labelling. The N-terminal amino-acid sequence can be generated by proteinase digestion. After blocked primary amines or guanidination of Lys within the neo-tope, the sample is trypsinised, followed by purification of peptides through N-terminal pullout and analysis or removal of internal tryptic peptides. The remaining peptides represent the N-terminome of the sample and include the protease-generated N-terminopes. MuDPIT (multidimensional protein identification technology) and MS/MS then can be used to complete the identification. In summary, all the technologies provide high selective, high efficient methods to investigate and identify proteolytic substrates in connective tissue.

Tony Day (Manchester, UK) described the roles of TSG-6 (tumour necrosis factor stimulated gene-6). He explained how the protein is not constitutively expressed in adults but levels of expression are increased in the context of inflammatory disease states such as arthritis and inflammatory-like processes such as ovulation. TSG-6 null mice are infertile and studies have highlighted that the protein is crucial for the formation of the cumulus ECM which is produced around the oocyte prior to ovulation. A potential mechanism for the involvement of TSG-6 was described involving the use of TSG-6 as a catalyst and cofactor for the transfer of heavy chains from the proteoglycan inter- α -inhibitor onto hyaluronan (HA). This transfer allows the formation of a stable HA matrix around the oocyte, which is believed to be essential for ovulation and fertilisation. More recent data were also presented highlighting dual roles for TSG-6 in bone metabolism. TSG-6 was found to inhibit RANKL-induced osteoclast activation which is consistent with a role in the erosion of bone. However other data generated from the study of the long bones of unchallenged TSG-6 null mice suggested that in the absence of inflammation TSG-6 may have a role in bone homeostasis.