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YSAS–YOUNG SCIENTIST AWARD SESSION

YSAS.1

Telomere shortening in subjects with impaired glucose tolerance and patients with diabetic macro-angiopathy

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Background

Telomeres, composed of large arrays of short guanine-rich sequence, TTAGGG, are essential and dynamic regulators of cellular life span and chromosome integrity. The biology of telomeres has long been the focus of numerous investigations related to cancer. However, it is now realized that telomeres might also play primary or secondary roles in complex genetic disorders, including essential hypertension, atherosclerosis and diabetes. It is well known that increased risk for coronary artery disease (CAD) is seen not only in subjects with Type 2 diabetes but also at the stage of pre-diabetes or impaired glucose tolerance (IGT). We recently demonstrated the occurrence of telomere shortening in leukocytes from Asian Indian Type 2 diabetic patients thus raising the possibility that telomere shortening might be a long-term risk marker of diabetes and macro-vascular complications. There are no studies to our knowledge that have looked at telomere shortening in pre-diabetes i.e. at the stage of IGT. The present goal of this study was to therefore examine the telomere length in subjects with IGT. Additionally, we also studied Type 2 diabetic subjects with and without atherosclerotic plaques (carotid/femoral) to see whether telomere shortening was greater in Type 2 diabetic subjects with atherosclerosis.

Methodology

Subjects with IGT ($n=30$), non-diabetic control subjects ($n=30$), type 2 diabetic patients without ($n=30$) and with atherosclerotic plaques ($n=30$) were selected from the Chennai Urban Rural Epidemiology Study (CURES). Southern-blot analysis was used to determine mean terminal restriction

fragment (TRF) length in leukocyte DNA. Levels of TBARS (thiobarbituric acid reactive substances), protein carbonyl content (PCO) and high sensitive C-reactive protein (hs-CRP) were measured by standard methodologies. Carotid intima-media thickness (IMT) was assessed by high resolution B-mode ultrasonography.

Result

Compared to control subjects, TRF lengths were significantly lower in IGT subjects and diabetic subjects without atherosclerotic plaques and still lower in diabetic subjects with atherosclerotic plaques. Age-adjusted telomere lengths were significantly ($p=0.004$) shorter in men vs women in both control (7.36 ± 0.5 kb vs 10.52 ± 0.9 kb) and IGT (6.57 ± 0.2 kb vs 8.74 ± 0.7 kb) subjects. There was no significant gender difference (6.1 ± 0.5 kb vs 6.88 ± 0.2 kb) in TRF lengths in diabetic subjects without atherosclerotic plaques. However, in those diabetics with atherosclerotic plaques, men had significantly shortened telomeres than women (4.14 ± 0.3 kb vs 5.2 ± 0.2 kb, $p=0.02$). Plasma levels of lipid peroxidation (TBARS), PCO and hs-CRP were significantly ($p<0.05$) higher in IGT subjects, diabetic patients without and with plaques compared to control subjects. In IGT subjects, TRF length was positively correlated to HDL cholesterol and negatively correlated to HbA_{1c}, TBARS, PCO, HOMA-IR and IMT. In regression analysis, presence of diabetes, HDL cholesterol and increased TBARS levels appear as significant determinants of telomere shortening.

Conclusion

Our study shows that telomere shortening occurs in subjects with IGT (pre-diabetes) and is progressively greater in subjects with type 2 diabetes and those with diabetes and atherosclerosis. Our results support that chronic burden of oxidation/inflammation could be one of the major mechanisms of telomere shortening. Since pre-diabetics are more prone for cardiovascular diseases, the shortening of telomeres could be a long-term biomarker and predictor of future coronary heart disease events. It would be important for the future studies to look for the impact of telomere shortening on the regenerating and/or

functional capacity of insulin responsive tissues (pancreas, adipose and muscle).

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YSAS.2

The influence of nanoscale biomimetic structures on osteoblast adhesion

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Introduction

Topographical modification of a biomaterial may be exploited to enhanced cytocompatibility at the cell substrate interface. Regulation of cell adhesion in orthopaedic design can lead to improved osseointegration and implant stability or prevent undesirable osseointegration, preferable if the device is to be removed. Cellular-biomaterial adhesion is mediated through transmembrane integrin receptors coupled to the extracellular matrix (ECM). These adhesion complexes contain structural and secondary signalling molecules crucial to cell adhesion and function. Experimentally, nanogrooves¹ nanopits² and nanoislands³ have been shown to affect contact guidance *in vitro* and directly influence cellular behaviour. Here experimental nanotopographies of varying order and dimension were fabricated in polymethylmethacrylate (PMMA) by electron-beam lithography (EBL), photolithography (PL) and polymer demixing. Work to date has concentrated on the effects these nanotopographies have on adhesion formation and cytoskeletal organisation in S-phase osteoblasts.

Materials and methods

Nanopit topographies were fabricated by EBL. Ordered arrays of pits in both a square and hexagonal conformation were fabricated with 300 nm pitch, as well as random nanopit array with a controlled degree of disorder. All pits were 100 nm deep and 120 nm in diameter. Grooved substrates produced by PL were 327 nm deep, and 10, 25 and 100 µm wide. Polymer demixed nanoislands and nanolacuni were fabricated by controlling Poly(styrene) and poly(bromostyrene) concentration and rate of spincoating. All experimental substrates were embossed into PMMA. Primary human osteoblasts (HOBs) were used as a cell model for adhesion quantification. HOBs were serum starved to induce synchronisation and bromodeoxyuridine (BrdU) was used to label S-phase cells. Immunocytochemistry was used to observe S-phase nuclei, vinculin, stress fibre associated actin, tubulin and fibronectin in HOBs, by both fluorescent and scanning electron microscopy. Image analysis[#] was then used to quantify adhesion size and number.

Results

Nanopits were seen to reduce adhesion formation and disrupted cytoskeletal and ECM organisation relative to planar controls. Here, actin and tubulin elements in S-phase HOBs presented decreased structural organisation and adhesions were observed to form predominantly at interpit regions. Reducing pit order resulted in increased adhesion formation. Grooved substrates were seen to

induce contact guidance and cellular alignment only at low cell densities. Increased cell–cell contact seemed to override the topographical influence at these groove depths irrespective of pitch. The 100 µm wide grooves did not influence cellular alignment but adhesion and cytoskeletal orientation were affected. 10 µm wide grooves greatly influenced cell alignment and induced contact guidance at low cell densities. This increased contact guidance however, was associated with reduced adhesion formation. 25 µm wide grooves produced an intermediate effect. Topographical fabrication via polymer demixing resulted in random arrays of ‘islands’ and ‘lacuni’ possessing microscale widths and heights/depths of approximately 40 nm. Both topographies were seen to induce increased cell spreading, cytoskeletal organisation, and the formation of large well-defined adhesion plaques.

Discussion

S-phase cells have been shown to undergo increased spreading and possess reduced intercell variation in adhesion numbers. In this study cells were identified as being within S-phase by the incorporation of a thymidine analogue, BrdU into their total DNA. HOB adhesion and morphology was dependant on feature symmetry and conformation — parameters that can be manipulated in order to direct osteoblast adhesion. Ordered pit arrays resulted in perturbed adhesion formation, cytoskeletal disruption and reduced cellular flattening. Such anti-adhesive properties are of particular importance in the design of temporary orthopaedic devices and can aid in the successful removal of such. A reduction in pit order was seen to reduce the anti-adhesive properties of this substrate. Adhesion formation and cytoskeletal development was seen to be most pronounced on random features fabricated by polymer demixing, and 100 µm wide nanogrooves, possibly as a result of increased protein adsorption at these features. These substrates were associated with increased numbers of large ‘fibrillar’ adhesions, indicating increased HOB adhesion. Increased contact guidance in HOBs was seen as groove width decreased and was associated with a reduction in adhesion formation and HOB spreading possibly due to increased motility. All grooved substrates were seen to influence adhesion orientation. Adhesions were influenced to align either parallel to, or perpendicular to the groove edge with adhesions occurring predominantly on the raised portion of the grooves.

Conclusion

This study implicates the relevance of nanotopographical ordering and conformation in elucidating desired cellular response at the cell–substrate interface by regulating adhesion formation, cell morphology and adhesion orientation.

Acknowledgements

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References

- [1] Zhu (2004) *Biomaterials* **25**, 4215–23.
- [2] Biggs (2006) *J Orthop Res* **25**, 273–282.
- [3] Dalby (2002) *Exp Cell Res* **276**, 1–9.
- [4] Diener (2005) *Biomaterials* **26**, 383–392 [#]<http://rsb.info.nih.gov/nih-image>

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YSAS.3

Comparison of xylem flow velocities determined by MRI and a non-invasive heat pulse technique in Golden Alder and Silver Birch

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Motivation

To date, magnetic resonance imaging (MRI) is the only truly non-invasive, repeatable technique for the measurement of xylem and phloem sap flow velocities in woody and herbaceous plants. However, the existing MRI equipment is not suited to field measurements. Heat pulse techniques are routinely used in the field to estimate xylem flow rates but, being invasive, they come at the price of damage to the plant. We have therefore developed a non-invasive and portable laser-based heat pulse system. Although phloem flow detection has been achieved with our system [1], most work to date has been focused on xylem flow as the latter is easier to measure in the small saplings used (ca. 1 cm in diameter) and reference transpiration rates data using weighing lysimetry are readily available.

Experimental setup

Work has been carried out on potted broadleaf saplings of stem diameter not exceeding 2 cm. A small area of the surface of the stem is heated with a laser pulse ($\lambda=812$ nm, typically 1–6 s duration and beam dimensions at the sample 1 mm high \times 5 mm wide). The optical power at the sample is typically 500 mW. The temporal evolution of the temperature is monitored 4–5 mm downstream from the point of heating by an IR camera with a spatial resolution of 0.2 mm/pixel. Flow velocities are determined from temperature profiles by applying Eq. 1, which was adapted from the point source solution of the heat equation for conduction in a homogeneous but thermally anisotropic medium [2] by including a convective term (velocity v in the x -direction). The analytical model describes thus a wood- and water matrix where heat travels radially through conduction (dominant radial thermal conductivity k) and longitudinally through forced convection in the fluid (longitudinal thermal conductivity k_1).

$$v = \sqrt{\frac{\frac{3}{2} \ln\left(\frac{t_2}{t_{\max}}\right) - \ln(2)}{\frac{\rho C}{4k_1}(t_{\max} - t_2)} + \frac{k_1 z^2}{k} + x^2}$$

Here v is the flow velocity, x is the distance between the point of heating and the point of measurement, z is the heat penetration depth into the stem, ρ and C are the overall density and specific heat of the wood-and-water matrix, t_{\max} is the temporal position of the maximum temperature rise with respect to the onset of the laser pulse, and t_2 is the second occurrence of the temperature half maximum.

Comparison of heat pulse technique with MRI flow imaging

Xylem and phloem flow velocities can be accurately monitored by MRI [3]. The custom-built 0.72 T Nuclear Magnetic Resonance (NMR) system at Wageningen University allows for the study of plants of diameter up to 2.5 cm. The frontal access to the plant provided by the planar geometry of the NMR system allowed for simultaneous MRI and heat pulse measurements (Fig. 1). We were able to demonstrate a near 1:1 agreement between water flow velocities determined by MRI and our heat pulse technique over two full day and night cycles (Fig. 2). The sapling studied was a 1.8 m tall golden alder (*Alnus Incana Aurea*) of mean stem diameter 1 cm at the point of heating; similar results were obtained with a birch sapling (*Betula Pendula Zwitsens Glorie*) of comparable dimension.

Conclusions and future work

Although a satisfactory agreement was obtained between MRI and our non-invasive heat pulse technique, flow velocity calculations from Eq. (1) rely on the knowledge of the significant heat penetration depth (z) which cannot, at present, be determined directly from surface temperature data. Numerical modelling of a simplified system consisting of water flow between two planar walls is used in conjunction with experimentation to understand the thermal dynamics of such a structure. Work with this model suggests that the thickness of the solid wall separating the surface from the fluid sets a limit for the fastest detectable flow velocity. This implies that xylem flow velocities in large trees might be underestimated by the current approach. In addition, heating was found to induce a local circulation of the fluid; this effect is proportional to the amount of heat applied and inversely proportional to the thickness of the wall and the flow velocity (Fig. 3). This effect is believed to hamper the resolution of phloem flow in small saplings. We are consequently working towards a modulated short pulse solution capable of systematically tracing phloem flow even in small saplings.

Possible applications

Phloem flow monitoring can make a significant contribution to a detailed understanding of the terrestrial carbon cycle

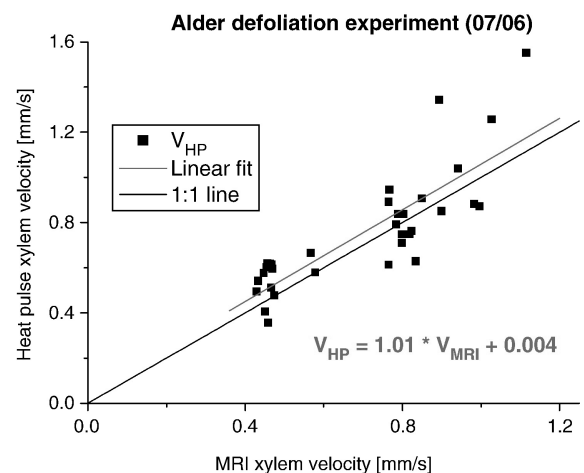


Fig. 1.

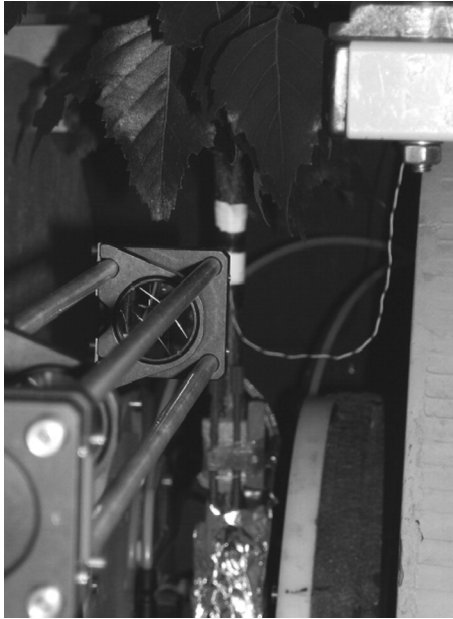


Fig. 2.

and hence the dynamics of climate change. It will complement atmospheric and soil measurements of CO₂, providing information on climate change feedback mechanisms caused by a plant's response to stress and environmental changes.

References

- [1] C. Helfter, J.D. Shephard, J. Martinez-Vilalta, M. Mencuccini, D.P. Hand (2007), *Tree Physiology* 27 (2), 169–179.
- [2] H.S. Carslaw, J.C. Jaeger, *Conduction of Heat in Solids*, 2nd Ed., Oxford at the Clarendon Press (1959).
- [3] C.W. Windt, F.J. Vergeldt, P.A. de Jager, H. Van As (2006), *Plant, Cell and Environment* 29 (9), 1715–1729.

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YSAS.4

Life within insect antennae: Symbiotic bacteria protect wasp larvae against fungal infestation

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Symbiotic associations between different organisms are of great importance for evolutionary and ecological processes. Bacteria are particularly valuable symbiotic partners due to their huge diversity of biochemical pathways that may open entirely new ecological niches for higher organisms.

Here I report on a unique and highly specialized association between a digger wasp, the European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae), and actinomycete bacteria. In contrast to all other known symbioses, the beewolf bacteria are cultivated in the reservoirs of unique antennal glands in female beewolves. The female secretes the bacteria into its subterranean brood cells prior to oviposition. Several days later, the beewolf larva takes up the bacteria and applies them to the cocoon silk during the spinning process. On the cocoon, the symbionts play an important role in reducing the incidence of fungal infestation and thereby significantly enhance the survival probability of the larva in the cocoon. Presumably, the bacteria are later taken up from the cocoon during eclosion and incorporated into the antennal gland reservoirs of the adult female.

Using PCR-based methods, 16S rDNA sequencing and fluorescence in-situ hybridization (FISH), the bacteria were identified as a novel species of the genus *Streptomyces*. Closely related endosymbionts were found in 28 *Philanthus* species and subspecies, but no symbionts could be detected in closely related genera of the subfamily Philanthinae (*Aphilanthops*, *Clypeadon*, *Cerceris*). Based on almost complete 16S rRNA gene sequence data, the symbionts of all analyzed *Philanthus* species formed a monophyletic clade within the genus *Streptomyces*, indicating that the symbiosis is highly specific and most likely the product of a long history of coevolution and cospeciation. Sequence divergences among symbionts suggest an origin of the *Philanthus*–*Streptomyces* association about 26–67 Ma ago, which may have coincided with the origin of the genus *Philanthus*. On the basis of 16S rDNA sequences and ultrastructural data, the new taxon '*Candidatus Streptomyces philanthi*' is proposed for the antennal symbionts of *Philanthus* species, with symbionts from different host species being treated as ecotypes and named according to their hosts (e.g. '*Candidatus Streptomyces philanthi triangulum*').

It is not yet clear how the bacteria benefit from the association with *Philanthus*. Certainly, they obtain an unoccupied and presumably competition-free niche in the beewolf antennae and a reliable transmission route to the next generation. Additionally, several pieces of evidence suggest that they may also receive nutrients from their host: (1) Females secrete massive amounts of bacteria into each brood cell and sometimes construct several brood cells per day; thus, the bacteria have to grow quickly inside the antennal gland reservoirs to replenish the stock for

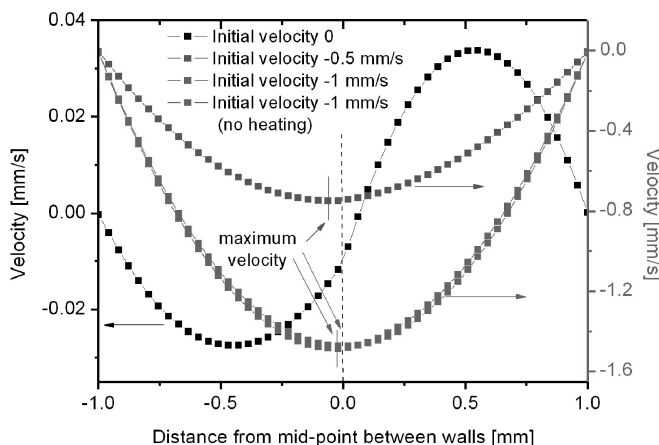


Fig. 3.

further brood cells. (2) The reservoirs are surrounded by class 3 gland cells that may supply the bacteria with nutrients (e.g. amino acids). (3) The epithelium lining the reservoir contains numerous vesicles suggesting that active transport of substances from the hemolymph into the reservoir occurs. This possibility is further substantiated by chemical analyses of the hydrocarbon profile of the antennal gland secretion and female hemolymph, which revealed very similar compositions.

The beewolf–*Streptomyces* symbiosis constitutes the first known case of bacteria being cultivated in insect antennae and one of the few examples involving the pharmaceutically important group of actinomycete bacteria as insect endosymbionts. Further studies on ecological and evolutionary aspects of the symbiosis will provide valuable insights into the importance of actinomycete bacteria for pathogen defense in insects and may also identify novel secondary metabolites with antibiotic properties that might prove useful for human medicine.

Publications resulting from this work:

Kaltenpoth, M., Schmitt, T. and Strohm, E., in preparation. Chemical composition of the antennal gland secretion of female European beewolves, *Philanthus triangulum*.

Strohm, E., Herzner, G., Kaltenpoth, M., Boland, W., Schreier, P., Peschke, K. and Schmitt, T., in preparation. The chemistry of the postpharyngeal gland of female European beewolves (Hymenoptera, Sphecidae) supports a homology with this gland in ants.

Goettler, W., Kaltenpoth, M., Herzner, G. and Strohm, E., 2007. Morphology and ultrastructure of a bacteria cultivation organ: the antennal glands of female European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae). *Arthropod Structure and Development* 36: 1–9.

Kaltenpoth, M., 2006. Symbiotische *Streptomyces*-Bakterien in Grabwespen. *Naturwissenschaftliche Rundschau* 59 (11): 618–619.

Kaltenpoth, M., Göttler, W., Dale, C., Stubblefield, J.W., Herzner, G., Roeser-Mueller, K., and Strohm, E., 2006. 'Candidatus *Streptomyces philanthi*', an endosymbiotic streptomycete in the antennae of *Philanthus* digger wasps. *International Journal of Systematic and Evolutionary Microbiology* 56 (6): 1403–1411.

Kaltenpoth, M., 2005. Bakterien schützen Wespen-Nachwuchs vor Pilzbefall, *Naturwissenschaftliche Rundschau* 58, 329–330.

Kaltenpoth, M., Göttler, W., Herzner, G. and Strohm, E., 2005. Symbiotic bacteria protect wasp larvae from fungal infestation, *Current Biology* 15, 475–479.

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YSAS.5

The functional involvement of lamin A and LAP α in human ageing: The role for lamina protein redox modifications in senescence signaling

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Mutations in the gene encoding a nuclear intermediate filament protein, lamin A, cause a spectra of human age-related diseases and premature ageing syndromes, collectively termed laminopathies. Current research suggests that A-type lamins may affect longevity and the maintenance of a number of human somatic tissues and cell types. These findings prompted us to explore whether lamin A plays a role in normal cellular ageing process. As a model cellular system we used human diploid fibroblasts (HDF), which are known to undergo a progressive accumulation of irreversibly arrested senescent cells during ageing in vitro. The experimental approach involved cell culture of HDF and analysis of protein expression, localisation, solubility properties and oxidative modifications of lamin A and its binding partners LAP2 α (lamina-associated polypeptide 2 α) and retinoblastoma protein (pRb) during ageing of HDF in vitro using a range of biochemical, microscopy, flow cytometry and in vitro nuclear assembly techniques.

Our results obtained by confocal microscopy show that human fibroblasts aged in vitro acquire a range of aberrant nuclear phenotypes characteristic of progeroid human fibroblasts. These include dysmorphic nuclei and defective nuclear assembly of both lamin A and its binding partner LAP2 α , all of which strongly correlate with cell cycle arrest. Interestingly, protein sequence analysis revealed that the C-terminal specific domains of lamin A and LAP2 α contain multiple cysteine residues. Moreover, we show using three different biochemical techniques, including 2D-gel electrophoresis and mass spectrometry, glutathione blot assays and immunoprecipitation methods, that the C-terminal specific cysteine residues in both lamin A and LAP2 α undergo oxidative modifications (S-glutathiolation and other irreversible oxidative modifications) in senescent fibroblasts. These modifications inhibit the formation of higher-order disulphide-linked structures in both proteins in senescent fibroblasts as shown by non-reducing gel electrophoresis in the presence of alkylating agents. In addition, during biochemical fractionation of senescent fibroblasts, these modifications led to a partial proteolysis of lamin A within its C-terminal tail domain and an accumulation of a soluble lamin A proteolytic fragment whilst LAP2 α became lost from the detergent/salt-resistant nucleoskeleton. Consequently, lamin A and LAP2 α fail to tether the nucleoplasmic forms of retinoblastoma protein (pRb) within the nuclei of senescent fibroblasts, which we have recently reported to be required for maintaining a proliferative state in early-passage human fibroblasts. Indeed, we have reported that a targeted RNAi-knockdown of either lamin A or LAP2 α in human fibroblasts led to a rapid de-phosphorylation and a loss of nucleoplasmic forms of pRb and irreversible G1 phase arrest as assessed by FACS analyses and confocal microscopy using proliferation markers. Furthermore, consistent with these findings, addition of extracts from senescent fibroblasts to a *Xenopus* in vitro nuclear assembly system caused oxidative modifications to C-terminal cysteine residues in *Xenopus* lamin LIII and inhibited nuclear lamina assembly and DNA replication.

Our data show that lamin A and LAP2 α become oxidatively modified in senescent fibroblasts, which induces changes in their structural conformation leading to their destabilisation and a less efficient assembly in the nucleus. This in turn leads to a de-phosphorylation of retinoblastoma protein and pRb-dependent senescent arrest. Therefore, our findings suggest that lamin A and LAP2 α act as oxidative stress sensors and are central components of senescence pathways. We propose a novel model for ageing of human fibroblasts in vitro whereby the accumulation of oxidative damage to lamin A and LAP2 α contributes to senescence signalling by destabilising the nuclear architecture.

Publications resulting from this work:

Kaltenpoth, M., Schmitt, T. and Strohm, E. (in preparation). Chemical composition of the antennal gland secretion of female European beewolves, *Philanthus triangulum*.
Strohm, E., Herzner, G., Kaltenpoth, M., Boland, W., Schreier, P., Peschke, K. and Schmitt, T. (in preparation). The chemistry of the postpharyngeal gland of female European beewolves (Hymenoptera, Sphecidae) supports a homology with this gland in ants.
Goettler, W., Kaltenpoth, M., Herzner, G. and Strohm, E. (2007). Morphology and ultrastructure of a bacteria cultivation

organ: the antennal glands of female European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae). *Arthropod Structure and Development* 36: 1–9.

Kaltenpoth, M. (2006). Symbiotische *Streptomyces*-Bakterien in Grabwespen. *Naturwissenschaftliche Rundschau* 59 (11): 618–619.

Kaltenpoth, M., Göttler, W., Dale, C., Stubblefield, J.W., Herzner, G., Roeser-Mueller, K., and Strohm, E. (2006). ‘*Candidatus Streptomyces philanthi*’, an endosymbiotic streptomycete in the antennae of *Philanthus digger* wasps. *International Journal of Systematic and Evolutionary Microbiology* 56 (6): 1403–1411.

Kaltenpoth, M. (2005). Bakterien schützen Wespen-Nachwuchs vor Pilzbefall, *Naturwissenschaftliche Rundschau* 58, 329–330.

Kaltenpoth, M., Göttler, W., Herzner, G. and Strohm, E. (2005). Symbiotic bacteria protect wasp larvae from fungal infestation, *Current Biology* 15, 475–479.

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