Department of Immunology
Medical University of Warsaw
Banacha 1a, 2-097 Warsaw, Poland
Tel. +48 22 599 21 98

BASTION – FROM BASIC TO TRANSLATIONAL RESEARCH IN ONCOLOGY

Report on the visit of Dr Lech Trzeciak to the University of Cologne, Institute of Virology (and the German National Reference Center for Human Papilloma Viruses) , within 7PR21/BASTION/WP1 (Twinning, T1.8)

Recipient/host name: Prof. Herbert Pfister

Goal of the visit

Oncogenesis in humans is generally driven by mutations; however, in up to 20% of human cancers a critical role at early stage of disease development is played by infectious agents. An important group of thee are human papillomaviruses (HPVs), strictly epitheliotropic viruses that infect keratinocytes. Genus A generally prevails in mucosal areas and includes several types known for their substantial oncogenic potential (such as HPV16 or HPV18); genome of these viruses encodes for proteins interacting with and inactivating well-known tumor suppressor proteins. HPVs from B group invade skin and usually cause short-lived benign skin changes. However, a deficiency of an immune system (whether inherited or acquired) enables B type infection to persists, what – combined with a prolonged exposure to ultraviolet - leads to a non-melanoma skin cancer (NMSC). An example of such a deficiency, with extreme and exquisite susceptibility to HPV beta infections, is epidermodysplasia verruciformis (EV). It is a rare disease caused by mutations in either of two closely related neighbouring genes, *TMC6* and *TMC8.* Resulting persistent cutaneous HPV infections lead slowly to NMSC. EV is thus regarded as a natural model for HPV-associated cutaneous oncogenesis.
During the first part of my twinning in Institute of Virology in Cologne I have performed an extensive genotyping of the course of HPV infection in an atypical case of EV resulting from yet-unreported mutation in *TMC8*. We observed frequent co-existence of two (or more) types of HPVs in various areas. My second visit to Cologne, as signaled in the report from the first part of the twinning, was therefore devoted mostly towards establishing an in-situ hybridization method (ISH) in order to gain a better insight into (co?)localization of HPVs in normal and diseased tissue. Additionally, I also set to learn how to isolate and culture human keratinocytes that could be indispensable in functional studies of proteins involved in HPV infection leading to carcinogenesis.

Short description of performed studies

In situ hybridization

For establishing an in situ hybridization method we have choosen to use biotin-labeled nick-translated DNA probes and tyramide-based amplification of signal with streptavidin-horseradish peroxidase conjugate as a detector and AEC-based colour reaction to report detection. Based on the reports in the literature, this method

is sufficiently sensitive, requires neither isotopes nor fluorescene microscopy and produces relatively weak background. I have performed a series of optimization experiments, testing the effects of various adjustements on overall sensitivity of ISH – aiming also at decreasing the cost of the assay without compromising its sensitivity. The following aspects were tested: nick-translation time (probe size), probe composition (full-length genome vs part of genome), dilution of streptavidin conjugate used before and after signal amplification, composition of hybridization buffer and, lastly, type of slide coating (unplanned; see below). The method developed is capable of reproducibly and unequivocally detecting HPV6 and HPV16-positive cells (staining with either diffuse or punctate pattern). Tests with other HPV probes will be conducted according to research needs; recently HPV11 probe was prepared and is being tested in Cologne.
Two problems arose during these tests:
1) selection of positive controls
The sensitivity limit of ISH for HPV is at best ~2-10 copies per individual cell, whereas for PCR-based methods it is well below 1 copy per averaged cell (i.e. most cells contain no virus at all). Thus, depending on the distribution pattern and load of viruses, one can therefore reasonably expect to get

- negative ISH despite evident viral load of at least up to 10 copies/cell
- positive ISH in few scattered cells in a sample with the load way below ~2-10 copies/cell.

The best positive control would be a sample with substantial and uniform virus integration – then qPCR load would correspond to actual load per cell. We have yet to find such human samples.

2) microscopic slide coating
This aspect, supposedly simple, was in reality difficult to control, because every pathology lab has its own long-lasting preferences compatible with downstream applications it routinly performs (mostly immunohistochemistry) and does not pay attention to this detail. I have initially worked with 3 types of slides and these differed only in minor handling issues (related mostly to the degree of hydrophobicity). However, when the method matured and we attempted to probe real samples, the whole set of slides from a new supplier, while handling very well, presented with a very strong red/pinkish wavy background that was obscuring the true staining and rendered the results unusable. Ad hoc-performed tests showed that this staining resulted from a direct binding of the streptavidin-HRP conjugate to the glass (or its coating). All these samples will have to be re-sliced and placed on the glass we recommend before we can reanalyze them with ISH.

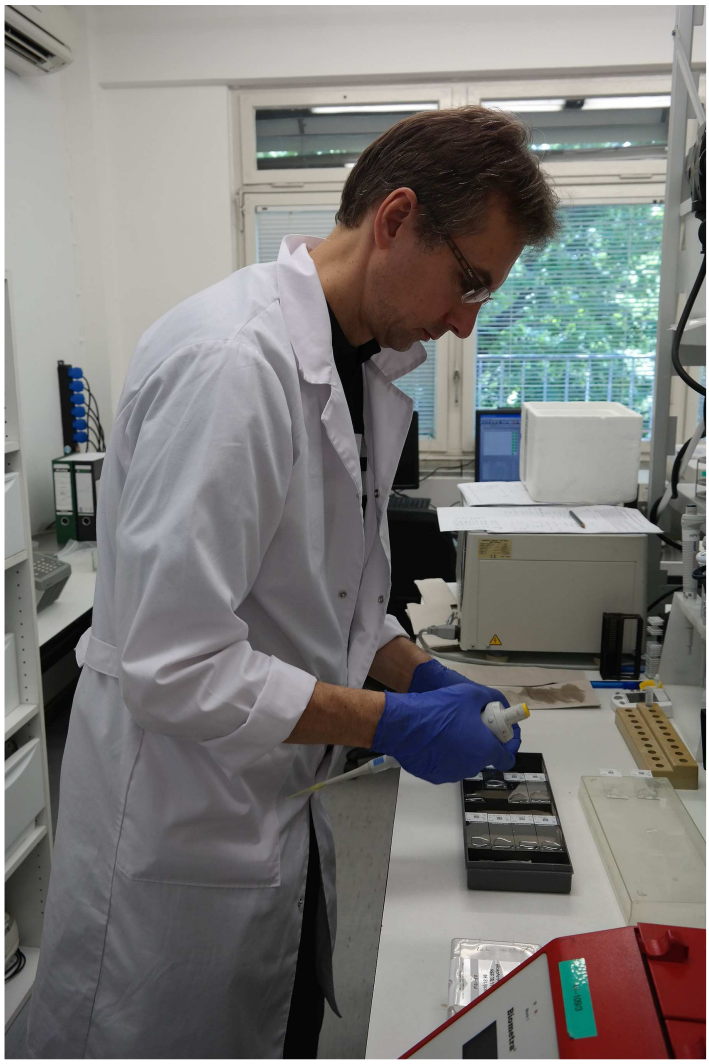
In addition to anti-HPV ISH, I had participated in performing in-situ hybridization for detection of miRNA (an assay utilized by Baki Akgül's group in the Institute). This method differs primarily in the type of probe utilized; miRNAs are very short and require locked nucleid acids(LNA)–based probe to facilitate hybridization. There are also some differences in composition of buffers and other conditions, but in general the execution of miRNA-ISH is sufficiently similar to HPV-ISH so that I would feel comfortable reestablishing it in Warsaw..

Primary keratinocyte cultures
Human primary keratinocytes are frequenty used in Baki Akgül's group. They are isolated from foreskin removed for clinical reasons and subsequently propagated for 10-14 days (co-cultured over a layer of 3T3 fibroblasts pre-treated with mitomycin) until <70% confluent and then harvested and frozen until needed. I have participated in an isolation, propagation and harvesting procedure twice and successfully processed one batch myself. I was also shown how to prepare organotypic cultures from these keratinocytes. This method can now be transfered to Warsaw.

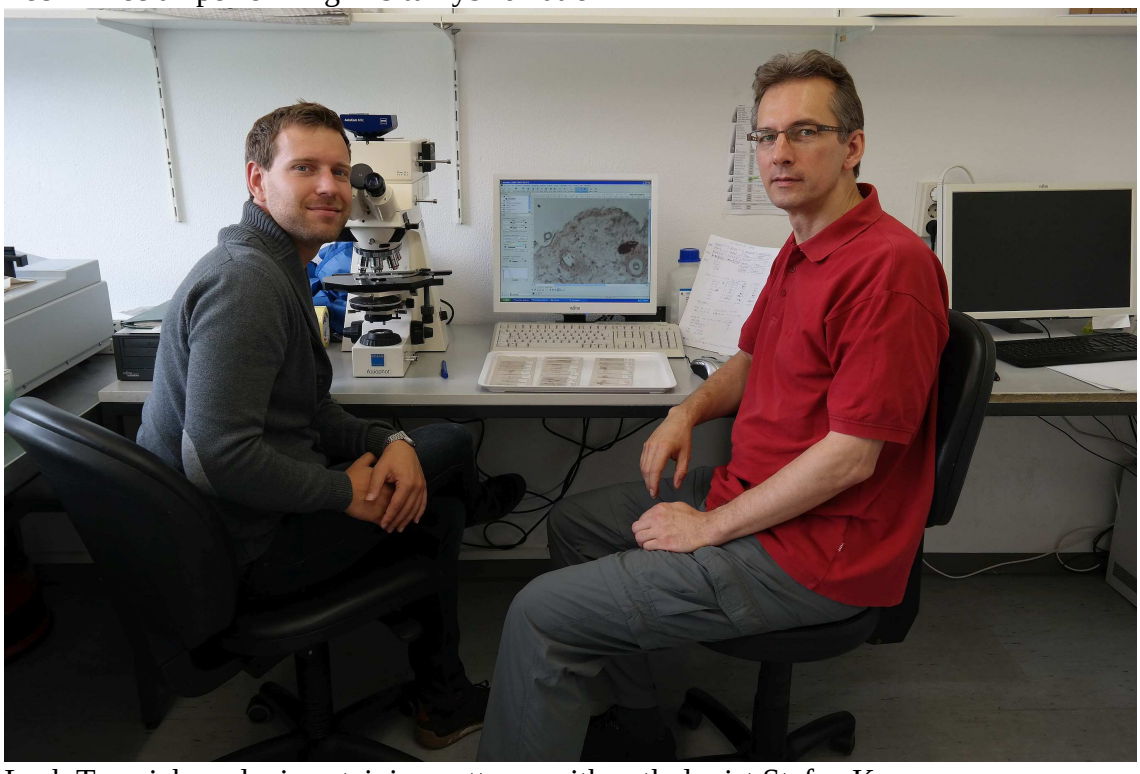
Workshop on modern microscopy techniques
During my stay in Cologne I also took an opportunity to participate in Leica Microscopy Workshop. The workshop and following hands-on demonstration focused on modern ultraresolution techniques: DLS, GSD and STED (2015.06.16-17.)

Manuscript preparation
I have also took a chance to discuss the scope of the manuscript that would report on the studies performed during my first stay in Cologne. We held two meetings with prof. Pfister regarding that matter and a revised version is being prepared.

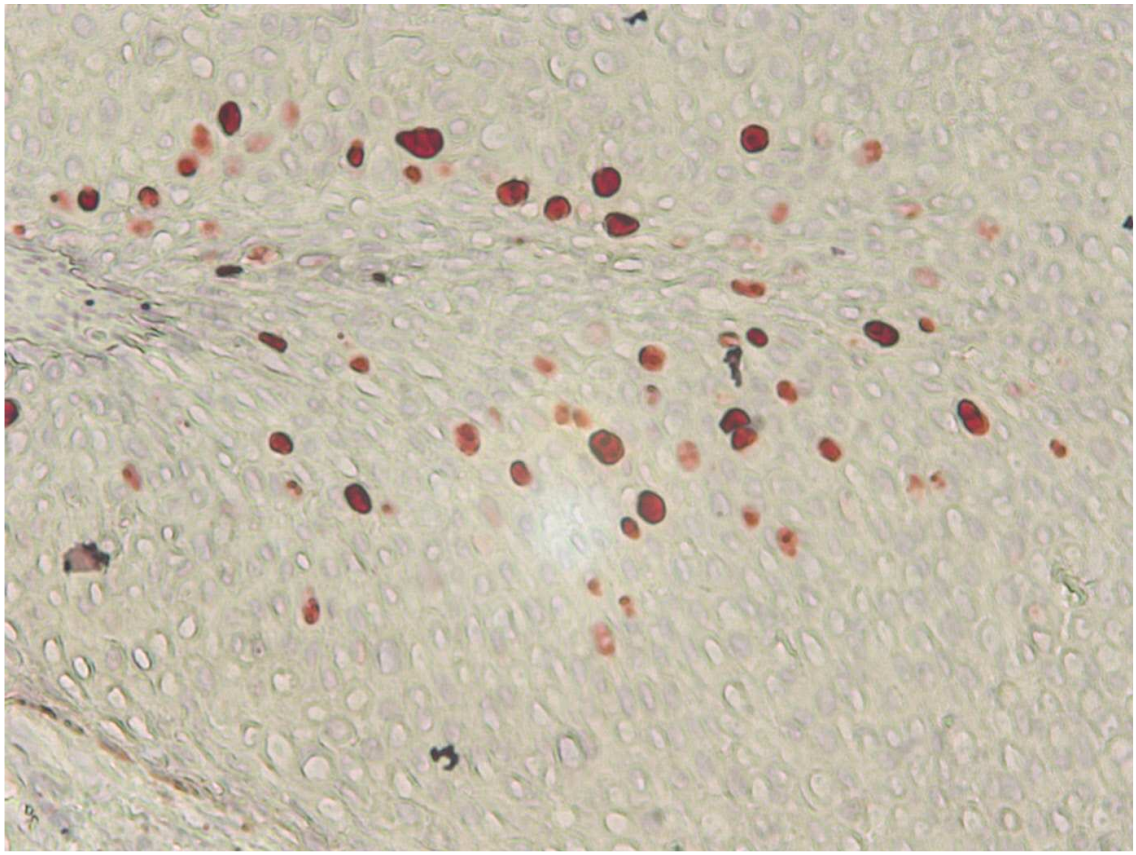
Photographic documentation



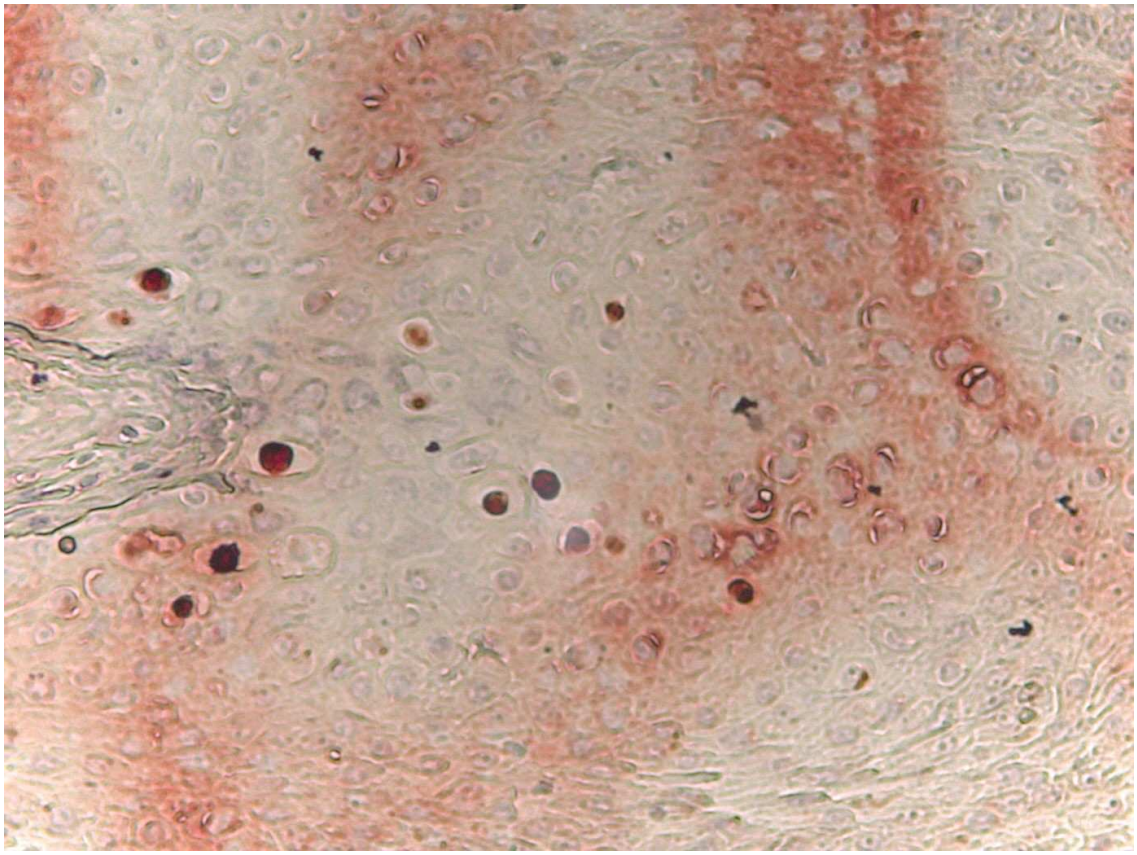
Lech Trzeciak performing in situ hybridization



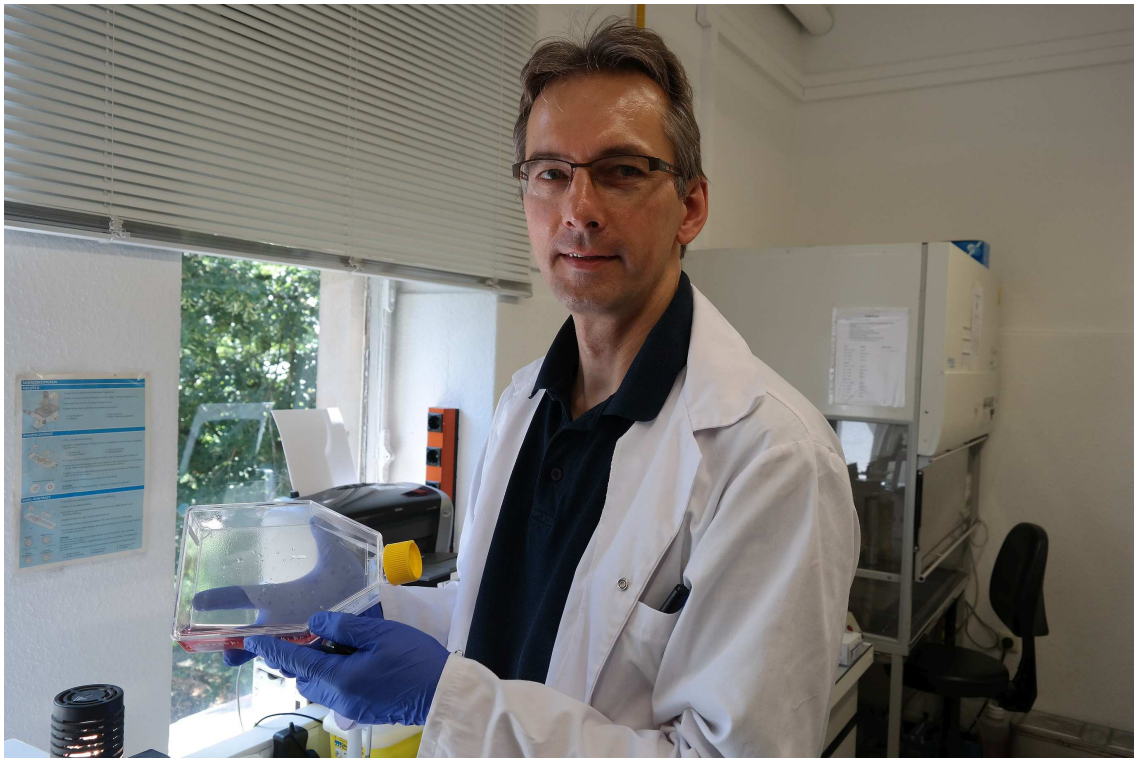
Lech Trzeciak analyzing staining patterns with pathologist Stefan Kocan.



Example of staining against HPV16.



Example of staining against HPV6 obscured by a strong wavy background caused by an incompatible microscopic slide coating



Lech Trzeciak with personally obtained culture of human keratinocytes on the feeding layer of 3T3 fibroblasts

Accessories necessary for organotypic culture of keratinocytes

Lech Trzeciak at a meeting with dr Steffi Silling and prof. Herbert Pfister (head of the Institute).

Future cooperation prospects

There are several areas of possible future cooperation. 1) the ISH method, established during this visit, will be useful in exploring cases with co-infection with various HPVs or with uncertainty about the precise localisation of a virus (such as cases of neoplasms with low viral loads). 2) we are now capable of establishing cultures of primary keratinocytes from our patients, whenever necessary. Then we can utilize methods and techniques used in the Institute for studies on molecular mechanisms of HPV-related carcinogenesis. 3) we can develop closer cooperation in the area of next generation sequencing (NGS) application in virological studies. We have already gained experience in detecting HPV susceptibility mutations in human DNA via the whole exome sequencing (WES; we have two novel mutations to report). Our deparment is also already involved in using NGS in searches for infectious agents in certain diseases. Unfortunately, the reciprocal twinning visit of German researcher to Warsaw has not occured.