Università degli Studi del Piemonte Orientale

Scuola di Medicina

Dottorato di Ricerca in Scienze e Biotecnologie Mediche (XXIX ciclo)

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PhD student annual report

# Defining the causal association between human beta papillomavirus infection, keratinocyte stem cells expansion and skin cancer development

## Introduction

Modern organ transplantation, which includes a multitude of different organs, is no longer encumbered by graft rejection but instead morbidity and mortality from cancer as a direct consequence of immunosuppressive therapy. The incidence of cancer in organ transplant recipients (s) is at least double that of the general population, while the risk of skin cancer (notably squamous cell carcinoma, SCC) is x60-250 (1-2) and is the most frequent form of malignant cancer in this individuals. In addition to the other well-known risk factors for skin cancer, numerous studies have also pointed to a possible causal role of *Betapapillomavirus* (β-HPV) in the pathogenesis of skin cancer in the immunocompromised host, either directly or in conjunction with sun exposure (3-4). Nearly all adults are persistently infected in the skin with many viruses belonging to the Human Papillomavirus (HPV) family. Although they appear not to cause overt clinical symptoms in a great majority of infected individuals, they can cause cancer, either in skin or in genital sites, particularly in subjects with impaired immune function. To date, more than 170 HPV types have been completely sequenced and classified into five genera based on DNA sequence analysis. Of these, *Alphapapillomaviruses* (α-HPVs) are associated with genital cancer and some genotypes with common skin warts. Skin-tropic *Betapapillomaviruses* (β-HPVs) are evolutionarily distinct from genus *Alpha* and appear to cause widespread unapparent or asymptomatic infections in the general population, but they have been associated with skin cancer in the immunocompromised host (5). Although the molecular mechanisms underlying β-HPV role in skin cancer development are not yet fully understood, both experimental and epidemiological evidence suggests a carcinogenic role of these viruses that takes place at the early stages in a manner that is different from that which has been worked out for high-risk AlphaPV; they generally do not integrate into the human cellular DNA and do not seem to be involved in maintenance of the malignant phenotype in Keratinocytes (6). Rather, β types have an established capability to impair DNA repair and apoptosis following excessive UV exposure. Recent work has suggested that overexpression of E6 and E7 oncoproteins from β-HPV types 5 and 8 can enhance the stem-like characteristics of transduced Keratinocytes (7). Another compelling evidence speaking in favour of β-HPV-induced transformation comes from the transgenic mouse model harbouring the complete early region of HPV8 under the control of the keratin-14 promoter. These mice express high levels of the viral early proteins in the skin and spontaneously develop benign skin lesions (>90% by 60 weeks) that progress to SCC in a significant proportion (6%) (8). Similar to OTR and EV patients, HPV8 transgenic mice develop skin keratosis, often referred to as field cancerisation. Field cancerisation is associated with the acquisition of a genetic change in a stem cell, with formation of an altered patch of epithelium which can be recognised histologically and reflect abnormalities of proliferation and/or differentiation of the epithelial cells (9). Thus, field cancerisation may be considered a manifestation of an initiation event. If followed by expansion of the initiated population (promotion), a population of cells are at high risk of developing further genetic changes, leading to invasive cancer (10-11). The human counterpart of the experimental animal model of initiation and promotion, and the stem cell origin of epithelial cancer, are supported by the phenomenon of field

cancerisation. Thus it is possible to hypothesize that β-HPV promotes field cancerisation by amplification of a keratinocyte stem cell pool through neutral drift, resulting in clinically apparent skin keratosis, which in turn predispose to skin cancer development (7-12).

**Aims**

During the 2 years of the Ph.D. course, I have been working on the following two Tasks:
-Task 1. Generation of *in vivo* orthotopic skin cancer xenografts in nude mice from kidney transplant recipients (KTR)-derived skin tumours and the identification of beta-HPV infection/expression in xenografts;
-Task 2. Define whether β-HPV transforming capability could be associated with keratinocyte stem cells (KSC) activation by identifying which population is targeted and expanded in HPV8 transgenic mice alongside with its self-renewal capacity.
An experimental approach that has been very successful for the investigation of tumour biology in general is the orthotopic xenograft model. We have successfully set up an innovative *in vivo* orthotopic xenograft assay using a "humanised" stromal bed repopulated with human fibroblasts, whereby primary human skin cancer tissue can be directly implanted for the routine growth of tumours.

**Materials and methods**

-HPV8 transgenic mice. These mice harbor the complete early region of HPV8 under the control of the keratin-14 promoter. They express high levels of the viral early proteins in the skin and spontaneously develop benign skin lesions (>90% by 60 weeks) that progress to SCC in a significant proportion (6%). These mice are used for whole mount and colony forming efficiency assay.
-Whole mount. Skin from HPV8 transgenic mice are peeled from the tail, cut into pieces (0.5x0.5 cm2) and incubated in dispase 2,5 U/ml in DMEM at 4°C over night. Forceps are used to gently peel the intact sheet of epidermis away from the dermis and the epidermal tissue is fixed in 10% neutral buffered formalin for 2 hours at room temperature. Fixed epidermal sheets are stored in PBS containing 0.2% sodium azide at 4°C until used.
-*In vivo* tumorigenicity assays of tumours. A stromal reaction is induced 2 weeks earlier by first implanting a sterilized gelfoam into the dorsal subcutaneous space of athymic Nude-*Foxn1*$^{nu}$ mice. Selected tumors are xenografted into the stromal bed in the form of intact tumor tissue (~0.5 cm$^3$). Tumors arising in nude mice are removed, FFPE or snap-frozen, and their sections are used for immunofluorescence and pathology evaluation.
- Colony forming efficiency assays (CFEA). Keratinocytes are isolated from the epidermis of HPV8 transgenic mice (n=10) and wild type (n=10) either pups or adult mice. The skin is harvested and incubated into a solution with DMEM and Dispase 2.5 U/ml O/N at 4°C. The day after, the epidermis is separated from the dermis and incubated in Tripsin-EDTA for 30' at 37°C in 5% $CO_2$. After repeated pipetting, the cells are resuspended in DMEM, centrifuged, seeded in 6 wheels plate, cultured for 15 days, and then stained with crystal violet 0.05%.
-Immunofluorescence analysis. Tissue sections are processed using antibodies against β-HPV E4 (kindly provided by J. Doorbar, dilution 1:500), L1 (in-house polyclonal antibodies), Ki67 (Abcam, dilution 1:200), LRIG1, (R&D System, dilution 1:100) LGR5 (novus biological, diluition 1:400), LGR6 (Abcam, diluition 1:50), MCM7 (Neomarkers, dilution 1:200), CD34 (BD, dilution 1:100), PCNA (Sigma, dilution 1:1000) and K15 (Thermos Scientific, diluition 1:50) using the immunofluorescence protocol currently used in our laboratory (15).

## Results

**Task 1**. Selected skin tumors from KTR attending the dermatology clinic in Novara e Turin have been xenografted into athymic nude mice (Athymic Nude-*Foxn1$^{nu}$*) in the form of intact tumor tissue (~0.5 cm$^3$). We have used athymic nude mice, instead of SCID mice, because they have been demonstrated to give the highest success rate in this model. Briefly, a stromal reaction has been induced 2 weeks earlier by first implanting a sterilized Gelfoam into the dorsal subcutaneous space together with $10^6$ primary human fibroblasts suspended in 100 µl of Matrigel and wounds have been closed with surgical staples. After 14 days, intact tumor tissue has been xenografted in the humanized stromal bed and 100µl Matrigel have been co-injected with an additional $10^6$ primary human fibroblasts. Tumors arising in nude mice have been removed, FFPE and their sections have been used for genotyping, immunofluorescence and pathology evaluation. Xenografts were initially harvested after six months as discussed with Girish Patel who developed the technique. Unfortunately, 7 xenografts implanted in this period showed cystic structures with dying epithelium. Five samples didn't give rise to viable tumors and 5 were lost because the mice died for other reasons. Based on this experience, we have decided to harvest them earlier approximately three months after grafting in nude mice. Following this procedure, 80% of the samples have given rise to viable tumors. Histologically, they displayed hyperplastic epithelium and in some cases also dysplastic areas. To date, 41 tumors, from 25 KTRs have been collected and implanted in nude mice. 26 xenografts have given rise to a tumor mass that allowed the preparation of FFPE blocks. They were established from tumors that were diagnosed as basal cell carcinoma (BCC, n=17), actinic keratosis (AK, n=1), seborrheic keratosis (SK, n=1), squamous cell carcinoma (SCC, n=2), and keratoacanthoma (KA, n=2) respectively. In 6 cases, the tumor mass was big enough and half of it has been frozen in RNA later to extract total RNA. These samples will be used for gene expression profiling. In 6 cases (3 SCC and 3 BCC), the xenografts have been reimplanted into nude mice for the second passage. Tissue sections from the FFPE blocks are being analyzed by immunofluorescence for the viral and cellular markers already used in the primary tumors, including the cellular proliferation marker minichromosome maintenance protein 7 (MCM7), and the viral protein E4 and L1.

As shown in the representative H&E images reported below (Figure 1), strong retention of overall tumour histology including cell morphology, stromal component architecture, and grade of differentiation of the epithelia have been observed between original patients' tumour and tumour grafts. Panels A shows a tumor from the hand of a female KTR who was transplanted for the first time in 1985. Due to her long lasting status of immunosuppression, the patient has developed an EV-like phenotype with many wart-like lesions. Consistent with her clinical picture, she has developed more than 15 skin cancers. Positivity for the viral markers was found in 3 blocks corresponding to an AK and the pathological border of a SCC.

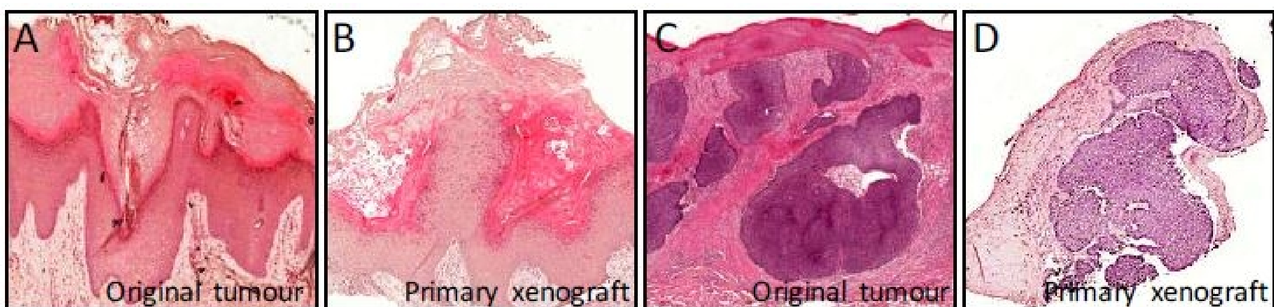


Figure 1. Histopathologic features of 2 patients' tumours and tumour grafts: A and B correspond to a well differentiated SCC from a female KTR with a 35 year history of iatrogenic immunosuppression who has developed an EV-like phenotype and many skin cancer where β-HPV infection was observed in many tumours; C and D correspond to a BCC from another KTR.

**Task2.** This part of the work has been performed in collaboration with Simone Lanfredini, a former PhD student from our laboratory who is currently working at the European Cancer Stem Cell Research Institute- Cardiff University-UK in the laboratory of Girish Patel. To determine whether the HPV8 transgenic mice keratinocytes have self-renewal capacity, we have performed a colony forming efficiency assay using keratinocytes isolated from whole HPV8 transgenic mice epidermis (n=10) and wild type (n=10). At the beginning, I used adult mice but the yield was very low. Then, we decided to use pups that have got hair-free skin. Freshly isolated keratinocytes have been plated and cultured for 15 days to perform an in vitro colony forming efficiency assay. This assay enables us to compare enumerated colonies from HPV8 transgenic and wild type mice. Cells from HPV8 transgenic mice showed greater colony forming efficiency in comparison with wt-derived cells, with a greater frequency of holoclones indicative of expanded keratinocyte stem cell numbers (Figure 2).

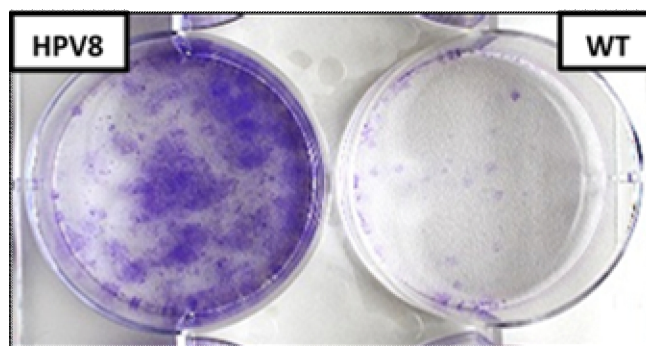


Figure 2. Representative data showing keratinocyte colony forming efficiency assay from whole skin extracts from wild type (WT, right) and HPV8 transgenic mice (left).

In order to understand the pattern of keratinocyte proliferation, we hypothesize that the proliferative effect of the HPV8 transgene may be restricted to a distinct hair follicle keratinocyte stem cell population. For viral infections to persist in the skin, against the tide of constant keratinocyte turnover, they must infect keratinocyte stem cells. There are at least 4 keratinocyte stem cell populations in the hair follicle that can recreate the entire interfollicular epidermis and hair follicle (16-20). In homeostasis, LGR5+ cells in the hair bulb and lower portion of the bulge support hair shaft formation (18), while CD34 and K15 positive cells of the bulge are involved in hair cycling, supporting regrowth of the hair follicle during anagen (16-17). LGR6 positive cells at the entry point of the sebaceous gland and within the sebaceous gland maintain sebaceous gland turnover (19). Lrig1 positive cells at the junctional zone support turnover of the infundibulum and adjacent overlying epidermis (20).

To determine which hair follicle keratinocyte population is expanded in βHPV8 transgenic mice compared wild type littermate, we have labelled skin sections in whole mount analysis. We observed no difference in hair follicle length, however the width and so overall area of the hair follicles was greater in the transgenic mice correlating with expansion of the LRIG1+ area (Figure 3). The mean area of the hair follicle LRIG1+ population was markedly increased in the βHPV8 transgenic compared to wild type mice (23,845+/-13,480 vs 14,907+/- 3,793 $\mu m^2$, $p<0.001$), compared to the CD34+ (8,356+/-1,465 vs 8,077+/- 1,510 $\mu m^2$, p=NS), LGR6+ (60,250 +/- 9,972 vs 49,216 +/- 13,540 $\mu m^2$, p=0.01), and LGR5+ (1,810 +/- 1,145 vs 1,735+/- 779 $\mu m^2$, p=NS) populations. In line with these findings hair follicle keratinocyte stem cell proliferation was greater

within the Lrig1+ (69 vs 55%, p<0.001), compared to the CD34+ (1 vs 1%), LGR5+ (1 vs 3%) and LGR6 (29 vs 40%) populations, of βHPV8 transgenic compared to wild type mice.

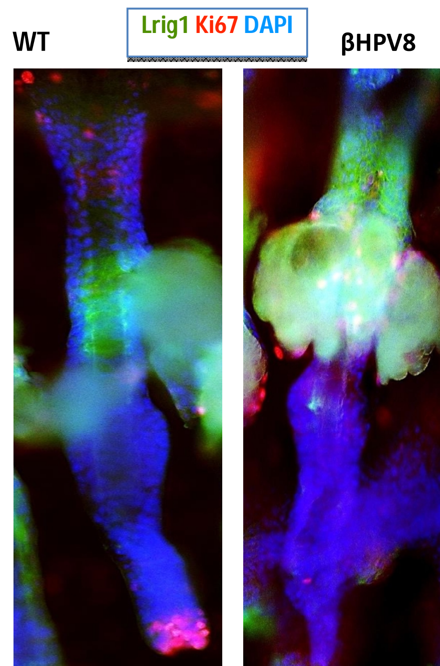


Figure 3. Whole mount staining of hair follicles from wild-type and HPV8 transgenic mice using antibody against Lrig1 (green) and Ki67 (red).

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**Meetings' participation**

- 42° congresso nazionale della Società Italiana di Microbiologia. Torino, Italy
  From 9-28-2014 to 9-1-2014
- 3rd Workshop on Emerging Oncogenic Viruses. San Pietro in Bevagna, Manduria, Italy.
  From 6-4-2014 to 6-8-2014.
- 7th International Conference on HPV, Polyomavirus and UV in Skin Cancer, Novara, Italy.
  From 4-9-2014 To 4-12-2014
- International *DNA Tumour Virus Meeting* 2015, Trieste
  From 21st to 26th July 2015

**Poster:**

- SIM Torino, Centro congressi 'Torino incontra' 28 settembre-1ottobre 2014

**Identification of the skin virome in a young boy with severe HPV2- positive warts that completely regressed after administration of tetravalent human papillomavirus vaccine.**
**Olivero C.**, Landini M.M., Peretti A., Matteelli A., Urbinati L., Mignone F., Lai A., Doorbar J, Borgogna C., Gariglio M., and De Andrea M.

- ICGEB *DNA Tumour Virus Meeting* 2015 Trieste, Italy, From 21 to 26 july 2015

**Different infection patterns in patients displaying abnormal susceptibility to HPV associated with development of multiple skin and mucosal lesions**
C. Olivero, F. Calati, A. Peretti, C Borgogna, M. De Andrea, J. Doorbar, D.V. Pastrana, C.B. Buck, and M. Gariglio.

## LESSONS

- "Tissue engineering: the state of the art" – 14 November 2014 – Dott.ssa Francesca Boccafoschi - Department of Health Sciences, University of Eastern Piedmont.
- "Regenerative Medicine" – 21 November 2014 – Prof. Maria Prat - Department of Health Sciences, University of Eastern Piedmont.
- "Ribosomopathies" – 25$^{th}$ May 2015 – Prof. Steve Ellis – Medical School, University of Louisville (Kentucky)
- "Basis of scientific research" – 10$^{th}$ June 2015 – Prof. Nicoletta Filigheddu – Università del Piemonte Orientale (Italy)

## SEMINARS

1. "Dysregulated antigen receptor signaling: molecular lessons from two congenital lymphoproliferative disorders" – 06 November 2014 - Prof. Andrew L. Snow - Department of Pharmacology Uniformed Services University of the Health Sciences Bethesda (Maryland, USA).
2. "Optical coherence tomography from bench to bedside shening the light during percutaneous vascular intervention" – 17 November 2014 - Dott. Secco Gioel Gabrio – Department of Health Sciences, University of Eastern Piedmont.

3. “La scoperta del bosone di Higgs” – 25 November 2014 - Dott. Roberta Arcidiacono - DiSCAFF, University of Eastern Piedmont - Dott. Marta Ruspa - Department of Health Sciences, University of Eastern Piedmont.
4. “Nuove sfide ed opportunità dell'epidemiologia molecolare per lo studio dei tumori” – 27 November 2014 - Prof. Laura Baglietto - Inserm - Centre for Research in Epidemiology and Population Health, Unit: Nutrition, Hormones and Women’s Health, Paris.
5. “Humoral responses to HCV infection and clinical outcomes” – 28 November 2014 - Dott. Arvind Patel - Programme Leader, MRC Centre for Virus Research, University of Glasgow (UK).
6. “Microglia microvescicles: messengers from the diseased brain” – 17 December 2014 - Dott. Roberto Furlan, San Raffaele University, Milan.
7. “Anticancer strategy Targeting cancer cell metabolism in ovarian cancer” – 19 January 2015 - Prof. Dr Yong-Sang Song, MD, PhD Director Cancer Research Institute, Gynecologic Oncology Chariman, Cancer Biology Interdisciplinary Program Professor, Obstetrics and Gynecology, College of Medicine Seoul National University.
8. “Different molecular mechanisms regulate hepatocyte differentiation during the transitions between epithelial and mesenchymal states” – 20 January 2015 - Dott. Tonino Alonzi, PhD, Lab. Of Gene Expression and Experimental Hepatology, Istituto Nazionale per le Malattie Infettive “L. Spallanzani” IRCCS, Rome.
9. “Targeting the liver to cure myocarditis: a lesson from a model of STAT3-dependent auto-immune myocarditis” – 21 January 2015 - Prof. Valeria Poli - Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology Center, University of Turin.
10. “Myeloid cells as therapeutic target in cancer” – 27 January 2015 - Prof. Antonio Sica - DiSCAFF, UPO, Novara.
11. “Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells” – 11 March 2015 – Prof. Darko Bosnakovski - Associate Professor, University "Goce Delcev" Stip, Faculty of Medical Sciences, Krste Misirkov bb, 2000 Stip R. Macedonia.
12. “Signal control in iNKT cell development and function” – 09 April 2015 - Prof. Xiaoping Zhong, MD, PhD - Associate Professor, Department of Pediatrics-Allergy and Immunology Duke University, Medical Center, Durham (North Carolina, USA).
13. “Actin-based mechanisms in the control of gene expression and cell fate” – 21$^{st}$ April 2015 – Prof. Piergiorgio Percipalle – Associate Professor, Department of Cell and Molecular Biology, Karolinska Institutet (Solns, Sweden).
14. “An integrated approach to the diagnosis and treatment of ovarian cancer” – 7$^{th}$ May 2015 – Prof. John McDonald, MD, PhD – Integrated Cancer Research Center, School of Biology and Parker H. Petit Institute of Bioengineering and Biosciience, Georgia Institute of Technology, Georgia Tech University, Georgia (Atlanta, USA).
15. “Conflicting interests and scientific communication” – 14$^{th}$ May 2015 – Prof. Kathleen Ruff – RightOnCanada Founder, Senior Advisor to the Rideau Institute (Ottawa, Canada).
16. “Recent developments in (cutaneous) Human Polyomavirus research” – 5$^{th}$ June 2015 – Mariet C.W. Feltkamp – Associate Professor of Medical Virology, Department of Medical Microbiology, Leiden University Medical Center (Leiden, The Netherlands).

17. “High-tech product preservation and operator protection: two apparently opposite requirements in different fields of medicine and biotechnology: the emerging glove box approach” – 15th July 2015 - Dr. Ing. Marco Fatta, Phd – COMECER Group (Italy).

18. “Le cellule staminali nel danno renale acuto e nel trapianto di rene” – 28th July 2015 - Dr. Vincenzo Cantaluppi, MD – Facoltà di Medicina e Chirurgia, Università di Torino (Italy).

19. “Cell based models for studying molecular mechanisms of Facioscapulohumeral Muscolar Distrophy (FSHD)” , “Toward animal model for Facioscapulohumeral Muscolar Distrophy (FSHD)” – 3rd September 2015 - Prof. Darko Boshnakovski, PhD – University Goce Delcev Stip, Faculty of Medical Sciences (Stip, R. Macedonia).

20. Miniworkshop on “Biotechnology for Dermatology” – 9th July 2015 - Dr Gwenaël ROLIN, PhD - Clinical Research Engineer - Thomas LIHOREAU - Ingénieur hospitalier, Research and Studies Center on the Integument (CERT), Department of Dermatology, Clinical Investigation Center (CIC INSERM 1431), Besançon University Hospital; INSERM UMR1098, FED4234 IBCT, University of FrancheComté, Besançon, France.