

## Presence of HPV 16 and HPV 18 in Spermatozoa and Embryos of Mice

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**Abstract.** *Background/Aim: Sperm cells are competent to integrate exogenous DNA into their genome. We sought to clarify Human Papilloma Virus (HPV) internalization in spermatozoa and early preimplantation embryos. Materials and Methods: Sperm was incubated with plasmid vectors containing the complete genome of human HPV 16 and HPV 18 tagged with the green fluorescent protein (GFP) gene, to investigate HPV 16 and HPV 18 integration in mouse spermatozoa. Oocytes were in vitro fertilized with preincubated spermatozoa to investigate HPV 16 and HPV 18 potential transfer to mouse embryos. Results: Spermatozoa were able to internalize constructs of cloned high-risk HPV either as integrated or as episomal DNA. Constructs of cloned HPV can also be transferred to mouse embryos, through in vitro fertilization of the oocytes by mouse spermatozoa. Conclusion: Viral DNA transmission to the early mouse embryo via sperm, highlights the effect of HPV in reproductive cells and preimplantation development.*

Human Papilloma Virus (HPV) is a non-enveloped, circular, double stranded DNA virus (1). The viral genome consists of a regulatory non-coding long control region, an early region encoding for E6, E7, E1, E2, E4 and E5 genes and a late region encoding for L1 and L2 genes (2). HPVs are generally classified as low and high risk, according to their

potential to cause genital cancer. The different types of papilloma viruses exhibit characteristic tropism and distinct life cycle features (3).

HPV 16 and HPV 18, as oncogenic types, are responsible for cervical cancer, the second most common cause of cancer-related death worldwide (4, 5). HPV 16 is considered to be the most frequently identified high-risk HPV genotype, followed by HPV 18. Approximately 70% of cervical cancer cases are linked with these two genotypes (6, 7).

Cancer of the uterine cervix is associated with high-risk HPV presence and increased HPV E6/E7 oncogene expression (8-11). Persistent infection with oncogenic HPV genotypes triggers HPV DNA integration into the host genome, eventually leading to chromosomal damage accumulation and genome destabilization in infected cells (12-17). The exogenous DNA insertion into the oocyte by sperm (18-20), as well as the mechanisms involved (21-28), have been extensively studied. In fact, living spermatozoa of almost all species are able to take up spontaneously exogenous DNA and internalize a part of it into their nucleus (24). The exogenous DNA fragments are localized at the postacrosomal and equatorial regions of the sperm head (22, 23) with 15-22% internalized into the sperm nuclei (24). A proportion of the internalized DNA is integrated at specific sites in sperm genome, probably at a nucleosomal subfraction of chromatin, suggesting a common site for exogenous DNA insertion (29, 30). Sperm has the capacity to actively take up exogenous DNA derived from HPV. In addition to HPV L1 gene, sperm probably take up DNA from HPV types 16 and 18 (31, 32).

Taking into account the capability of sperm cells to integrate exogenous DNA into their genome, we sought to clarify HPV internalization into sperm genome. In this study, we incubated mouse spermatozoa with plasmid vectors containing the complete genome of human HPV 16 and HPV 18 virus tagged with the green fluorescent protein (GFP)

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gene, to investigate HPV 16 and HPV 18 integration in mouse spermatozoa. We also *in vitro* fertilized oocytes with preincubated spermatozoa to investigate HPV 16 and HPV 18 potential transfer to mouse embryos.

## Materials and Methods

**Animals.** FVB/N male and female mice were provided by the Hellenic Pasteur Institute. Their health was verified by a quality control report. Mice were kept in the Breeding Laboratory Animal Institute (Ioannina University, Ioannina, Greece) under a controlled 12 h light and 12 h darkness life cycle and a standard nutritional program. All research protocols were in compliance with the European Union guidelines for animal use and were approved by the General Directorate of Agricultural Economics and Veterinary Medicine of Epirus Region, Greece, in accordance to principles of laboratory animal handling.

**Plasmids.** HPV 16 and HPV 18 full genome was cloned in plasmid vectors and tagged with green fluorescence protein (GFP) gene (GFP-HPV 16, GFP-HPV 18 - kindly provided by Professor Vincent C. Lombardi, Department of Microbiology and Immunology, University of Nevada, Reno, USA). A sufficient amount of plasmid vectors containing HPV 16 and HPV 18 genome was isolated from bacterial cell cultures.

**Sperm retrieval and preparation.** Male fertile mice, non-mated with female mice for 3-7 days, were sacrificed by cervical dislocation. Sperm was collected from cauda epididymis in culture medium (Sydney IVF Sperm Medium, COOK, Limerick, Ireland). Swim Up procedure was employed as to select for the most motile spermatozoa. Sperm cells were subsequently counted in a Neubauer chamber. Mouse spermatozoa were used for *in vitro* fertilization (IVF) procedure following fluorescence-activated cell sorting (FACS) and confocal microscopy.

In IVF experiments, sperm was placed in fertilization medium (Sydney IVF Fertilization Medium, COOK, Limerick, Ireland) for about 2 h (37°C, 5% CO<sub>2</sub>), so as to be capacitated and acquire fertilization ability. Semen samples were divided into a control group and a study group, where sperm was incubated for 30 min with GFP-HPV 16, GFP-HPV 18 plasmid vectors. All sperm samples were finally added to the fertilization plates (200 sperm cells/μl). As for fluorescence-activated cell sorting analysis, spermatozoa were incubated with plasmid vectors, containing HPV 16 and HPV 18 genome (50 ng/10<sup>6</sup> sperm cells), for 5 h under liquid culture conditions (37°C, 5% CO<sub>2</sub>). As for confocal microscopy analysis, sperm samples were placed on microscope slides and screened for GFP fluorescence.

**Oocyte retrieval.** For superovulation and multiple oocytes development, 8 IU of the follicle stimulating hormone FSH (rec-FSH, Gonaf-F, Merck Serono, London, UK) were intraperitoneally injected in 6-8 weeks old female mice. Forty-eight h later, 5 IU of the human chorionic gonadotropin hCG (hCG, Pregnyl, N.V. Organon, Oss, the Netherlands) were also injected to induce oocyte maturation. Female mice were sacrificed by cervical dislocation. About 12 h after hCG treatment, oocytes were collected from the oviducts in culture medium (Sydney IVF Follicle Flush Buffer & Fertilization Medium, COOK, Limerick, Ireland).

**In vitro fertilization.** Oocytes were placed in fertilization plates and incubated with sperm in suitable fertilization medium (Sydney IVF Fertilization Medium, COOK) for about 5 h. Mouse zygotes were cultured in cleavage and blastocyst medium (Sydney IVF Cleavage Medium & Sydney IVF Blastocyst Medium, COOK) in culture laboratory incubator (37°C, 5% CO<sub>2</sub>), up to the blastocyst stage. Fertilization outcome was checked by the second polar body extension and the zygote pronuclei formation. *In vitro* preimplantation development of mouse embryos was daily observed and recorded.

**Fluorescence-activated cell sorting (FACS).** Spermatozoa, after being incubated with the plasmid vectors, were washed, resuspended in PBS and analyzed by quantitative flow cytometry using the FACS Calibur cytometer. The measurements were performed on 10,000 spermatozoa per semen sample. Non incubated spermatozoa were used as controls to evaluate background fluorescence setting intensity thresholds. The number of HPV positive cells was determined using fluorescence distributions, characterized by specific fluorescence intensity thresholds, to distinguish positive from negative signal. In each assay, fluorescence limits were set so that 99.6% of the cells were considered as negative and 0.4% as false positive. Sample values above the 0.4% threshold were considered as positive. The percentage of virus-positive cells was calculated subtracting false positive from positive values percentage, after overlapping the distribution curves of the study and the control sample. Data analysis was performed by CellQuest software.

**Confocal microscopy.** GFP observation in mouse spermatozoa and embryos as well as preimplantation development evaluation, were performed through Leica X10 objective lens, using a Leica TCS SP5 confocal microscope equipped with an argon laser (excitation at 488nm), a 561 solid-state laser line and a helium neon laser (excitation at 633 nm). All images were obtained by LASAF Software.

**Statistical analysis.** FACS analysis was evaluated by the Kolmogorov-Smirnov test. All measurements with  $p \leq 0.001$ ,  $D/s(n) \geq 12$  and  $D \geq 0.12$  were set as statistically significant.

## Results

**HPV 16 and HPV 18 integration in mouse spermatozoa.** In order to study HPV 16 and HPV 18 integration to mouse spermatozoa, semen was incubated with plasmid vectors, containing the complete genome of human HPV 16 and HPV 18 virus tagged with the GFP gene. Spermatozoa were incubated with GFP-HPV 16 and GFP-HPV 18 constructs (50 ng/10<sup>6</sup> sperm cells) for 5 h under liquid culture conditions (37°C, 5% CO<sub>2</sub>). HPV 16 and HPV 18 integration in mouse spermatozoa was monitored by flow cytometry (Figure 1). A total of 26 sperm samples were studied. Twelve sperm samples (46.2%) were HPV positive. To further verify HPV 16 and HPV 18 presence, sperm samples were also screened for GFP expression by confocal microscopy. A GFP fluorescent signal was observed in HPV-positive spermatozoa. The results indicate that spermatozoa are able to internalize constructs of cloned high risk HPV viruses either as integrated or as episomal DNA.

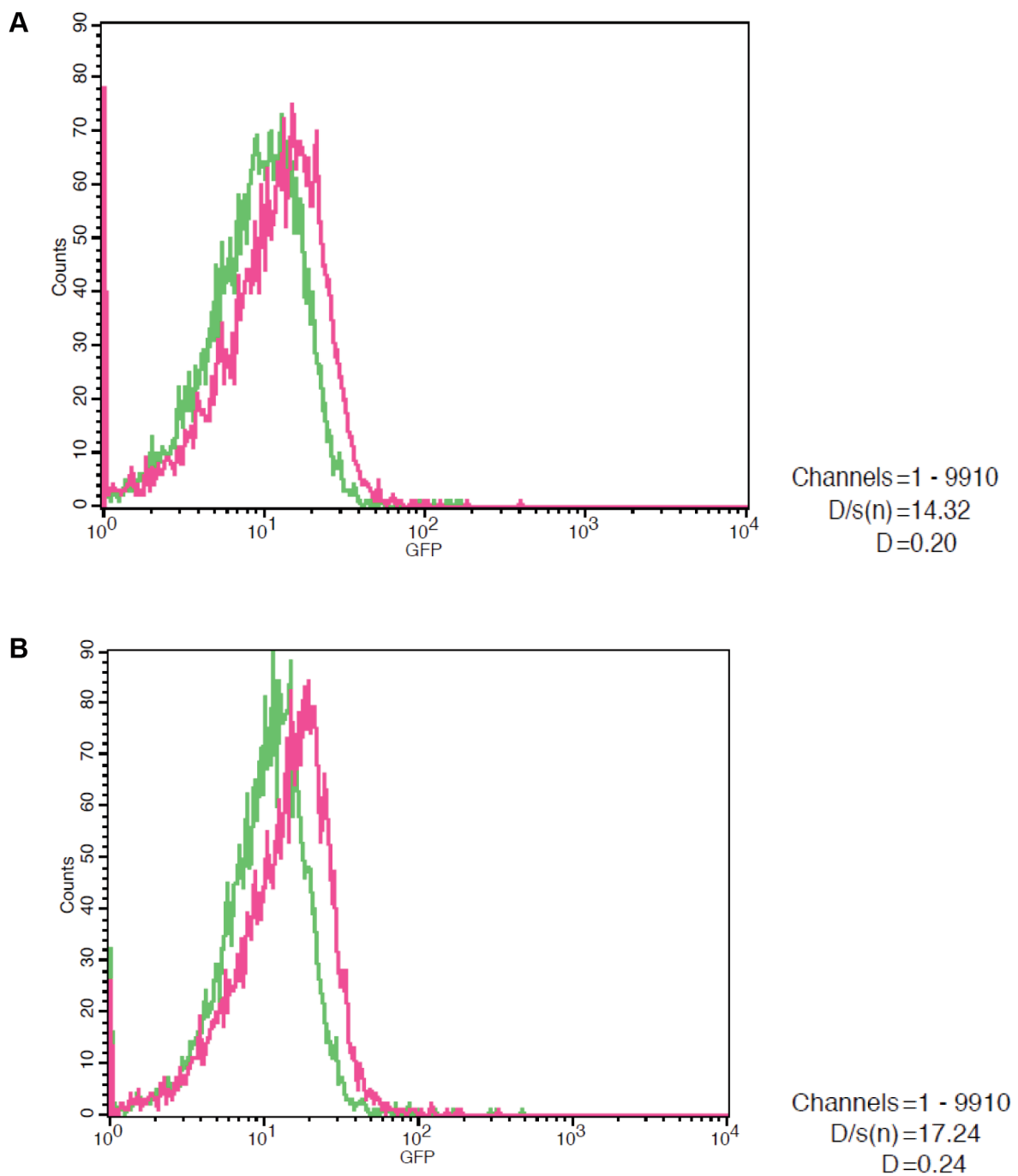


Figure 1. Fluorescence-activated cell sorting (FACS) analysis in mouse epididymal spermatozoa after a 5-h incubation with GFP-HPV 16 (A) and GFP-HPV 18 (B) constructs (50 ng/10<sup>6</sup> sperm cells). Spermatozoa are able to internalize constructs of cloned high risk HPV viruses.

**HPV 16 and HPV 18 transfer to mouse embryos.** In order to study the HPV 16 and HPV 18 presence in mouse embryos, exploring the effect of HPV DNA transfer to mouse oocytes by sperm during fertilization, oocyte fertilization by preincubated spermatozoa was conducted, as almost half of

them were GFP positive. Prior to *in vitro* fertilization process, spermatozoa were incubated with GFP-HPV 16 and GFP-HPV 18 plasmid vectors (50 ng/10<sup>6</sup> sperm cells) for 30 min. HPV 16 and HPV 18 presence in mouse embryos was evaluated through green fluorescence detection by confocal

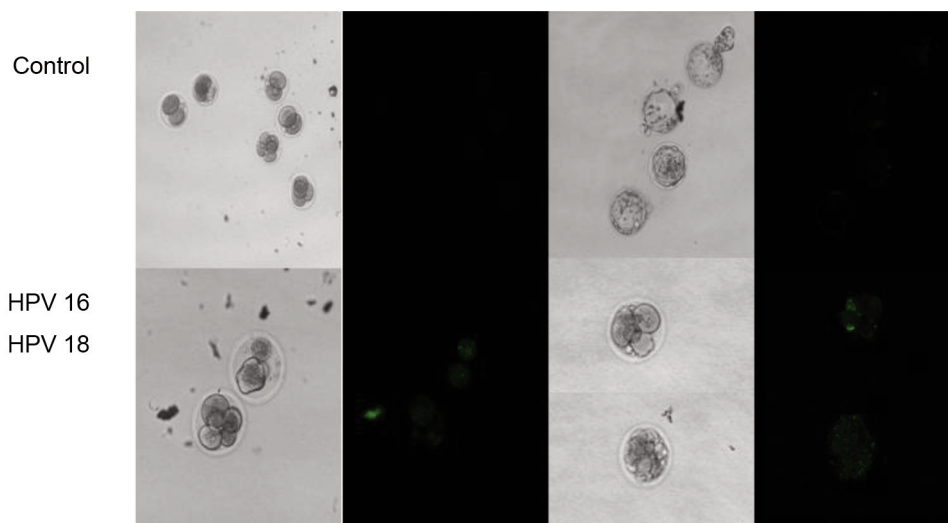


Figure 2. Confocal microscopy analysis of control embryos and HPV-positive embryos. HPV 16 and HPV 18 positive embryos present green fluorescence protein signal. Constructs of cloned HPV viruses can be transferred to mouse embryos, through oocyte *in vitro* fertilization by preincubated spermatozoa.

microscopy (Figure 2). The control group included 97 embryos. As for the HPV 16 and HPV 18 group, a total of 89 embryos were studied. A total of 36 embryos (40.4%) were HPV positive. The results indicate that constructs of cloned HPV viruses can be transferred to mouse embryos, through oocyte *in vitro* fertilization by spermatozoa.

## Discussion

HPVs constitute agents of one of the most common sexually transmitted diseases worldwide, affecting both males and females. Despite the fact that HPV is present in semen (33-36), the role of infected sperm cells in virus transmission remains unknown. In fact, it is not still clear whether HPV positive spermatozoa infect the partner, retain their fertilization ability or possibly interfere with *in vitro* fertilization procedure, embryo development and assisted reproduction techniques outcome. In the current study, we investigated HPV 16 and HPV 18 integration in mouse epididymal spermatozoa caused by cloned human HPV viruses and their potential transfer to mouse embryos. Taking into account the capability of mammalian sperm cells to bind exogenous DNA or RNA molecules and internalize them into their nucleus (24), we incubated mouse spermatozoa with plasmid vectors containing the complete genome of human HPV 16 and HPV 18 virus tagged with GFP gene.

Generally, the specific sites on the spermatozoon where the foreign DNA is localized have been reported to be at the equatorial and postacrosomal regions of the sperm head (24). The action of sperm cells as vectors or carriers and the integration of HPV DNA into the sperm nucleus are both not

well-defined, raising queries on whether viral DNA is just trapped in the membrane, is free in the cytoplasm, or is internalized into the nucleus and maintained as episomal. HPV probably binds to two distinct sites along the equatorial region of the spermatozoon's head. Glycosaminoglycans and soluble factors of spermatozoon's surface mediate the interaction between HPV and sperm (37-39). Specifically, glycosaminoglycan syndecan-1 and L1 HPV capsid protein co-localize in the equatorial region of the head (40). Although HPV DNA mainly localizes in the sperm head, its integration into the nucleus is unclear. In this study, HPV 16 and HPV 18 integration in mouse sperm cells was monitored by flow cytometry and verified through GFP expression screening by confocal microscopy, indicating that spermatozoa are able to internalize constructs of cloned high-risk HPV viruses either as integrated or as episomal DNA. Viral DNA integration into the sperm nucleus could not be directly demonstrated, but nevertheless integration is evident by GFP gene expression.

Sperm cells are considered to be transcriptionally and translationally silent, due to their compact DNA structure and their restricted cytoplasm. Keeping in mind spermatozoas' transcription and translation inactivation, the detection of viral RNA or protein in sperm suggests that HPV could infect the early stages of spermatogenesis. Spermatozoas' strongly compacted nucleus makes viral DNA penetration a demanding process. However, it was indicated that HPV 16 and HPV 18 E6 gene-specific mRNA is expressed in human sperm, assuming that HPV genes are actively expressed in infected sperm cells (41). It was also demonstrated that HPV is capable of transferring viral DNA



into sperm following incubation and that infected cells are able to deliver exogenous DNA to the cumulus cells surrounding ovulated oocytes at the moment of fertilization (42). In this study, HPV presence was confirmed in embryos, indicating that spermatozoa convey the exogenous human viruses to oocytes during *in vitro* fertilization. The exogenous DNA was possibly integrated into the sperm genome and consequently transferred by sperm in the genome of HPV-positive embryos.

Regarding HPV transmission and embryogenesis, spermatozoa carrying exogenous HPV DNA act potentially as vectors, transmitting HPV to the fetus through fertilized oocytes (43-46). In case that sperm cells carrying foreign DNA are allowed to contact directly oocytes, the DNA is transferred into the oocytes at the time of fertilization (21, 47). Additionally, in mouse models, HPV infected sperm successfully fertilized oocytes (48), followed by subsequent gene expression in the inner cell mass and the trophectoderm of preimplantation blastocysts (32). During the hamster egg-human sperm penetration test, the human sperm transferred both E6/E7 genes and L1 major capsid protein to oocytes (40), while increased DNA fragmentation and trophoblast death were observed in blastocysts after E6/E7 genes expression (49-51). Exposure of early mouse embryo to HPV 16 and HPV 18 DNA fragments, demonstrated embryo stage specific effects, showing a decrease in blastocyst formation (HPV 16) and hatching process (HPV 18) (51).

## Conclusion

This study highlights the possibility of viral DNA transmission to the early embryo *via* sperm, opening new perspectives on the effect of HPV in reproductive cells. It is of crucial importance to clarify HPV integration and address the impact of HPV expression in mouse early preimplantation development. HPV persistence may impair sperm parameters, suggesting caution in the use of these cells for assisted reproduction techniques or sperm banking. At present, it is unknown whether there are risks for embryos fertilized by infected sperm; however, HPV-positive spermatozoa injected in the oocyte cytoplasm could possibly interfere with fertilization, implantation, embryo development, premature abortion, and definitively with outcome of assisted reproduction techniques. More work needs to be carried out to refine the transmission process and to improve researchers' understanding on DNA transfection properties of sperm cells. Of course, further studies are necessary to discuss the possible clinical implications of the experimental findings.

## Conflicts of Interest

The Authors declare that they have no conflicts of interest regarding this study.

## Authors' Contributions

E.M., C.K. and I.G. conceived and designed the study; E.M. and C.K. conducted the experiments; E.M. and I.G. wrote the paper; E.M., C.K., T.E., A.Z., N.Z. and I.G. discussed the manuscript and provided important feedback; I.G. supervised the project.

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