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VLP production in *Leishmania tarentolae*: A novel expression system for purification and assembly of HPV16 L1

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ABSTRACT

Viral like particles (VLPs) have been used as immunogen for improvement of preventive vaccines against several viral infections in preclinical and clinical trials. These constructs can stimulate both cellular and humoral immunity. Two prophylactic HPV L1 VLP vaccines known as Gardasil and Cervarix were commercialized worldwide. However, there are main problems for expression and purification of VLPs in eukaryotic expression systems such as *baculovirus* and yeast leading to high cost of these vaccines. A novel *Leishmania* protozoan system has been applied to produce different recombinant proteins due to unique properties including generation of similar proteins with mammalian, easy handling, and large-scale culture. In the current study, we developed a novel strategy to produce HPV L1 VLP using stably transfected *Leishmania* cells. The positive transfectants were analyzed by SDS–PAGE and Western blot analysis. The assembly of purified L1 protein was detected by TEM microscopy. Finally, C57BL/6 mice were immunized by crude VLPs and antibody responses were assessed. The results of electronic microscopy revealed average 55–60 nm for L1 VLP. Furthermore, high IgG1 and IgG2a antibody responses were generated by L1 VLPs in mice similar to L1 VLPs produced in *baculovirus*-infected insect cells. Regarding the results, the amount of recombinant protein generated by *Leishmania* was 2–3 mg/500 ml media, suggesting further optimization of this system for using in large animals and human.

1. Introduction

Viral like particles (VLPs) are produced by structural proteins of viruses which have an inherent property for self-assembly, and imitate the morphology of the pathogen [1,2]. Generally, human papillomavirus (HPV) virions contain 360 copies of L1 and up to 72 copies of L2, which assemble into an icosahedral structure with one L2 molecule being at the central opening of each capsomere [3]. VLPs demonstrate a main development in subunit vaccines with enhanced immunogenicity [1,2]. VLPs possess the size and morphology of viruses (~22–200 nm), depending on the specific viral proteins being included [4]. Up to now, two HPV vaccines (quadrivalent Gardasil and bivalent Cervarix) based on the VLP structures were approved by FDA for human use. In these vaccination strategies, the recombinant HPV L1 major capsid protein was self-assembled into VLPs in yeast and/or insect cell expression systems. The size of VLPs was ~60 nm in diameter, similar to native virions [2,5]. Regarding some studies, there are different recombinant protein expression systems such as *E. coli*, yeast, insect, plant, and mammalian cells for generation of VLP-based candidate vaccines targeting various viral, bacterial, parasitic and fungal pathogens, as well as non-infectious diseases [5,6]. These expression systems have shown some disadvantages including lack of post-translational modifications, improper protein folding, and formation of inclusion bodies in prokaryotic systems; contamination, high cost, low yield, hyper-glycosylation in eukaryotic systems [4,6]. Recently, the ability of the eukaryotic parasite "*Leishmania tarentolae*" was confirmed to express different recombinant eukaryotic and biomedical proteins [7]. *Leishmania tarentolae* (*L. tarentolae*) shows some benefits compared to the mammalian cell lines such as: (a) the parasite is non-pathogenic for human; (b) it is fairly easy to cultivate; (c) it produces suitable recombinant protein yields (0.1–30 mg/L or more than), and (d) it can easily be improved to an industrial scale [8–11]. Furthermore, development of constitutive and inducible-integrative expression vectors for *Leishmania* led to the generation of secretory or intracellular recombinant proteins. By homologous recombination, the expression cassette is

Abbreviations: HPV, Human papillomavirus; VLP, Viral like particles; *odc*, Ornithin decarboxylase; DAB, Diaminobenzoic Acid; TEM, Transmission electron microscopy; OPD, O-Phenylenediamine; LM, Laminin; EMCV, Encephalomyocarditis virus.

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either inserted into the chromosomal 18S ribosomal RNA locus (*ssu*: constitutive system) or into the ornithin decarboxylase locus (*odc*: inducible-integrative system) of *L. tarentolae*. *Ssu* is a repetitive locus of the *L. tarentolae* genome with high rates of transcription by the host RNA polymerase I, whereas the *odc* locus is transcribed by RNA polymerase II [12]. In current study, we attempted to generate the recombinant L1 protein of HPV16 in the *L. tarentolae* expression system and also its assembly as viral like particle. In addition, its immunogenicity was studied in C57BL/6 mice model compared to VLPs produced in insect cells. The production of VLP in parasite (*L. tarentolae*) was done for first time in the world.

2. Material and methods

2.1. Gene construct

HPV16 L1 gene (Accession number: AJ313179.1) from pUF3-L1 vector (provided kindly by Prof. Martin Muller) was cloned into a cloning vector pGEM-7Zf (+) and sequenced. Then, the correct insert was subcloned into *Xho*I and *Kpn*I sites of an inducible LEXSY expression vector pLEXSY-I-blecherry3 (JenaBioscience). The resulting construct was designated as pLEXSY-I-L1 (Fig. 1). The plasmid was produced at high concentration using Midi DNA extraction kit (Qiagen).

2.2. Generation of stable Leishmania clones expressing HPV16 L1

Digestion of 15 μg pLEXSY-I-L1 was performed by *Swa*I restriction enzyme. This treatment generated a 2000 bp fragment representing the *E. coli* part and a large fragment containing the linearized pLEXSY-I-L1 integrated into the chromosomal *odc* locus of the LEXSY host (T7-TR). The EGFP control plasmid (pLEXSY-I-egfp-blecherry3: Cat. No. EGE-246) was used as a positive control. Gel extraction of the linearized plasmid was performed with an agarose Gel Extraction Kit (Qiagen). Then, the promastigotes of *L. tarentolae* (*L. tar*: T7-TR) were grown in Brain–heart infusion medium (BHI, Sigma) and also Medium 199 (M199, Sigma) at pH = 7.2 and 26 °C for comparison of culture medium efficiency. For transfection, the linearized pLEXSY-I-L1 (8–10 μg) was electroporated into 4×10^7 log phase parasites in 2 mm cuvettes at 450 V and 500 μF using Bio-Rad Gene PulserEcell. After two pulses, the recombinant stable transfectants were selected on two different solid media (BHI-agar and M199-agar) containing 50 μg/ml of bleomycin (Jena Bioscience, Germany). For maintaining T7 polymerase and TET repressor genes in the host genome, two antibiotics (LEXSY NTC and LEXSY Hygro) were added in final concentration of 50 μg/ml for each marker. For genomic analysis, transcription and expression assays, the recombinant clones were cultured in BHI and also M199 media containing 50 μg/ml of bleomycin at 26 °C.

2.3. Confirmation of genomic integration

The genomic DNA of recombinant *L.tar*-L1 from 1 ml of culture was prepared by the DNeasy® Blood and Tissue Kit (GF-1). Integration of pLEXSY-I-L1 into the genome was performed by diagnostic PCR using L1 primer pairs (L1F/L1R: below) and also 5′*odc* forward/

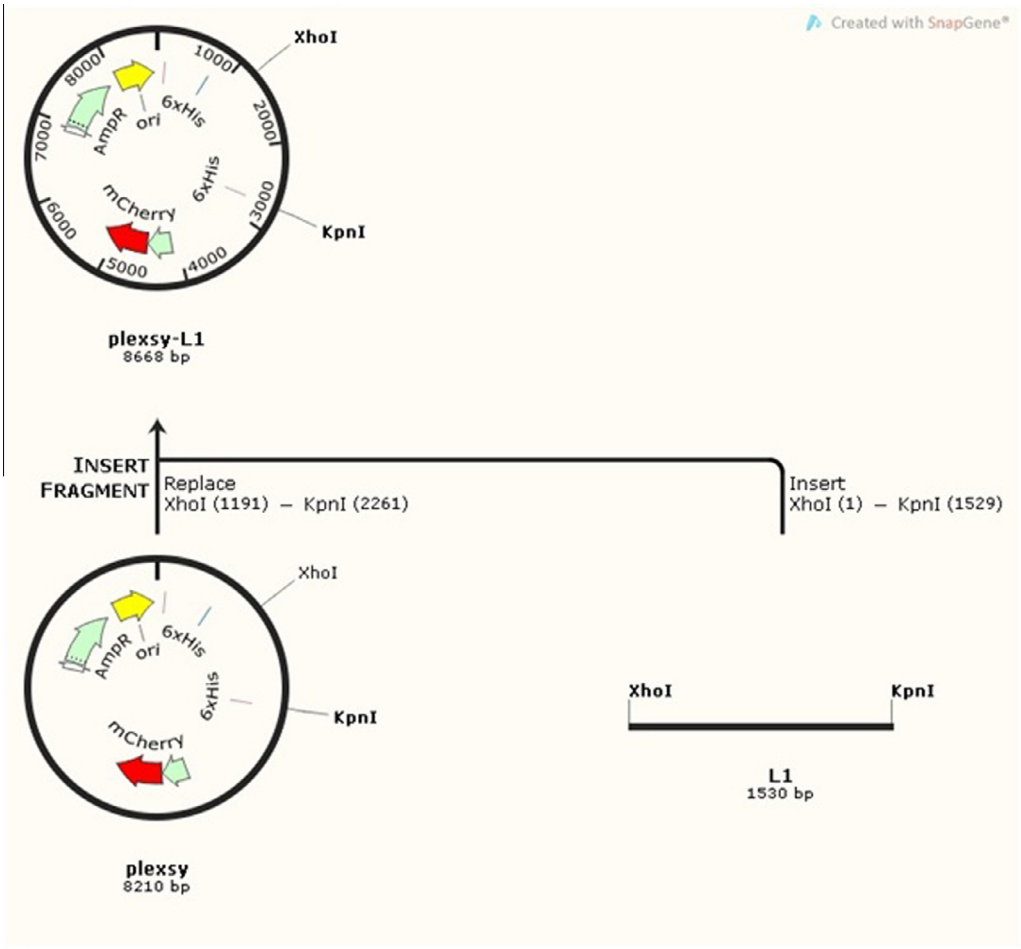

Fig. 1. Schematic model of pLEXSY-I-L1 construct by SnapGene.