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# Enhanced Production of Herpes Simplex Virus 1 Amplicon Vectors by Gene Modification and Optimization of Packaging Cell Growth Medium

Iván Fernández-Frías,[1,2] Sara Pérez-Luz,[1,2] and Javier Díaz-Nido[1,2]

[1]Departamento Biología Molecular and Centro de Biología Molecular "Severo Ochoa" (UAM-CSIC), Universidad Autónoma de Madrid, 28049 Madrid; [2]Instituto Investigación Sanitaria Puerta de Hierro-Majadahonda, Madrid, Spain

**Herpes simplex virus 1 (HSV-1)-derived amplicon vectors are unique in their ability to accommodate large DNA molecules allowing whole genomic loci to be included with all of their regulatory elements. Additional advantages of these amplicons include their minimal toxicity and ability to persist as episomes, with negligible risk of insertional mutagenesis, being particularly well-suited for gene therapy of neurological disorders due to their outstanding ability to deliver genes into neurons and other neural cells. However, extensive gene therapy application has been hindered by difficulties in vector production. This work improved HSV-1 amplicons production by genetic modification of the packaging cell line and optimization of the culture medium. A stably-transfected Vero 2-2 cell line overexpressing the anti-apoptotic Bcl-2 protein was generated, exhibiting an increased resistance to apoptosis, prolonged culture duration, and a significant improvement in viral vector production. Additionally, supplementation of the growth medium with antioxidants, polyamines, amino acids, and reduced glutathione further increased the yield of packaged amplicon vectors. With these modifications, HSV-1 amplicons could be isolated from culture supernatants instead of cell lysates, leading to vector preparations with higher titer and purity and paving the way for generation of stable cell lines that are capable of continuous herpesviral vector production.**

## INTRODUCTION

Many efforts have been made to improve gene therapy of genetic disorders.[1,2] An ideal gene therapy protocol consists of single-administration of the therapeutic gene, which subsequently persists and segregates alongside chromosomes, maintained in episomes to avoid risk of insertional mutagenesis.[3,4] One of the main challenges from the beginning of gene therapy research was targeting genes of interest to specific cells, tissues, or organs, which was resolved using the natural ability of viruses to infect cells.[5,6] Viruses are appropriate tools for gene therapy because of their high efficiency in infecting cells, and different viral vector systems have been developed as vehicles to deliver DNA "in vitro,"[7] including adenovirus, retrovirus (lentivirus), and adeno-associated viruses. Each system has inherent advantages and disadvantages related to their own viral characteristics, but they all share one common inconvenience: the amount of packaged exogenous DNA is limited to the viral genome size. To overcome this hurdle, most constructs used for gene therapy are based on cDNAs controlled by heterologous promoters, though their improper regulation may cause variable expression or even complete silencing,[8] restricting their usefulness.

Alternatively, inclusion of entire genomic loci provides all necessary regulatory elements to ensure controlled, physiological expression of the transgene,[9] already shown for mice transgenesis technology.[10,11] Use of large fragments of genomic DNA permits delivery of intact mammalian genes with all introns, promoters, enhancers, and long-range controlling elements, providing near-endogenous levels of expression[12] and tissue specificity.[13] In order to accommodate such large DNA fragments (up to 150 kb) in a viral vector, viruses with larger genomes must be used, such as herpes simplex virus 1 (HSV-1). Other features that make HSV-1 suitable as a delivery particle are its broad cell tropism and the fact that its genome doesn't integrate into the host cell chromosomes.[14] One strategy for the use of HSV-1 in gene delivery is through amplicon vectors, which are plasmids or bacterial artificial chromosomes (BACs) that contain only two sequences of viral origin: the *oriS* element, which allows replication in packaging cells, and a *pac* sequence for packaging into HSV-1 viral particles.[15–17] Amplicons are packaged into viral particles using a "helper virus-free" packaging system (from transient cotransfection with an oversized helper HSV-1 genome lacking *pac* and ICP27 sequences),[18] which reduces the possibility of cytopathic effects.

The strategies used to make viral-based vectors safer vehicles for gene therapy are directly associated with lower virus production,[19] which complicates translation of this technology into the clinical setting. As interest in the use of viral particles in gene therapy increases, so do efforts to achieve higher titers and purity of the

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**Correspondence:** Sara Pérez-Luz, PhD, Departamento Biología Molecular and Centro de Biología Molecular "Severo Ochoa" (UAM-CSIC), Universidad Autónoma de Madrid, 28049 Madrid.
**E-mail:** spluz@cbm.csic.es

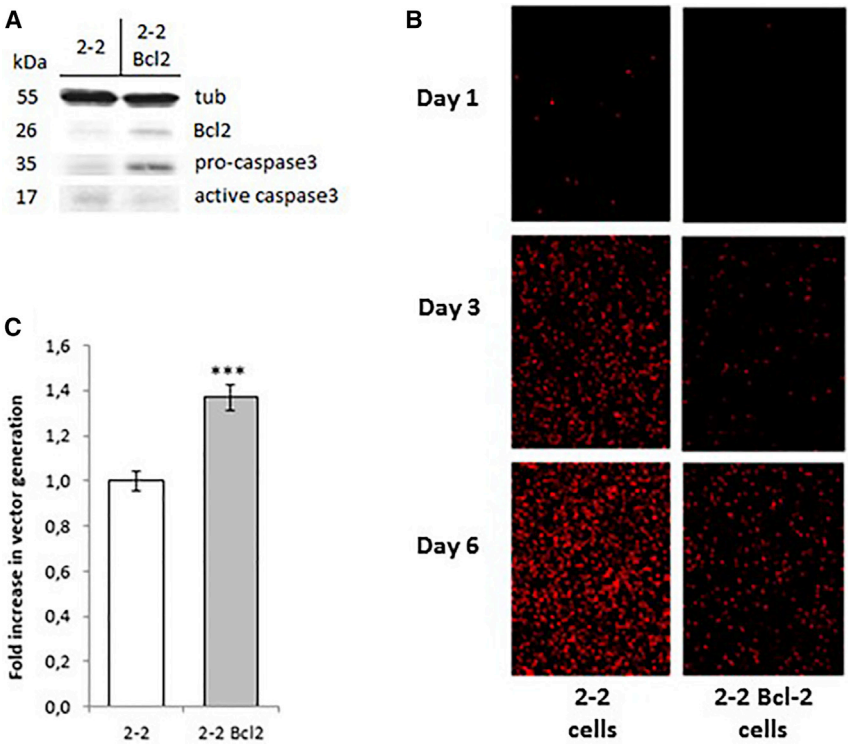


**Figure 1. Characterization of the New Packaging Cell Line, 2-2 Bcl2**

(A) Western blot analysis of cell culture extracts. SDS-PAGE was performed using cell lysates from parental Vero 2-2 (2-2) and novel Vero 2-2 cells constitutively expressing the anti-apoptotic Bcl-2 protein (2-2 Bcl2). Bcl-2, pro-caspase-3, and caspase-3 were detected in western blots by specific antibodies. (B) Cell death during generation of HSV-1 vectors at 1, 3, and 6 days. Scale, 10 μm. (C) Maximum infectious titer of HSV-1-based amplicon vectors in 2-2 Bcl2 cells. After production of amplicon vectors in parental Vero 2-2 (2-2) and novel 2-2 Bcl2 cells, infectious titers (IU/mL) were determined in G 16-9 cells. Vertical axis represents infectious titer relative to that produced in Vero 2-2 cells; values presented are mean ± SEM; n = 3. Student's t test was used to compare amplicon vector generation in both cell lines (***$p < 0.001$).

*in vitro* preparations and in consequence the production of most viral particles (adenovirus, adeno-associated, and lentivirus-derived vectors) has undergone a considerable improvement over time.[20] In the case of HSV-1 amplicon vectors, however, such improvements in their production has been more limited, especially for helper-free herpesviral amplicon vectors bearing large DNA constructs.[21,22]

The higher nutritional demands required of virus-producing cell lines forces myriad physiological changes during viral packaging. Indeed, a recent report identified several metabolic pathways such as polyamine and glutathione biosynthesis as limiting the production of recombinant enveloped viruses.[23] Multiflux analysis has revealed that several metabolic pathways are altered when comparing virus production with normal cell growth, and metabolite supplementation in growth media can result in huge improvements in titer.[24,25]

At the same time, introduction of foreign DNA into packaging cell lines via lipofection induces the apoptotic cascade,[26] leading to increased cell mortality and lower titers of the viral suspension. Apoptosis is a natural cellular process regulated by different factors, including Bcl-2, an anti-apoptotic protein.[27] Overexpression of anti-apoptotic proteins has been used to prolong viability of cell cultures, enhancing industrial production of recombinant proteins.[28] Interestingly, overexpression of Bcl-2 has additional metabolic effects that may be useful to enhance viral production in mammalian cell cultures.[29]

In this study, the efficiency of production of HSV-1 amplicon vectors was enhanced using a novel packaging cell line stably expressing Bcl-2 and optimization of growth medium by adding different nutritional supplements such as antioxidants, polyamines, reduced glutathione, and an amino acid mixture, already shown to increase virus production.[23] Both strategies helped to avoid cytotoxicity and massive cell death during the packaging process, leading to enhanced production of vectors *in vitro*.

## RESULTS

### The Novel Packaging Cell Line 2-2 Bcl2 Shows Apoptosis Resistance and Increased Production of HSV-1 Amplicon Vectors

During the packaging process, massive cell death is triggered by production of viral particles and transfection of exogenous DNA.[26] Generating HSV-1 amplicon vectors at high titers is difficult when transfecting BAC-sized DNA, so it was imperative to optimize protocols. A novel packaging cell line, 2-2 Bcl2 (stably expressing the anti-apoptotic Bcl-2 protein), was generated and found to express higher levels of pro-caspase-3 and lower levels of active caspase-3 (Figure 1A), which would suggest that the new cell line has a resistance to the apoptotic process. Indeed, this was confirmed by propidium iodide assays highlighting lower levels of cell death in 2-2 Bcl2 cells compared to the original Vero 2-2 cells (Figure 1B). A boost in HSV-1 vector production was also observed in 2-2 Bcl2 cells, with a 40% increase ($2.88 \times 10^5$ versus $3.9 \times 10^5$ infective units per milliliter (IU)/mL) in generation of viral particles with respect to the parental cell line (Figure 1C). Thus, the new, genetically modified cell line is a suitably improved platform to generate HSV-1 amplicon vectors.

### Supplementation of Packaging Cell Growth Medium Increases Yield of HSV-1 Amplicon Vectors

In order to offset the increased nutritional demands of viral production, cell growth media was supplemented with amino acids,

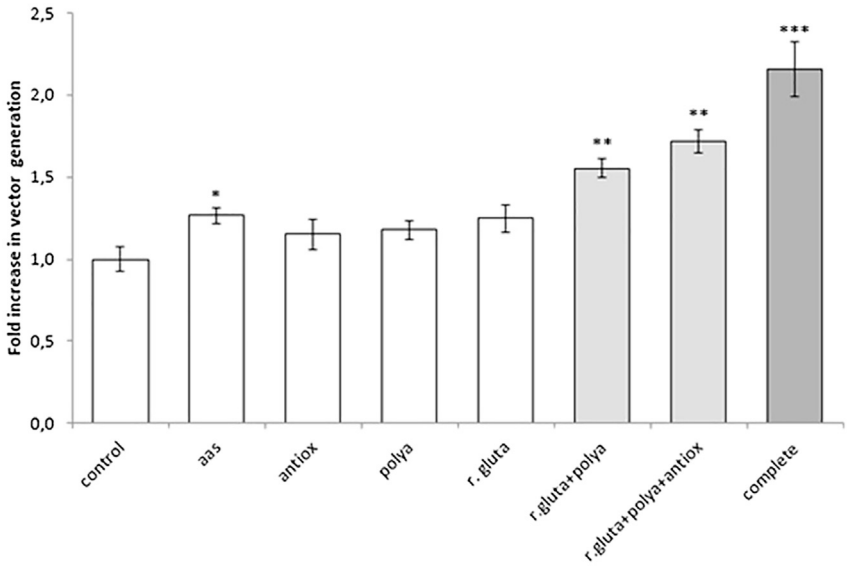


**Figure 2. Optimization of the Packaging Cell Growth Medium**

Quantification of vector production in Vero 2-2 cells expressing Bcl2 grown in standard medium (control) or supplemented alone or in combination with amino acids (aas), antioxidants (antiox), polyamines (polya), and reduced glutathione (r.gluta), or containing all the supplements (complete). Values are presented as mean ± SEM; n = 3. Student's t test was used to compare amplicon vector generation in the different media (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

antioxidants, polyamines, and reduced glutathione, targeting those pathways more affected during viral vector generation.[23] The supplements were added to the media alone or in combination after transfection of exogenous DNA for packaging, and supernatants were collected after 3 days. Separately, each showed a tendency to increase the efficiency of viral vector production, although only amino acid supplementation gave a statistically significant result (Figure 2). Addition of polyamines or antioxidants yielded a 1.2-fold improvement in viral titer, whereas reduced glutathione had a 1.3-fold increase. When multiple supplements were combined, there was a 1.8-fold increase, and use of all four supplements resulted in 2.3-fold enhancement ($8.06 \times 10^4$ versus $1.97 \times 10^5$ IU/mL) with respect to the non-supplemented medium (Figure 2).

Current packaging protocols are limited to 3 days[18] after which yield drops dramatically. Once the optimal medium was established, the maximum duration of packaging was determined in the 2-2 Bcl2 cells. As decreases in viral yield may be due to inactivation of particles by accumulation of cellular waste, culture media was replaced every 48 h, and amplicon vectors were harvested from the supernatant. Neither the new cell line nor the enriched growth medium was successful in lengthening the period of virus production, probably suggesting that low yield of virus production after 3 days is due to the transient transfection (data not shown).

### 2-2 Bcl2 Cells Enhance Yield of Large Plasmid Packaging

All previous packaging experiments to check the efficiency of the new Bcl-2-expressing cell line and medium supplementation were carried out with the 8 kb plasmid pHSV-lac. However, as one of the most interesting features of HSV-1 vectors is their large packaging capacity, able to harbor exogenous DNA up to 150 kb, the yield of packaging a 140 kb BAC containing a whole genomic locus was tested using the updated protocols. Use of the optimized media in parental 2-2 Vero cells resulted in a 3-fold increase in packaging process, whereas the new 2-2 Bcl2 cells cultured in standard and optimized growth medium had a 4-fold ($7.83 \times 10^3$ versus $3.03 \times 10^4$ IU/mL) and 4.7-fold increase ($7.83 \times 10^3$ versus $3.72 \times 10^4$ IU/mL) in virus production, respectively (Figure 3). Curiously, the effect of medium supplementation was greater in parental Vero 2-2 cells than the 2-2 Bcl2 cells, perhaps due to metabolic changes triggered by Bcl-2 overexpression.

## DISCUSSION

Gene therapy research has made remarkable strides over the last 30 years, with some success treating cancer, metabolic disorders, and several neurological diseases.[30,31] A number of new vector systems have been developed with broad potential; however, commercialization of gene therapy is in its infancy, and different issues must be resolved before it joins standard medical practice.[1] Viral vectors are the most suitable for use in gene therapy, proven by the fact that nearly two-thirds of gene therapy clinical trials are being performed with them (http://www.abedia.com/wiley). Retrovirus and lentivirus-based approaches are by far the most popular, as these vectors integrate into the host genome and provide lifelong expression of the therapeutic gene. Yet, this can become disadvantageous due to associated insertional mutagenesis events.[32] The main way to avoid this problem is the use of a non-integrating virus, such as HSV-1, which also has the advantage of a large storage capacity. The ability to package complete genomic loci with all regulatory sequences[13] or to produce different gene products[9] in general allows a more physiological expression of the therapeutic gene. Herpesviral vectors are therefore considered to have great potential for gene therapy and other applications,[33] but major hurdles remain for optimization of production.

Working with HSV-1-based vectors is quite cumbersome, as they are mainly generated using BACs containing genomic loci to benefit from their "storage" capacity. During the packaging process, the therapeutic gene-containing BACs, as well as a BAC containing the viral genes needed to form viral particles, are lipofected into a special cell line. As transfection of BACs is not as efficient as for small plasmids, and because the process triggers apoptosis, production yields low viral titers. In order for HSV-1 vectors to become true alternatives for therapeutic use, it is necessary to achieve higher titers and purity.

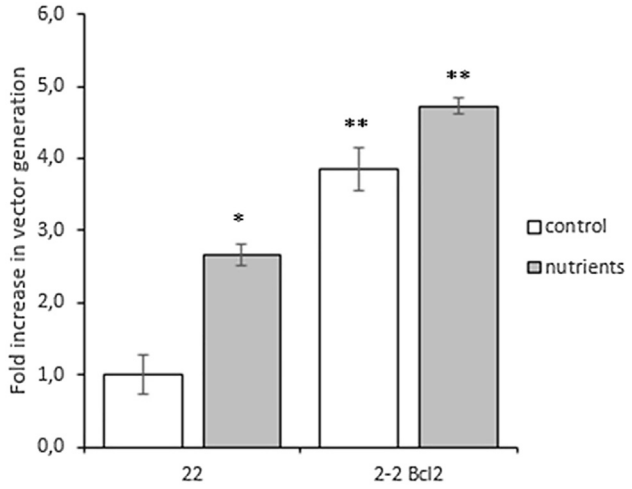


**Figure 3. Comparison of Large-Plasmid Packaging Efficiency in Cell Lines, with and without Optimized Cell Culture Media**

Packaging of a 140 kb BAC (pEHHG-FXN) was compared in parental Vero 2-2 (2-2) and novel 2-2 Bcl2 cells grown in standard (control) or supplemented growth media (nutrients). Vertical axis represents vector production relative to that of control cells grown in standard, unsupplemented growth media; values are means ± SEM; n = 4. Student's t test was used to compare amplicon vector generation (**$p < 0.01$; *$p < 0.05$).

In this work, viral particles were obtained without breaking cells in order to prevent generation of cellular debris and to get higher purity preparations, which will be essential for future clinical trials. However, as viral particles are mainly concentrated within cells, it is necessary to seek solutions to increase the titer in the supernatant. Bearing in mind that different factors play a role in cellular death, various approaches were used to address the problem. First, programmed cell death was reduced in a new packaging cell line expressing anti-apoptotic Bcl-2 protein, with a clear effect of increasing the production of HSV-1 vectors. A previous study reported that overexpression of Bcl-2 in HEK293 cells resulted in improved cell resistance to apoptosis and prolonged culture duration but reduced production of adenoviruses.[34] The different outcome observed in this study may be due to the different virus assembly machinery of adenovirus and herpesvirus or the distinct cell lines tested.

Unfortunately, viral production dropped dramatically after 3 days of culture in Bcl-2 overexpressing cells, probably due to the transient nature of transfection. The fact that Bcl-2 expression protects packaging cells from apoptosis may be used to attempt stable transfection of with the packaging construct, since it is known that packaging BACs is cytotoxic by itself.

Animal cell culture is a powerful tool for production of biologically important molecules such as monoclonal antibodies, vaccines, and recombinant proteins, though protocols must be optimized to maximize production. Metabolic studies have indeed concluded that addition of different nutrients to the culture medium is a rapid and effective way to improve these products.[25,35,36] Specific nutritional requirements vary by cell line, but supplementation with amino acids, polyamines, reduced glutathione, and antioxidants has been shown to be effective regardless of the cell type or production process. Several studies have shown how viral production imposes stress on packaging cells, and expression profile studies have demonstrated a higher demand on diverse pathways of cellular metabolism in packaging cells with respect to their parental cell lines.[23] Taking this into consideration, the growth medium of the packaging cells was supplemented with different nutrients that may be limiting during viral production. In our experimental model, addition of amino acids alone produced a significant increase in viral production, possibly indicating that this pathway is more affected during viral packaging. However, as each of the supplements produced a visible increase in virus production, the results clearly suggest scarcity of all of them, and indeed the effect was strongest when all the supplements were included together in the packaging medium.

In conclusion, our study improved HSV-1 virus production by genetic modification of the packaging cell line and supplementation of the culture medium. These changes allow HSV-1 amplicons to be isolated from culture supernatants instead of cell lysates leading to purer viral preparations, a good starting point for production of therapeutic herpesviral products with higher titers. With additional studies to improve the packaging process, the use of herpesviral vectors in gene therapy may become feasible for the treatment of human diseases in the near future.

## MATERIALS AND METHODS

### Cell Culture

Parental Vero 2-2 cells and the novel Vero 2-2 cells stably expressing Bcl2 (2-2 Bcl2) were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, Madrid, Spain), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 37°C in 5% $CO_2$.

The G16-9 cell line is derived from the human Gli-36 glioma cell line and expresses the HSV-1 VP16 protein to enhance activity of the pIE4/5-LacZ promoter cassette. G16-9 cells were cultured in DMEM supplemented with 10% FBS, hygromycin-B (200 μg/mL), 100 U/mL penicillin, and 100 μg/mL streptomycin.

For generation of the 2-2 Bcl2 cell line, Vero 2-2 cells were transfected using Lipofectamine and PLUS reagent (Thermo Fisher Scientific, Waltham, MA, USA) with a plasmid containing the gene, a kind gift from Belén Sanz-Castillo, and cells incorporating the plasmid were selected in medium supplemented with puromicin (1 μg/mL).

Specific supplementation of growth medium was as described previously.[23] Briefly, DMEM with 10% FBS and 2 mM L-glutamine was supplemented with 1X antioxidants (Antioxidant Supplement 1000X, #A1345), 1X polyamines (Polyamine Supplement 1000X, #P8483), 2 mM reduced glutathione (L-glutathione reduced, #G6013), all from Merck Chemicals & Life Science S.A., Madrid,

Spain; and an amino acid mixture prepared in the laboratory as follows: serine (400 mM), asparagine (400 mM), arginine (100 mM), methionine (100 mM), phenylalanine (100 mM), leucine (300 mM), valine (300 mM), and isoleucine (300 mM).

### Production and Titering of HSV-1 Amplicon Vectors

HSV-1 amplicon vectors were packaged using a helper virus-free packaging system as described previously.[18] Briefly, $3 \times 10^6$ of 2-2 and 2-2 Bcl2 cells were cotransfected in a 100 mm tissue culture plate with 5.4 μg of amplicon DNA, 6 μg of fHSVΔpacΔ27 0+, and 0.6 μg of ICP27 using Lipofectamine and PLUS reagent (Thermo Fisher Scientific). Plasmids pHSV-LacZ (8 kb) and pEHHG-FXN BAC (140 kb)[37] were used, each containing the elements required to be packaged into herpesviral particles. After 72 h, HSV-1 amplicons were concentrated by centrifuging viral preparations through a 25% sucrose cushion at 100,000 × *g* for 4 h at 4°C, and the pellet was re-suspended in Hank's balanced salt solution (HBSS) (Life Technologies). Infectious titers (IU/mL) were determined on confluent G16-9 cell monolayers by X-gal staining for pHSV-LacZ or GFP expression using a Axioskop2 fluorescence microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) for pEHHG-FXN BAC.

### Western Blot Analysis

Protein production was analyzed in both cell lines, Vero 2-2 and the new generated Vero 2-2 Bcl2. For protein extracts, cells were washed once with PBS, placed on ice, and then homogenized in a buffer containing 20 nM HEPES, pH 7.4, 100 mM NaCl, 100 mM sodium fluoride, 1% Triton X-100, 1 mM sodium orthovanadate, 5 mM EDTA, and the complete protease inhibitor cocktail (Roche, Barcelona, Spain). After determination of the protein concentration using the Bradford assay (Bio-Rad, Hercules, CA, USA), samples with equal amounts of protein (10–15 μg) were combined with electrophoresis buffer containing sodium dodecyl sulfate (SDS), boiled for 5 min, and separated by electrophoresis in 10% and 15% acrylamide gels in the presence of SDS. Proteins were transferred to nitrocellulose membranes following standard procedures and blocked with 2% FBS and 0.1% Tween-20 in PBS. Membranes were incubated overnight at 4°C with primary antibodies diluted in blocking solution, then washed three times in PBS with 0.1% Tween-20, and finally incubated with the corresponding secondary antibody for 2 h at room temperature. After three further washes in PBS, immunofluorescent proteins were visualized using the Odyssey System (LI-COR, Lincoln, NE, USA). The monoclonal antiserum directed against Bcl-2 (1:1,000) was from Santa Cruz Biotechnology (Dallas, TX, USA). The monoclonal antibody specific for β-tubulin (1:5,000) was from Sigma, and the antibody against caspase-3 (1:1,000) was from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies (1:5,000) used for Odyssey were from LI-COR (Lincoln, NE, USA).

### Cell Viability

Cell viability was assessed by calcein-propidium iodide uptake.[38] Calcein/acetoxymethyl ester is taken up and cleaved by esterases present in living cells, yielding yellowish-green fluorescence. In contrast, propidium iodide is taken up only by dead cells, which then exhibit orange-red fluorescence. Briefly, cells were incubated at 37°C for 30 min with 8 μM propidium iodide (Merck) and 1 μM calcein/acetoxymethyl ester (Molecular Probes, Eugene, OR, USA). The cells were stained at 1, 3, and 6 days, and visualized by a Axioskop2 fluorescence microscope (Carl Zeiss Microscopy).

## AUTHOR CONTRIBUTIONS

I.F.-F. performed the experiments. J.D.-N. conceived and designed the study. I.F.-F., S.P.-L., and J.D.-N. contributed to the interpretation of results. I.F.-F. and S.P.-L. wrote the first version of the paper. All the authors read and approved the final version of the manuscript.

## CONFLICTS OF INTEREST

The authors declare no competing interests.

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