Appendix-Construction of G47Δ-mAngio and G47Δ-empty

We have engineered the G47Δ-mAngio virus and a matching G47Δ-empty virus using the flip-flop method previously described.[1]

The cDNA coding for the mouse angiostatin under the control of the IL-2 secretory sequence was purchased from Invivogen (pbla-mangio, San Diego, CA) and tagged with a hemagglutinin (HA) epitope through primer-extension PCR with the following primers: Forward:ccgcggccgc**CTAAGCGTAATCTGGAACATCGTATGGGTA**actccctcctgtctctgagca Reverse:ggatatcttatcatgtcgagctagctaactcc. The primer-extension PCR reaction was also used to engineer the BamH1 and Stu1 restriction sites into the resulting chimeric gene so that it could be ligated into the pVec92 vector (Figure S1 upper row).

The resulting chimeric IL2-mAngio-HA gene was ligated into the pVec92 shuttle vector [2] using the BamHI and StuI sites engineered into the PCR product and found in the pVec92 (Figure S1 bottom row). Digestion of pVec92-mAngioHA clones with NheI/ScaI confirmed the insertion of the mAngioHA sequence (expected fragment ~1603bp) in 6 of 10 clones tested (clones # 1, 2, 3, 5, 7, 9- Figure S2).

Expression of the chimeric mouse angiostatin transgene tagged with HA was analyzed by standard Western blot using the HA-epitope as antigen, a rat monoclonal anti-HA (11867423001, Roche, Basel, CH) primary antibody, and a horseradish peroxidase-conjugated (HRP) anti-rat IgG (ECL NA935V, Amersham Biosciences Ltd, Little Chalfont, Buckinghamshire, UK) secondary antibody. Vero cells were transfected with 100 ng of the pVEC92-mAngioHA clones selected above using the lipofectamine^{TM} 2000 agent (11668-019 Invitrogen), which yield to about 30% of cells transfected. 24 After 24 hours supernatants and cells were collected separately to distinguish the intracellular mAngioHA protein from the secreted one. The intracellular mAngioHA was released by lysing the cells in RIPA buffer (R0278, Sigma, St. Louis, MO) through three freeze-thaw-sonication cycles, whereas the supernatants were analyzed without modification. Protein concentration in each sample was measured

with the BCA kit (23227 Pierce, Rockford, IL) and the samples were then run on a 12% SDS polyacrilamid gel for Western blot analysis (Figure S3). We have confirmed expression of the chimeric mouse angiostatin protein in 5 of these clones (clones # 2, 3, 5, 7, 9), but the protein in the supernatants was below the detectable levels, probably because these supernatants were not concentrated and the protein was not immuno-precipitated before loading. Because pVec92-mAngioHA clone # 3 presented also correct sequencing of the transgene (data not shown) it was selected for the next engineering steps.

The chimeric angiostatin gene was then transferred to the G47Δ-BAC vector through the CRE recombination system and the resulting DNA was electroporated in the Escherchia *coli* DH10B strain (Figure S4). The bacterial colonies carrying the G47Δ-BAC-mAngio vector were selected by growing them on LB plates containing chloramphenicol (Cm) and kanamycin (Kan). Further analysis for correct insertion of the transgene was done by digesting the G47Δ-BAC-mAngio vector with restriction endonucleases (10 clones were tested and 5 presented correct restriction pattern- Figure S5a). The correct sequence of the transgene was verified in clones 3, 6 and 9, which were thus selected for the final step of virus engineering. G47Δ-BAC-mAngio exceeded the limiting size for efficient HSV packaging. To remove the excessive sequences G47Δ-BAC-mAngio was co-transfected with the FLPe expression plasmid into VERO cells (Figure S4). FLPe-mediated recombination excised the sequences between the FRT sites (BAC, antibiotic resistance and stuffer), thus allowing packaging of the G47Δ-mAngio viruses. The viruses obtained through this method express the bacterial β-galactosidase (β-gal) and not GFP (bottom left). Such virus can replicate in VERO cells forming a GFP^{-}/$b\text{-}gal^{+}$ plaque that can be easily selected (bottom right). The matching control virus G47Δ-empty was constructed using the same procedure in absence of the transgene.

We have selected and amplified viral plaques # 3.4, 6.2, and 9.2, and analyzed their capacity to express and secrete the chimeric angiostatin protein (Figure S5b). VERO cells were infected with G47Δ-

mAngio (clones 3.4, 6.2 and 9.2) or G47Δ-empty at 1 multiplicity of infection (MOI) for 12 hours. The supernatants were then collected and the secreted mAngioHA was precipitated using the anti-HA immunoprecipitation kit (IP0010-IKT Sigma, St. Louis, MO) and run on a 12% polyacrilammid gel for standard Western blot analysis using the same antibodies described above. Supernatants from all three clones presented the chimeric viral protein IL2-mAngio-HA

We have then compared the replication efficiency of G47Δ-mAngio clones 3.4, 6.2 and 9.2 and G47Δ-empty in vitro through a single time-point burst assay. Vero and U87 cells were infected with either G47Δ-mAngio or G47Δ-empty at an MOI of 0.1 for 1 hour at 37^{o}C with DMEM medium supplemented with 10% FCS. The infection medium was then removed and cells were grown in fresh 10% FCS-DMEM medium. The infection medium with the input virus was stored at -80^{o}C while cells infected with virus and their supernatants were harvested at 24 (VERO) or 48 (U87) hours after virus infection. Each time point was tested in triplicates. The virus was then released from the cells through 3 cycles of freeze-thaw-sonication and titered on VERO cells. The burst of each virus was determined by the ratio of viral plaque forming units (pfu) obtained from each cell line versus the pfu of the input virus. Figure S6a) shows that G47Δ-mAngio clone 3.4 presented a replication capacity (burst) similar to the control virus over 24 hours (in VERO cells) or 48 hours (in U87 cells). This virus was selected for the subsequent experiments and be referred to as G47Δ-mAngio.

We verified whether the mouse angiostatin expressed by G47Δ-mAngio had anti-angiogenic properties in vitro through an endothelial tube formation assay. Supernatants from U87 cells infected with G47Δ-mAngio, G47Δ-empty (MOI=1) or equal volume of PBS for 12 hours were collected and tested for their capacity to inhibit the formation of tubular structures from endothelial cells. The presence of replicating viral particles in the supernatants was prevented by replacing the infection medium with 2%-FCS DMEM medium containing 1% of HSV-neutralizing IgG one hour after the infecion. 7-10×10^{4} endothelial cells (mouse MS1 or human HUVECs) were plated on 24 wells plates

over a matrigel coat (356237, BD Biosciences, Bedford MA) and treated with the supernatants described above. The number of tubes was determined by counting the number of tube junctions in 5 different microscope fields for each well. Each supernatant was tested in triplicates. Figure S6b and S6c demonstrate that supernatants collected form G47Δ-mAngio-infected cells strongly inhibited the formation of endothelial tubes.

References

1. Kuroda, T, Martuza, RL, Todo, T, and Rabkin, SD (2006). Flip-Flop HSV-BAC: bacterial artificial chromosome based system for rapid generation of recombinant herpes simplex virus vectors using two independent site-specific recombinases. *BMC Biotechnol* **6**: 40.
2. Fukuhara, H, Ino, Y, Kuroda, T, Martuza, RL, and Todo, T (2005). Triple gene-deleted oncolytic herpes simplex virus vector double-armed with interleukin 18 and soluble B7-1 constructed by bacterial artificial chromosome-mediated system. *Cancer Res* **65**: 10663-10668.

Figure Legends

Figure S1. Representation of the chimeric IL2-mAngio-HA gene transfer into pvec92 vector.

The upper row shows a representation of the chimeric IL2-mAngio-HA gene; the bottom row shows the p92 vector before (left) and after (right) insertion of the transgene. IL2-mAngio-HA was inserted into the BamH1-Stu1 sites of the vector and has generated a new restriction site into the vector (Sca1) that was used to check correct insertion of the transgene.

Figure S2. Endonuclease digestion analysis of pVec92-mAngioHA clones.

10 pVec92-mAngioHA clones were digested with NheI/ScaI to confirm correct mAngioHA insertion after ligation. The size of the expected fragment following endonuclease digestion is ~1603bp and was found in clones # 1, 2, 3, 5, 7, 9. Correct transgene insertion was confirmed in Clones 1 and 3 by sequence analysis.

Figure S3. Western blot analysis for expression of mAngioHA.

The supernatants (SN) and cells (C) for non-transfected VERO cells (VERO alone) and for each transfected clones (Clone 1-9) were run on an SDS polyacrilamid gel together with a protein size marker (M). pVec92-mAngioHA clones 2-9. present the mAngiHA protein.

Figure S4. Reppresentation of the G47Δ-mAngio construction procedure.

mAngioHA gene transfer from the shuttle vector pVec92 to the G47Δ-BAC through the cre-recombinase system (upper row) results in BAC clones (center) presenting a kanamicine (KAN) resistance that are easily selected. The sequence located between the FRT recombination sites of the G47Δ-Bac-mAngio were excised through the FLPe recombinase system during co-transfection of the G47Δ-BAC-mAngio clones with the FLPe expression vector in VERO cells. This generates G47Δ-mAngio viruses that do not express GFP (bottom left) and replicate in VERO cells forming a non-GFP plaque (bottom right).

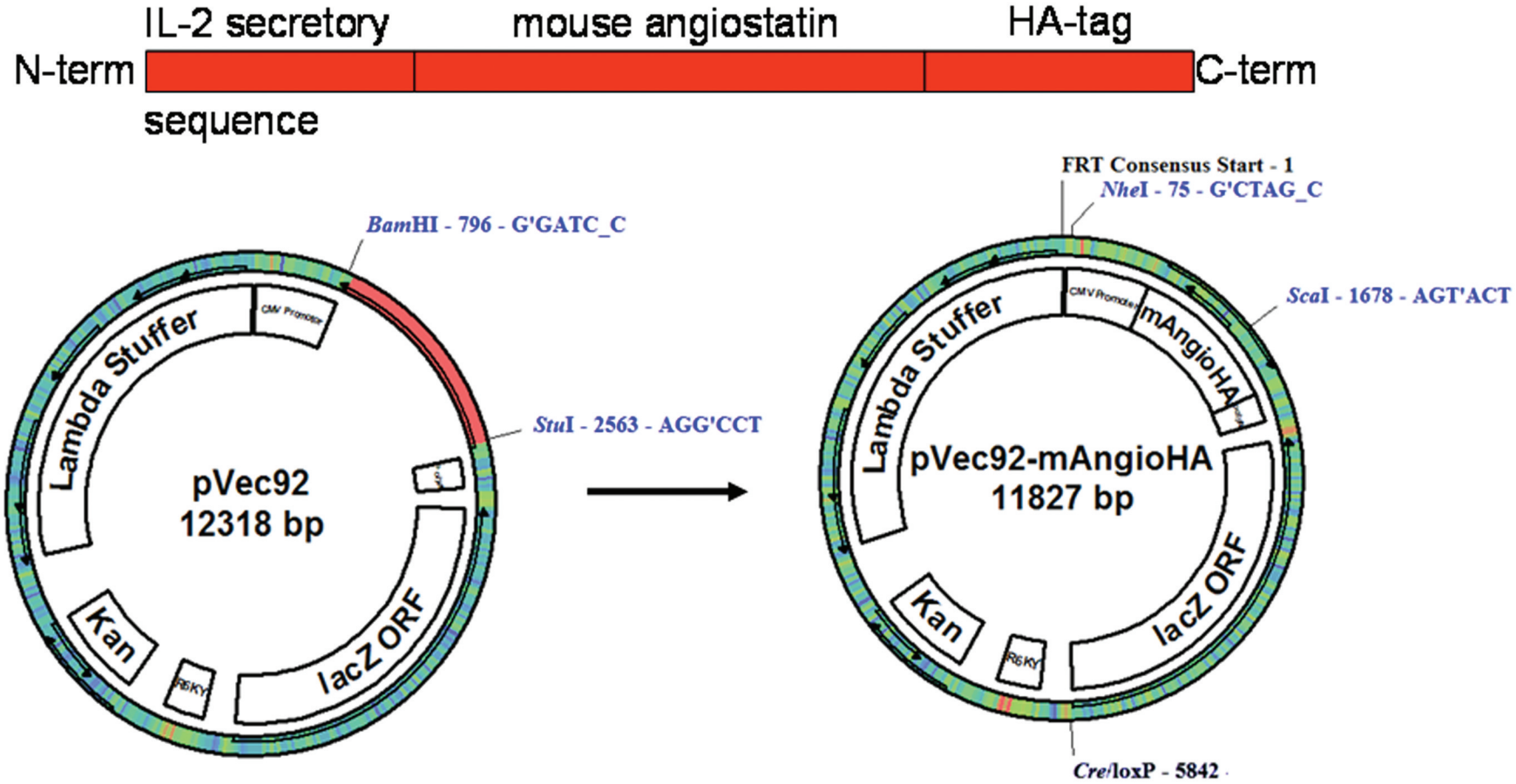
Figure S5. Endonuclease digestion analysis of G47Δ-BAC-mAngio clones and Western blot analysis for expression of viral mAngioHA protein.

a) The arrows show the appearance of new bands after digestion with BAMH1 in the clones that have inserted correctly the mAngioHA transgene (clones # 3, 4, 5, 6, 9) when compared to the parental G47Δ-BAC vector. L=DNA ladder. **b)** Suprnatants (S.N.) collected from Vero cells infected with G47Δ-mAngio clones 3.4, 6.2, 9.2, or with the G47Δ-empty control virus (Empty) were analyzed for presence of the chimeric mAngioHA product (~60kD). For positive control we used lysates of VERO cells transfected with the pVEC92-mAngioHA shuttle vector, whereas blank corresponds to immunoprecipitation performed with empty cell-culture medium. All the G47Δ-mAngio clones show high expression and secretion of the mouse angiostatin. The empty virus and blank lanes present a faint band corresponding to background carry-over of the anti-HA antibody during the immunoprecipitation.

Figure S6. Single virus burst assay and endothelial cells tube formation assay.

a) Comparison of the burst in VERO and U87 cells of G47Δ-mAngio clone 3.4 (mAngio) and G47Δ-empty (empty). **b)** Representation of the tubular structures formed by HUVECs when grown on a matrigel substrate with supernatants collected from U87 cells infected with PBS, G47Δ-empty (empty) or G47Δ-mAngio (mAngio). **c)** The graph shows the number of tubes counted when MS1 and HUVECs were grown in presence of supernatants collected from U87 cells infected with PBS, G47Δ-empty (empty), or G47Δ-mAngio (mAngio). The bars indicate the average number of tubes counted from three independent wells +/- standard deviation. Treatment of endothelial cells with supernatants derived from G47Δ-mAngio-infected VERO cells significantly decreased the number of tubes compared to treatment with supernatants form G47Δ-empty infected cells (*p*-values on top of bars).

Figure S1


IL-2 secretory sequence
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HA-tag
N-term
C-term
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StuI - 2563 - AGG'CCT
Lambda Stuffer
CMV Promoter
pVec92
12318 bp
Kan
R6KY
lacZ ORF
FRT Consensus Start - 1
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ScaI - 1678 - AGT'ACT
mAngioHA
pVec92-mAngioHA
11827 bp
Cre/loxP - 5842

Figure S2

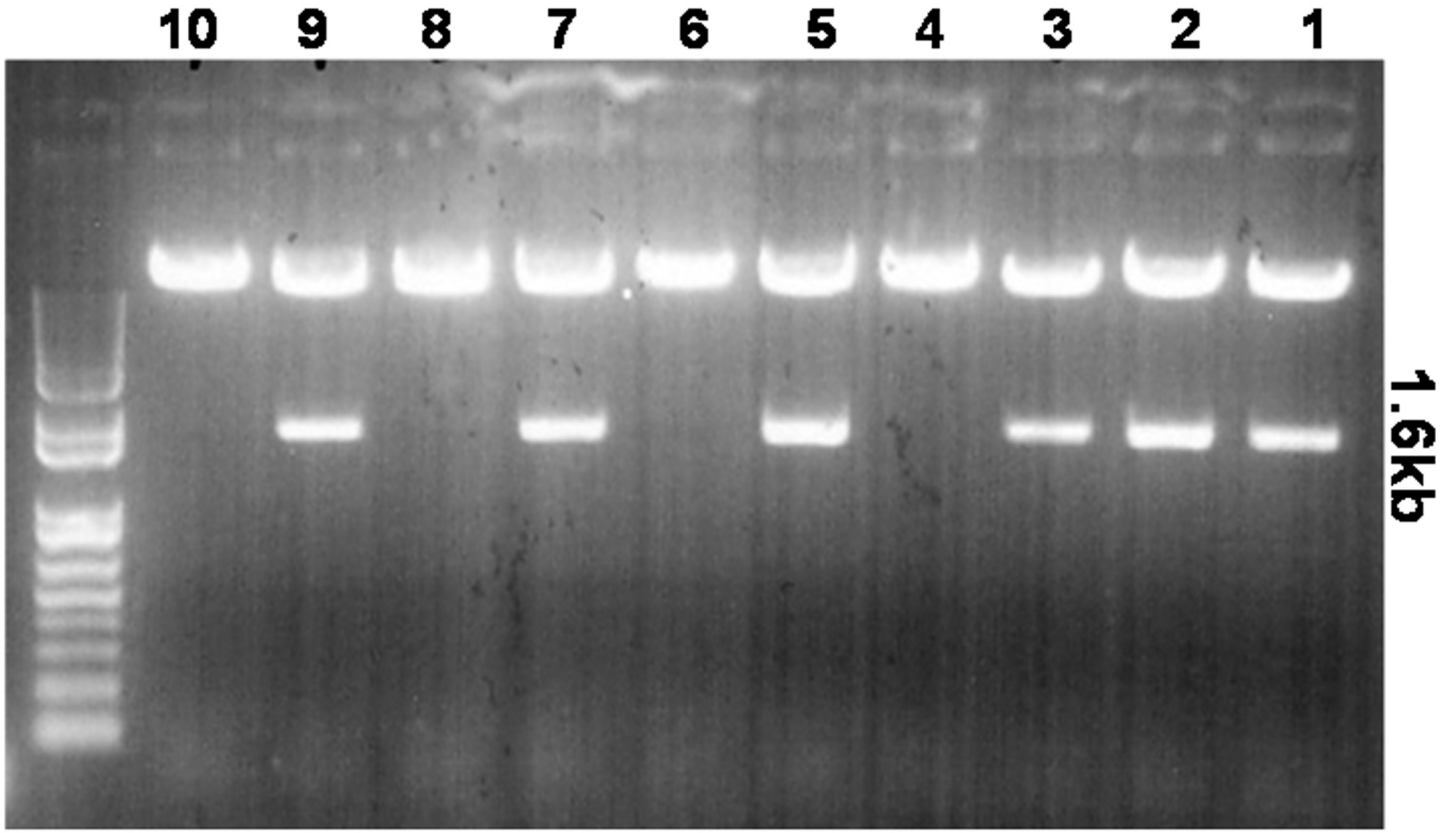


Figure S3

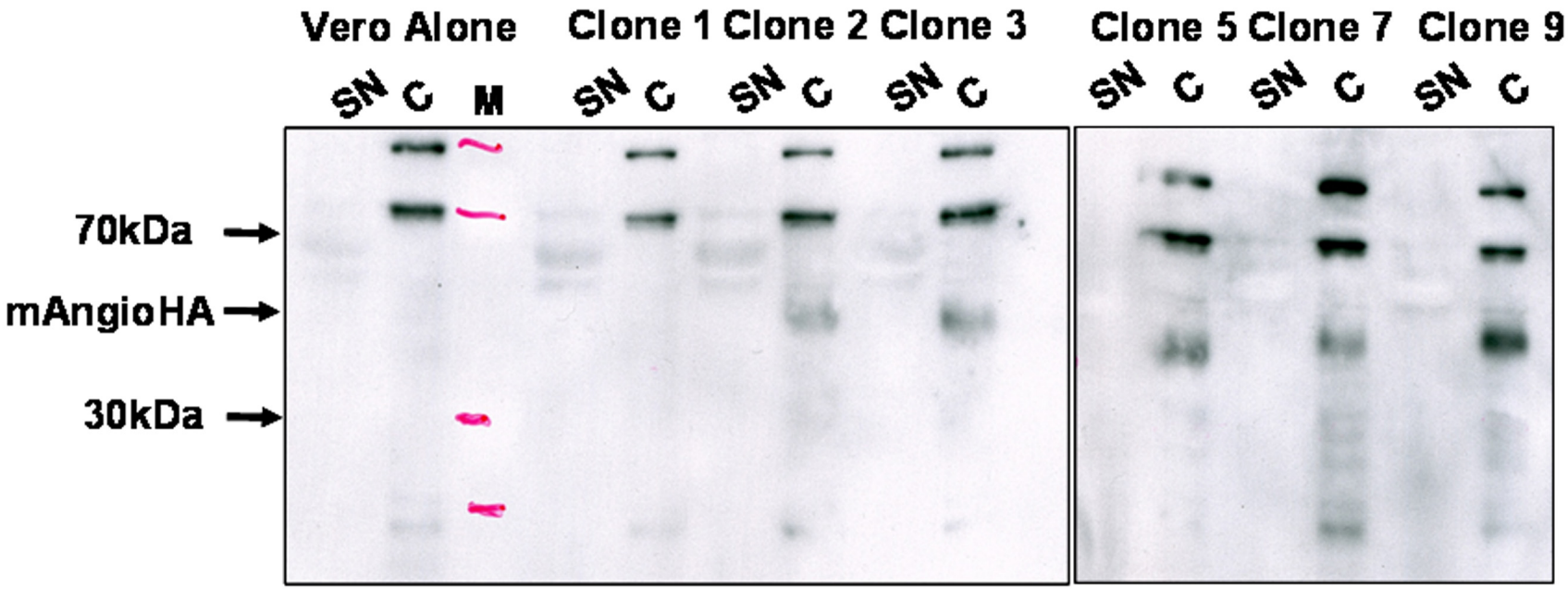
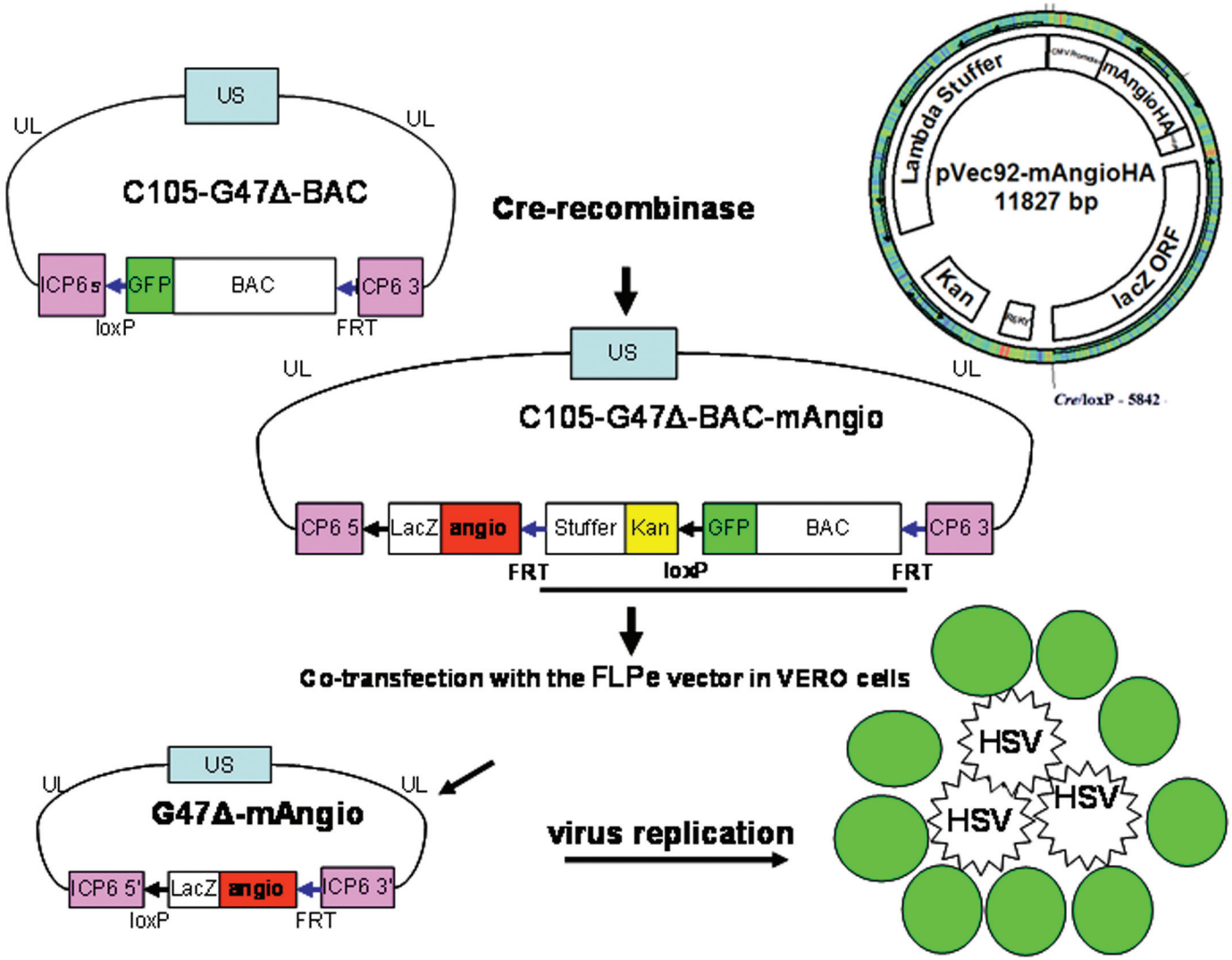

Vero Alone
Clone 1
Clone 2
Clone 3
Clone 5
Clone 7
Clone 9
SN
C
M
70kDa
mAngioHA
30kDa

Figure S4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UL
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GFP
BAC
CP6 3
loxP
FRT
Cre-recombinase
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11827 bp
Lambda Stuffer
mAngioHA
Kan
lacZ ORF
CreloxP - 5842
US
UL
UL
C105-G47Δ-BAC-mAngio
CP6 5
LacZ
angio
Stuffer
Kan
GFP
BAC
CP6 3
FRT
loxP
FRT
Co-transfection with the FLPe vector in VERO cells
US
UL
UL
G47Δ-mAngio
ICP6 5'
LacZ
angio
ICP6 3'
loxP
FRT
virus replication
HSV
HSV
HSV

Figure S5

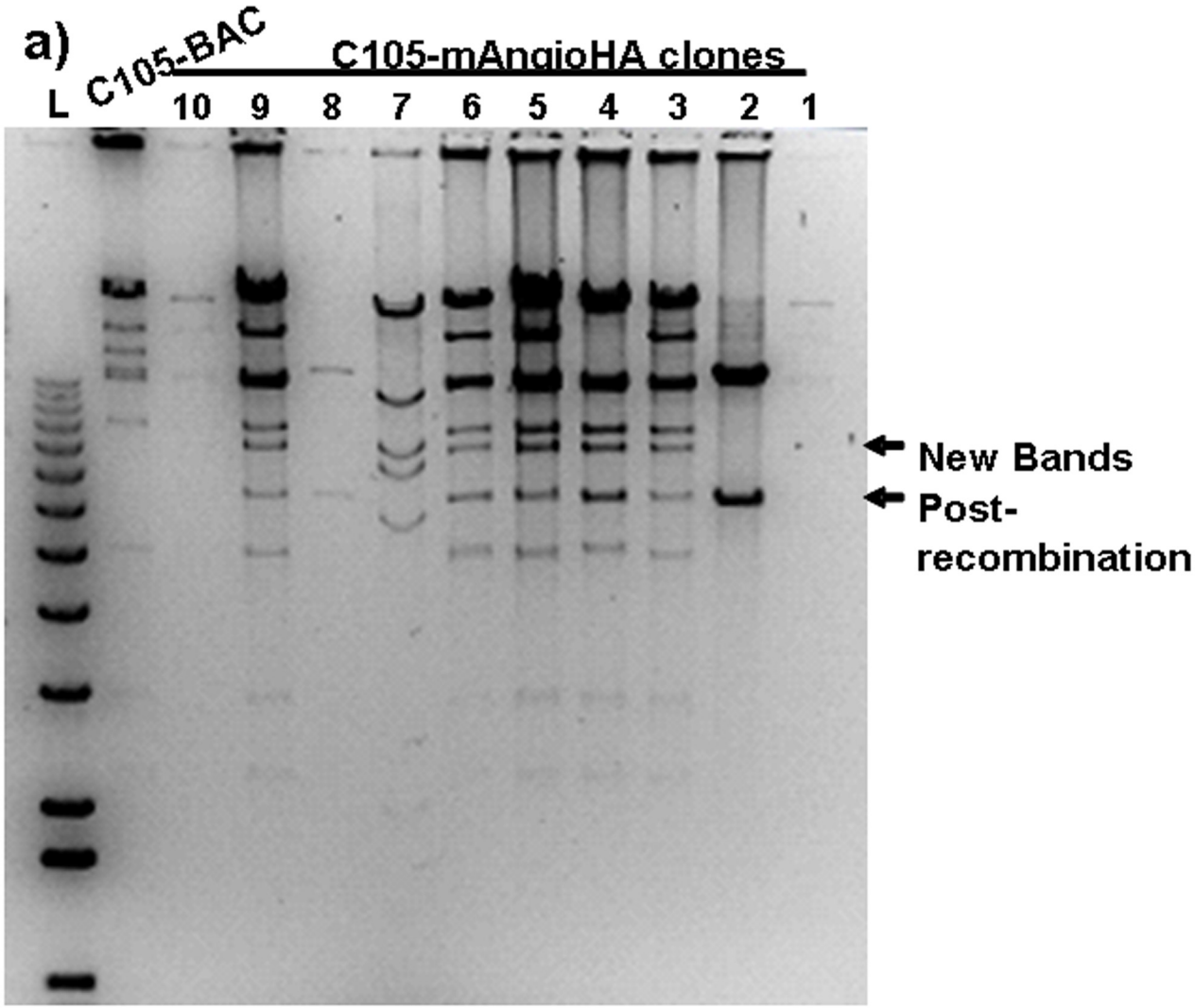

a)
C105-BAC
C105-mAngioHA clones
L
10
9
8
7
6
5
4
3
2
1
New Bands
Post-recombination

Figure S5

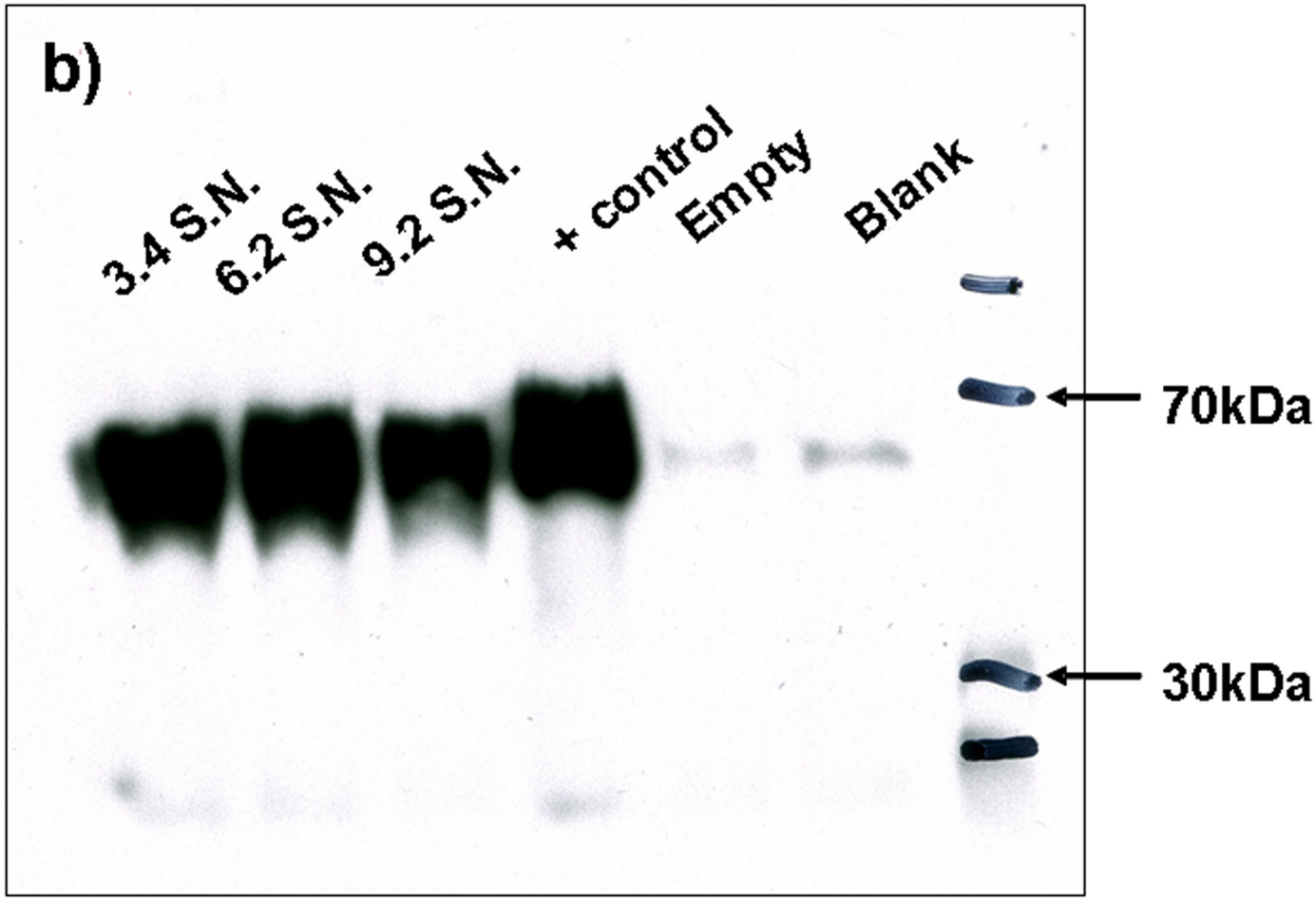
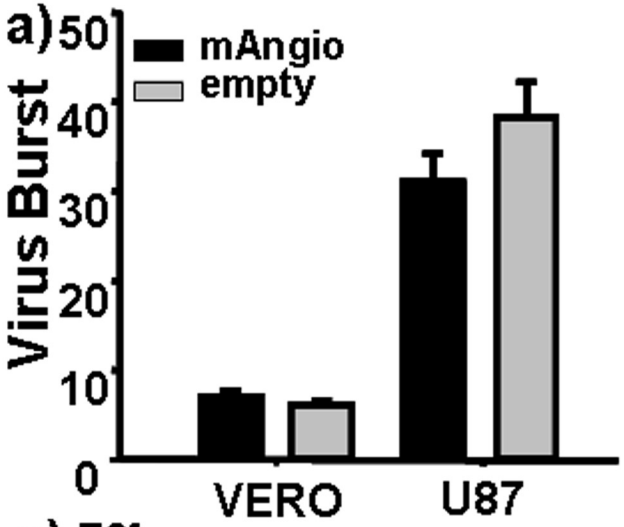
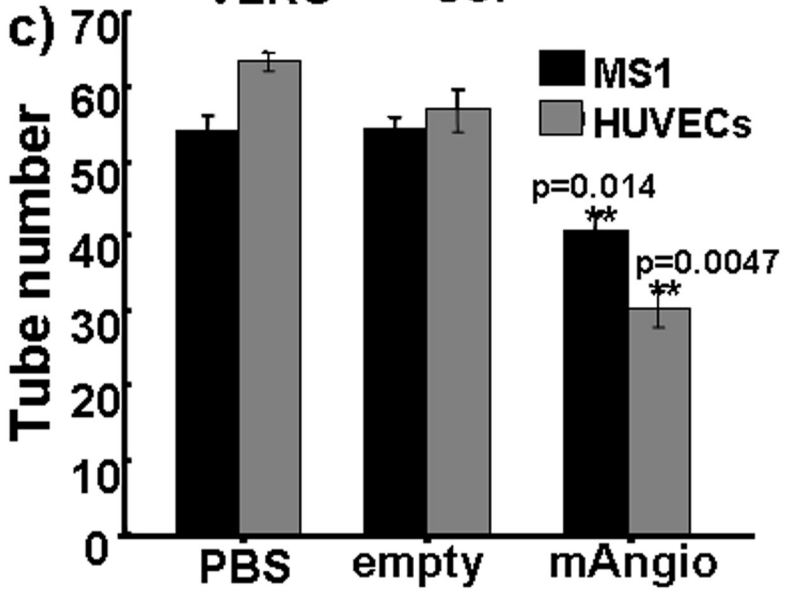
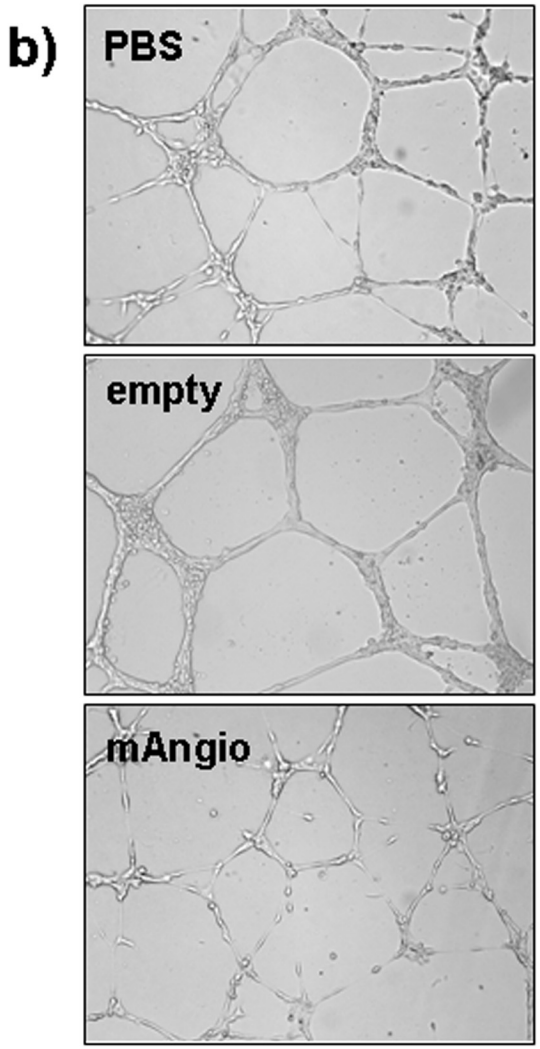


Figure S6


a)
Virus Burst
50
40
30
20
10
0
mAngio
empty
VERO
U87


c)
Tube number
70
60
50
40
30
20
10
0
MS1
HUVECs
p=0.014
**
p=0.0047
**
PBS
empty
mAngio


b)
PBS
empty
mAngio