Hermonat Laboratory Research Interests

1) AAV genetics, molec biol and biochem
2) Helper genes: making more AAV
3) AAV cardiovascular gene therapy
4) AAV anti-cancer immuno-gene therapy
5) AAV skin gene therapy
6) Research on other viruses

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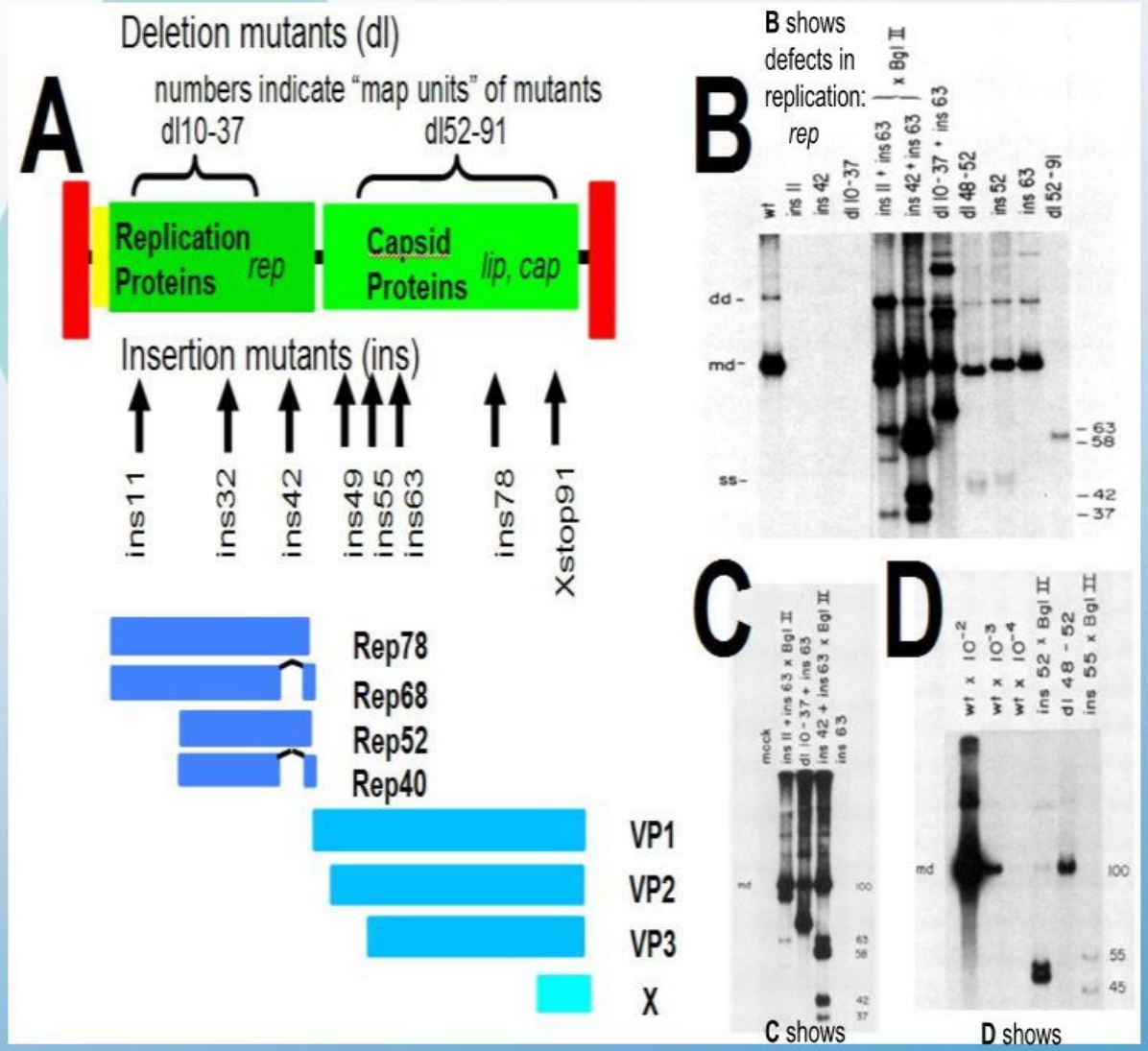
1) Genes / phenotypes, biochem ofAAV2



C shows defects in virus particle production ***cap***

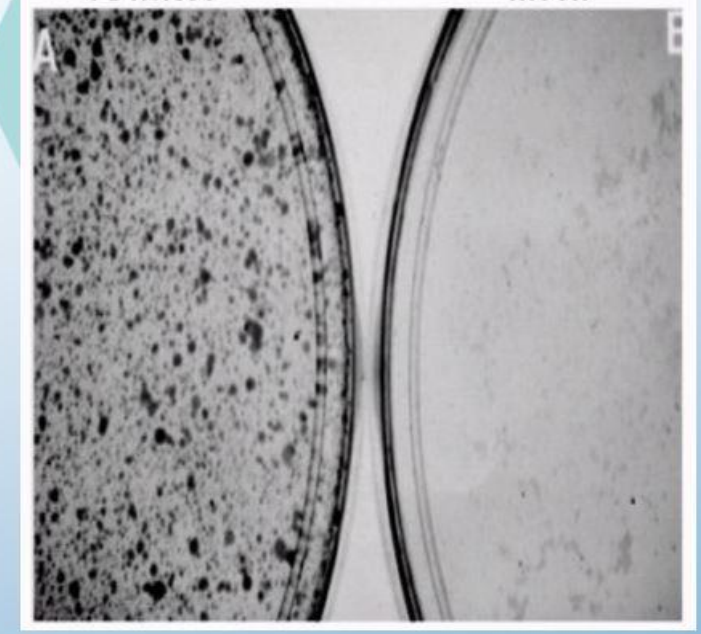
D shows virion production but of low infectivity. ***lip***

1) **Hermonat, P.L.**, Labow, M.A., Wright, R., Berns, K.I., and Muzyczka, N. (1984) Genetics of adeno-associated virus: isolation and preliminary Character-I zation of mutants in adeno-associated virus type 2. J. Virology 51(2):329-339.

2) Labow, M.A., **Hermonat, P.L.**, and Berns, K.I. (1986) Positive and negative a utoregulation of the adeno-associated virus type 2 genome. J. Virology 60(1):251-258.

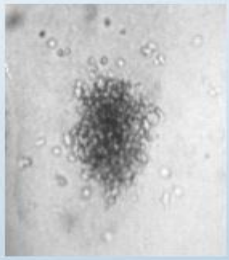
AAV is a natural at gene delivery.

1983, the first AAV gene transfer experiment Detroit 6 cells infected, then G418 selected

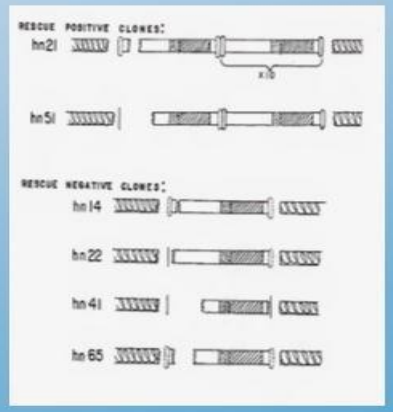


First transduction of hematopoietic progenitor cells or of primary explanted cells:

G418- resistant granulocyte colony after AAV/Neo infection



LaFace, D., **Hermonat, P.L.**, Wakeland, E.K., and Peck, A.B. (1988) Gene transfer into hematopoietic progenitor cells mediated by an adeno-associated virusvector. Virology 162:483-486.



There are many AAV proviral structures that result from AAV chromosomal integration

Hermonat PL. **Genetic analysis and utilization of adeno-associated virus as a mammalian cloning vector**. *University of Florida Dissertation*, copyright 1984, https://archive.org/details/geneticanalysisu00herm

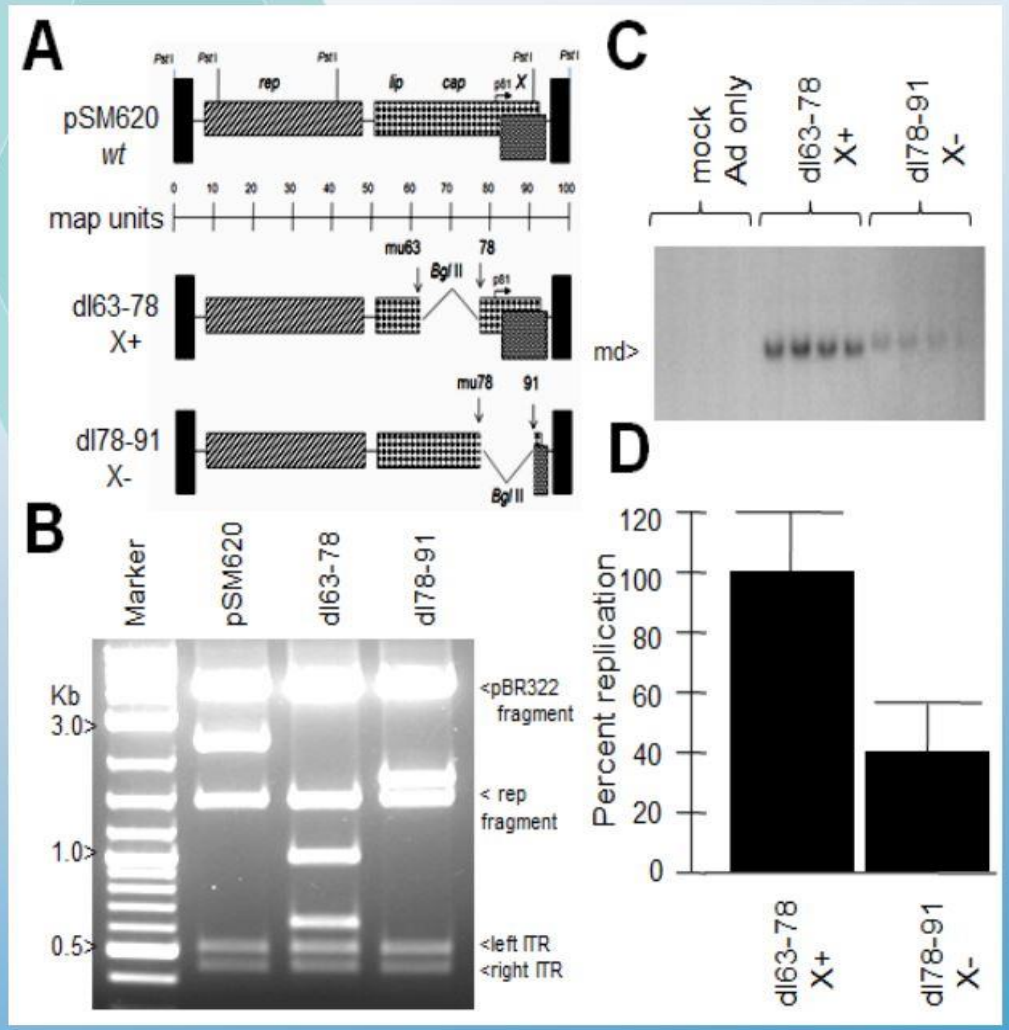
1) **Hermonat PL**, and Muzyczka N. **Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammalian tissue culture cells**. *Proc Natl Acad Sci USA*1984; 81:6466-6470, 1984.

2) **Hermonat PL** (2014) The first adeno-associated virus gene transfer experiment, 1983. Human Gene Therapy. 4: 135

3) **Hermonat, P.L.**, Labow, M.A., Wright, R., Berns, K.I., and Muzyczka, N. (1984) Genetics of adeno-associated virus: isolation and preliminary characterization of mutants in adeno-associated virus type 2. J. Virology 51(2):329-339

4) Labow, M.A., **Hermonat, P.L.**, and Berns, K.I. (1986) Positive and negative autoregulation of the adeno-associated virus type 2 genome. J. Virology 60(1):251-258.

However, there is a new AAV2 gene called X, and involved in DNA replication



Cao M, You H, **Hermonat PL**. (2014) The *X* gene of adeno- associated virus (AAV) type 2 is involved in viral DNA replication. *In press* PLoS ONE.

Some adeno-associated virus Rep78 biochemistry studies

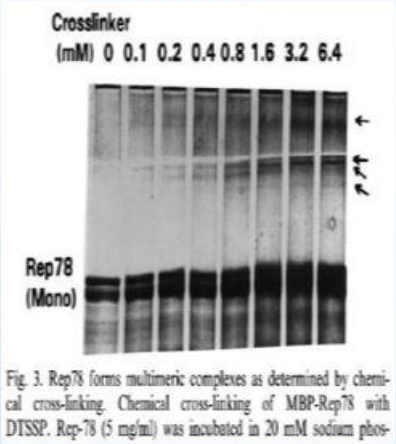
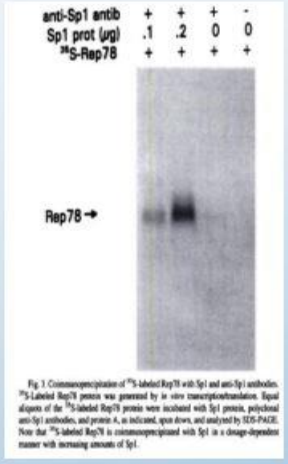
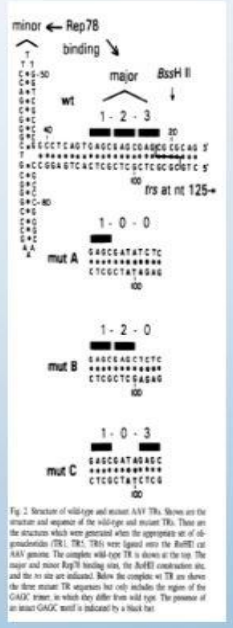


Fig. 3. Rep78 forms multimeric complexes as determined by chemical cross-linking. Chemical cross-linking of MBP-Rep78 with DTSSP. Rep-78 (5 mg/ml) was incubated in 20 mM sodium phos-

Hermonat PL, Batchu RB. The adeno-associated virus Rep78 major regulatory protein forms multimeric complexes and the domain for this activity is contained within the carboxy half of the molecule
FEBS Lett. 1997 Jan 20;401(2-3):180-4.



Hermonat PL, Santin AD, Batchu RB. The adeno-Associated virus Rep78 major regulatory/transformation suppressor protein binds cellular Sp1 in vitro and evidence for a biological effect. Cancer Res. 1996 Nov 15;56(22): 5299-304.



Bishop BM, Santin AD, Quirk JG, Hermonat PL. Role of the terminal repeat GAGC trimer, the major Rep78 binding site in adeno-associated virus DNA replication.
FEBS Lett. 1996 Nov 11;397(1):97-100.

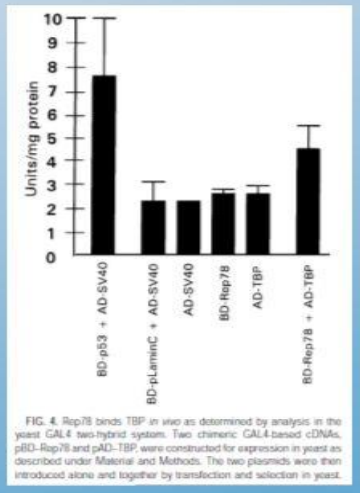
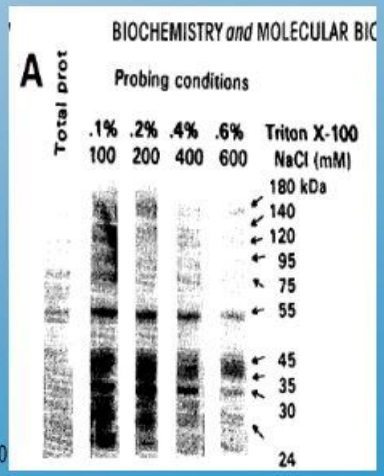


FIG. 4. Rep78 binds TBP in vivo as determined by analysis in the yeast GAL4 two-hybrid system. Two chimeric GAL4-based cDNAs, pBD–Rep78 and pAD–TBP, were constructed for expression in yeast as described under Material and Methods. The two plasmids were then introduced alone and together by transfection and selection in yeast.

Hermonat PL, Santin AD, Batchu RB, Zhan D. The adeno-associated virus Rep78 major regulatory protein binds the cellular TATA-binding protein in vitro and in vivo.
Virology. 1998 May 25;245(1):120-7.



Hermonat PL, Santin AD, Carter CA, Parham GP, Quirk JG. Multiple cellular proteins are recognized by the adeno-associated virus Rep78 major regulatory protein and the amino-half of Rep78 is required for many of these interactions.
Biochem Mol Biol Int. 1997 Oct;43(2):409-20

AAV2 biology

Packaging capacity of AAV2

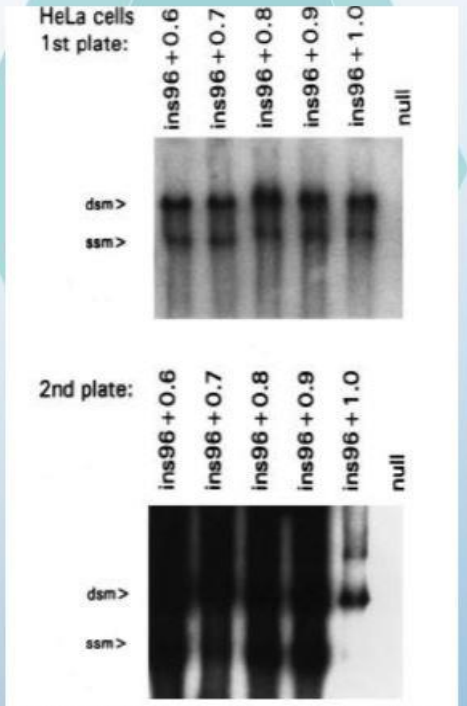


Fig. 4. Ins96+1.0 is packaged at a lower efficiency than smaller genomes in HeLa cells. Shown is a sequential, two plate Southern blot analysis for infectious virus production, similar to that described in Fig. 2. In this experiment, however, the stepped 100 bp large AAV genomes are analyzed and ^{32}P-labeled 100 bp ladder [insert] probe) is used to score for replicating AAV DNA. Note that all the AAV plasmids were able to replicate in the first plate; however, in the second plate ins96+1.0 was defective infectious virus production and subsequent DNA replication, when compared to the slightly smaller AAV genomes. These data indicate that the maximum packaging capacity of the AAV virion was exceeded by ins96+1.0. 'dsm' indicates double-stranded monomer DNA, and

Hermonat, P.L. , Quirk, J.G., Bishop, B.M., and Han, L. (1997) Packaging capacity of adeno-associated virus and the potential for *wild type-plus* AAV gene

therapy vectors. FEBS Letters 407:78-84.

OMG, AAV2 is an autonomous parvovirus !!!

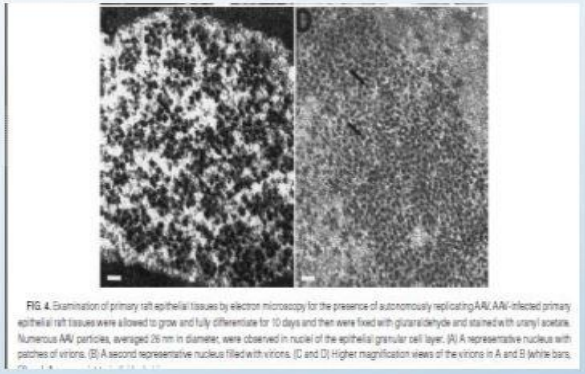


FIG. 4. Examination of primary raft epithelial tissues by electron microscopy for the presence of autonomously replicating AAV. AAV-infected primary epithelial raft tissues were allowed to grow and fully differentiate for 10 days and then were fixed with glutaraldehyde and stained with uranyl acetate. Numerous AAV particles, averaged 26 nm in diameter, were observed in nuclei of the epithelial granular cell layer. (A) A representative nucleus with patches of virions. (B) A second representative nucleus filled with virions. (C and D) Higher magnification views of the virions in A and B (white bars,

Meyers C, Mane M, Kokorina N, Alam S, **Hermonat** PL. **Ubiquitous** human adeno-associated virus type 2 autonomously replicates in differentiating keratinocytes of a normal skin model. Virology. 2000 Jul 5;272(2):338-46.

Human papillomavirus helps AAV

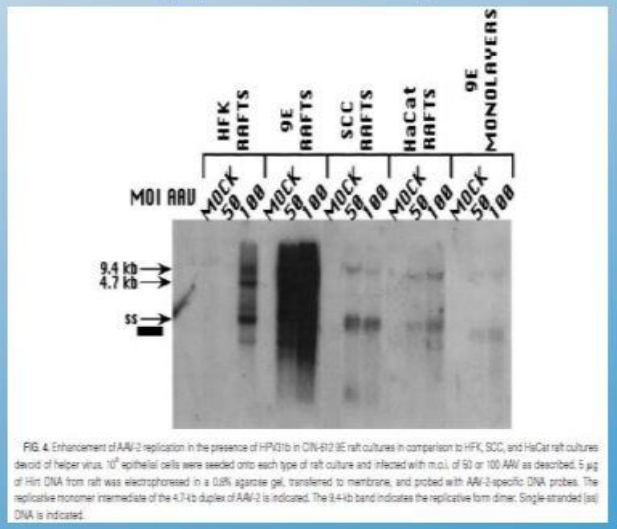


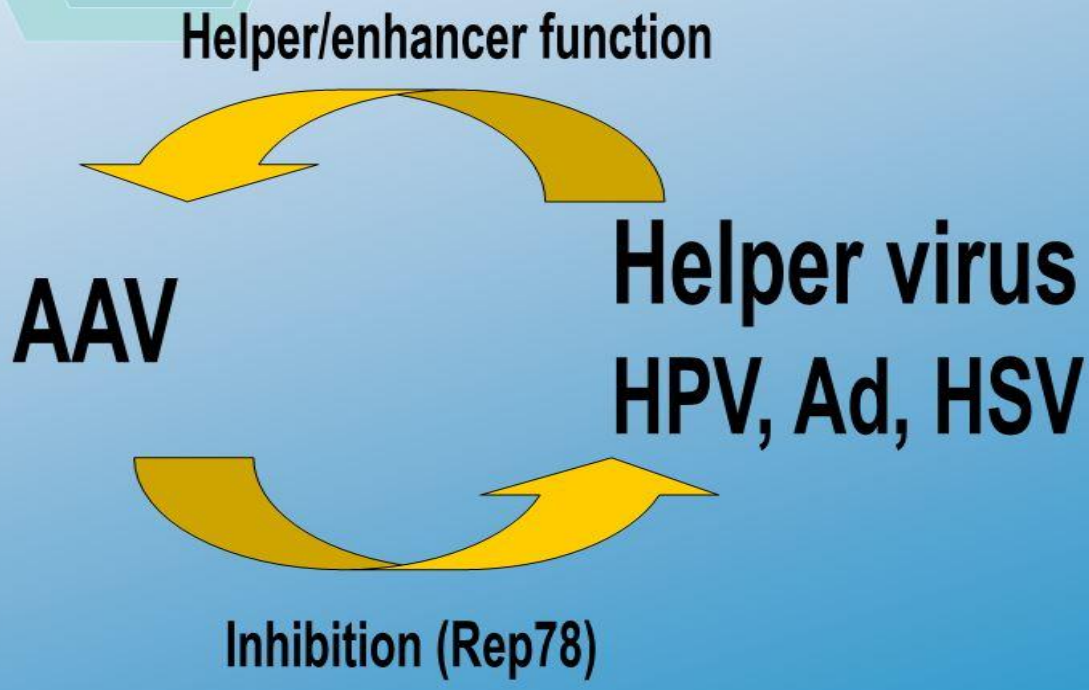
FIG. 4. Enhancement of AAV-2 replication in the presence of HPV31b in CIN-612 9E raft cultures in comparison to HFK, SCC, and HaCat raft cultures devoid of helper virus. 10^6 epithelial cells were seeded onto each type of raft culture and infected with m.o.i. of 50 or 100 AAV as described. 5 μg of Hirt DNA from raft was electrophoresed in a 0.8% agarose gel, transferred to membrane, and probed with AAV-2-specific DNA probes. The replicative monomer intermediate of the 4.7-kb duplex of AAV-2 is indicated. The 9.4-kb band indicates the replicative form dimer. Single-stranded (ss) DNA is indicated.

Meyers C, Alam S, Mane M, **Hermonat** PL. Altered biology of adeno-associated virus type 2 and human papillomavirus during dual infection of natural host tissue. Virology. 2001 Aug 15;287(1):30-9.

3) Making more AAV

Of what importance is HPV-AAV interaction?

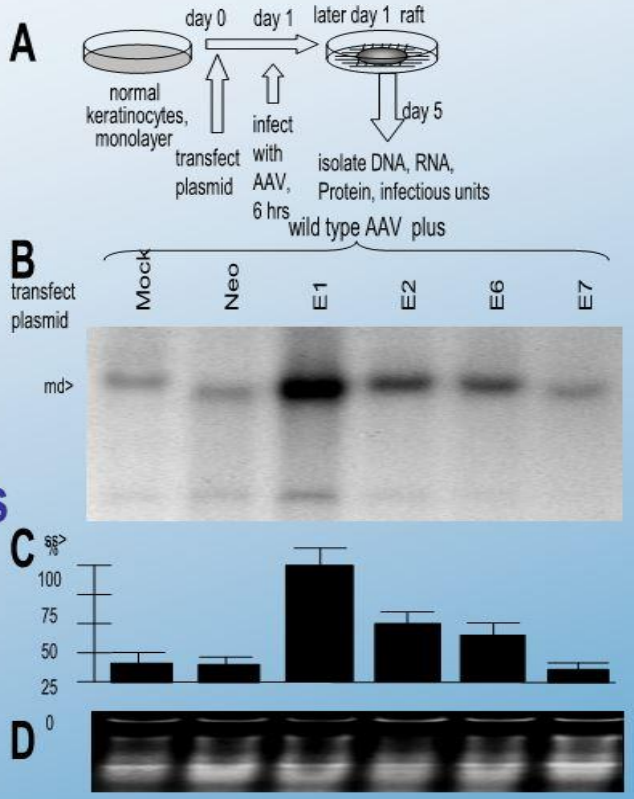
Adeno-associated Virus (AAV) is a "helper-dependent" parvovirus



Making more AAV

HPV E1, E2 and E6 help AAV replication

Adenovirus, herpesvirus and human papillomavirus (HPV) serve as helpers for AAV.



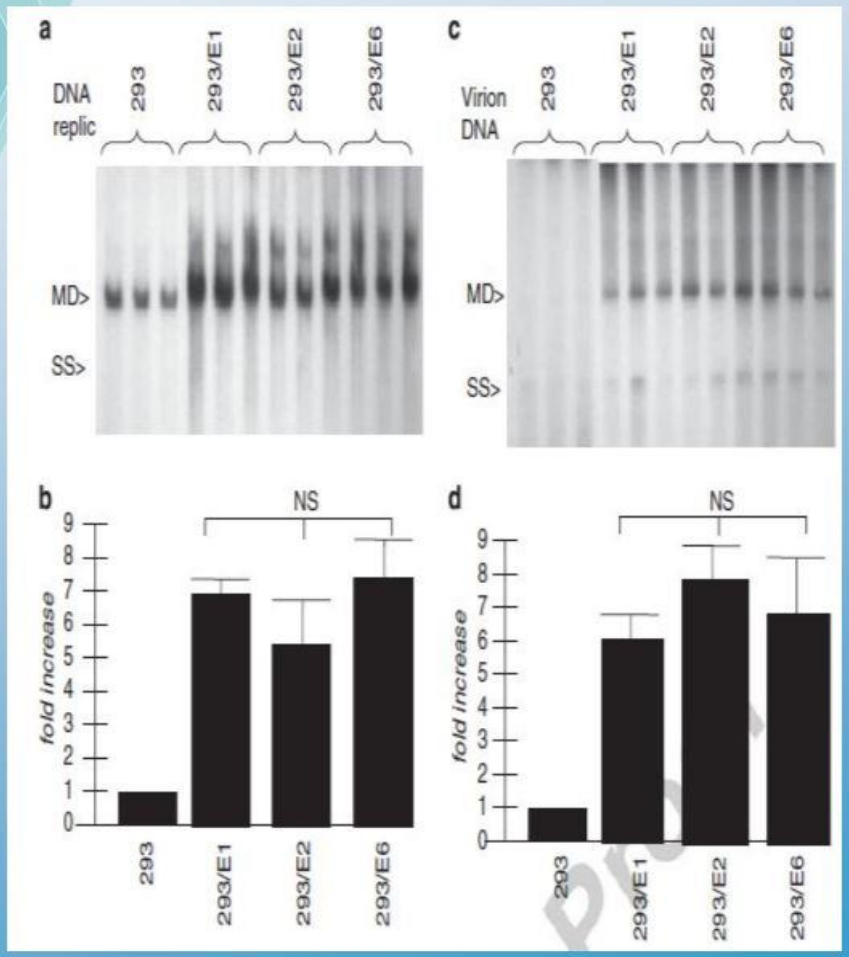
You, H., Liu, Y., Prasad, C.P., Agrawal, N., Zhang, D., Bandyopadhyay, S., Liu, H., Kay, H.H., **Hermonat, P.L.** (2006) Multiple human papillomavirus genes affect the adeno-associated virus life cycle. Virology 344(2):532-40.

Cao M, Zhu H, Bandyopadhyay S, You H and **Hermonat PL** (2012) HPV-16 E1, E2 and E6 each complement the Ad5 helper gene set, increasing rAAV2 and wt AAV2 production. Gene Therapy 19: 418-424

Making more AAV

Adenovirus *plus* HPV helper genes boost AAV Production

Cao M, Zhu H, Bandyopadhyay S, You H and **Hermonat PL** (2012) HPV-16 E1, E2 and E6 each complement the Ad5 helper gene set, increasing rAAV2 and wt AAV2 production. Gene Therapy 19: 418-424



These results suggest that combinations of helper genes derived from each of the three major helper viruses may give superior rAAV production than any one helper gene set.

3) Cardiovascular gene therapy,

List of genes:

Anti-atherosclerosis genes

TGFbeta1*
SMAD3*
IL10*
STAT3*
IL10 plus STAT3*
LOX1pr-IL10* disease-specific
IL13
AT2R*
B7-H1
Netrin-1*
PRDX6*
ApoA1Milano

Ineffective genes (controls)

CGRP *
Neo*
GM-CSF*

*published

Some of our papers on AAV cardiovascular gene therapy

1) Zhu H, Cao M, Chriva-Internati M, **Hermonat PL.** Comparison of efficacy of human interleukin 10, expressed from the disease-specific LOX1 or constitutive cytomegalovirus promoters, against atherosclerosis in mice using adeno-associated virus 2/8 delivery. *in press* PLoS ONE, 2014

2) Zhu H, Cao M, Figueroa JA, Cobos E, Uretsky BF, Chiriva-Internati M, **Hermonat, PL**. AAV2/8-hSMAD3 gene delivery attenuates aortic atherogenesis, enhances Th2 response, without inducing COL1A2/2A1 and CTGF (fibrosis) in LDLR-KO mice on high cholesterol diet. *In press* J Translational Medicine, 2014.

3) Hermonat PL. (2014) Adeno-associated virus-based transgene delivery for treating progressive vascular disease. *In press* Cloning and Transgenesis, 2014

4) Zhu H, Cao M, Straub KD, Hermonat PL. Systemic delivery of thiol-specific antioxidant hPRDX6 gene by AAV2/8 inhibits atherogenesis in LDLR KO mice on HCD. *In press* Gen Syndrom Gene Ther, 2013

5) Rhee SW, Hermonat PL, Rusch NJ. Methods of treating hypertension. *US Patent 8227445*, July 24, 2012 (AAV-based gene therapy for hypertension by delivering BK potassium channel gene)

6) Cao M, Khan JA, Kang BY, Mehta JL, Hermonat, PL. Dual AAV/IL-10 plus STAT3 anti-inflammatory gene delivery lowers atherosclerosis in LDLR KO mice, but without increased benefit. International Journal Vascular Medicine, 2012: 2012:524235.

7) Khan JA, Cao M, Kang B-Y, Liu Y, Mehta JL, and Hermonat PL. Systemic hNetrin-1 gene delivery by AAV8 alters leukocyte accumulation and atherogenesis in vivo. Gene Therapy, 2011, 18: 437-444.

8) Hermonat PL, Zhu HQ, Cao M, Mehta JL. LOX-1 transcription. Cardiovascular Drugs and Therapy, 2011. Epub ahead of print.

9) Khan JA, Cao M, Kang BY, Liu Y, Mehta JL, Hermonat PL. AAV/hSTAT3-gene delivery lowers aortic inflammatory cell infiltration in LDLR KO mice on high cholesterol. Atherosclerosis 2010, 213(1):59-66.

10) Hu C, Dandapat A, Chen J, Liu Y, Hermonat PL, Carey RM, Mehta JL. Over-expression of angiotensin II type 2 receptor (agtr2) reduces atherogenesis and modulates LOX-1, endothelial nitric oxide synthase and heme-oxygenase-1 expression. Atherosclerosis. 2008, 199(2):288-94.

11) Hu C, Dandapat A, Sun L, Khan JA, Liu Y, Hermonat PL, Mehta JL. Regulation of TGFbeta1-mediated collagen formation by LOX-1: Studies based on forced overexpression of TGFbeta1 in wild-type and lox-1 knock-out mouse cardiac fibroblasts. J. Biol. Chem. 2008, 283(16):10226-10231.

12) Dandapat A, Hu CP, Chen J, Liu Y, Khan JA, Remeo F, Carey RM, Hermonat PL, Mehta JL. Over-expression of angiotensin II type 2 receptor (agtr2) decreases collagen accumulation in atherosclerotic plaque. Biochem. Biophys. Res. Commun. 2008, 366(4): 871-877.

13) Dandapat A, Hu CP, Li D, Liu Y, Chen H, Hermonat PL, Mehta JL. Overexpression of TGFbeta1 by adeno-associated virus type-2 vector protects myocardium from ischemia-reperfusion injury. Gene Ther. 2008, 15(6): 415-423.

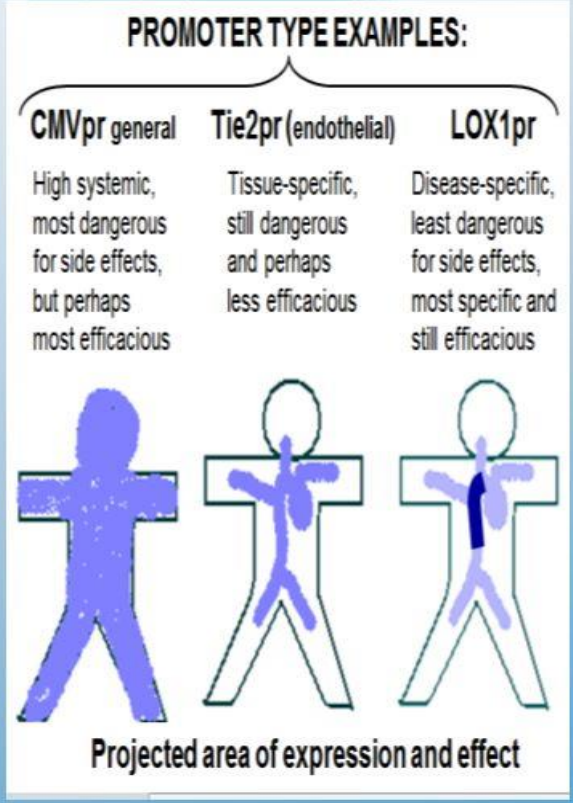
14) Hu CP, Dandapat A, Liu Y, Hermonat PL, Mehta JL. Blockade of hypoxia-reoxygenation-mediated collagen type 1 expression and MMP activity by overexpression of TGF-beta1 delivered by AAV in mouse cardiomyocytes. Am. J. Physiol. Heart Circ. Physiol. 2007, 293(3): H1833-8.

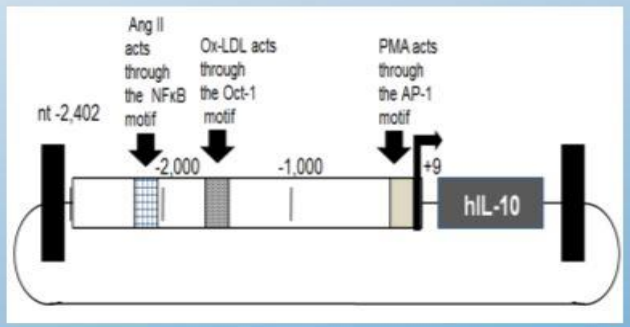
15) Liu, Y., Li, D., Chen; J. Xie, J., Bandyopadhyay, S., Zhang, D., Nemarkommula, A.R., Liu, H., Mehta, J.L., **Hermonat, P.L.** (2006) Inhibition of atherogenesis in LDLR knockout mice by systemic delivery of adeno-associated virus type 2-hIL-10. Atherosclerosis 188: 19-27.

Disease-specific Gene Therapy

Use of the LOX1pr

Our hypothesis is that disease-specific gene therapy, giving targeted expression at the site of disease, will be the most common approach used in gene therapy as it gives a built-in safeguard against adverse reactions, resulting in measured response and giving greater safety.





The LOX1 promoter is very big at 2.4 kb in length. However the fact that its expression is believed to pre-date visible disease makes it an outstanding candidate for disease-specific gene therapy.

Through the use of disease-specific gene therapy we can treat cardiovascular disease, yet at the same time, limit adverse effects due to overexpression of these strong therapeutic genes.

When the disease diminishes then so too will the expression of the LOX1pr and the therapeutic transgene.

1) Zhu H, Cao M, Chriva-Internati M, **Hermonat PL.** Comparison of efficacy of human interleukin 10, expressed from the disease-specific LOX1 or constitutive cytomegalovirus promoters, against atherosclerosis in mice using adeno-associated virus 2/8 delivery. *in press* PLoS ONE, 2014

Disease-specific gene delivery by LOX1pr gives efficacy with much lower overall transgene expression and built-in safeguard.

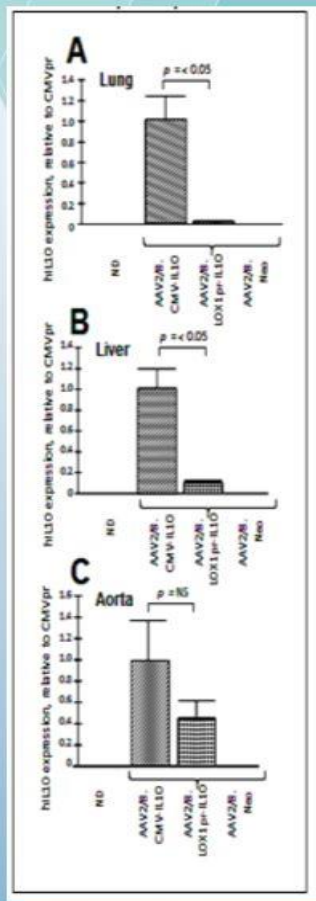


Figure 12: Expression of hIL10 using the LOX1pr shows targeted expression in the atherosclerotic aorta. These data show the advantage of the disease-specific gene therapy approach. This is vastly superior to the "everywhere, all the time approach" (here, showing CMVpr as an example). Relative expression of delivered hIL10 genes compared to endogenous βactin determined by real-time quantitative PCR from aorta of 6 mice in each group. Note that while CMVpr-IL10 and LOX1pr-IL10 treatments were statistically similar, LOX1pr-IL10 trended to be less than half the expression level of CMVpr-IL-10.

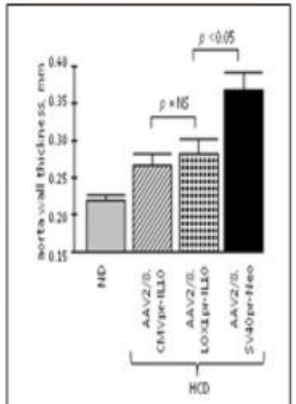


Figure 14: Analysis of the aortic wall thickness by HRUS. HRUS was used to measure the wall thickness of the aorta. Shown is a quantification of the thoracic region of the aortas in 8-10 animals from each animal group. Note that both CMVpr-hIL10- and LOX1pr-hIL10-HCD-treated animals had a significantly thinner aortic wall than the AAV/Neo-HCD-treated animal.

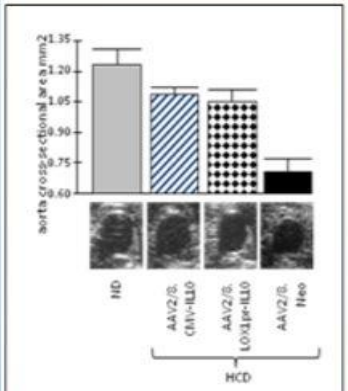
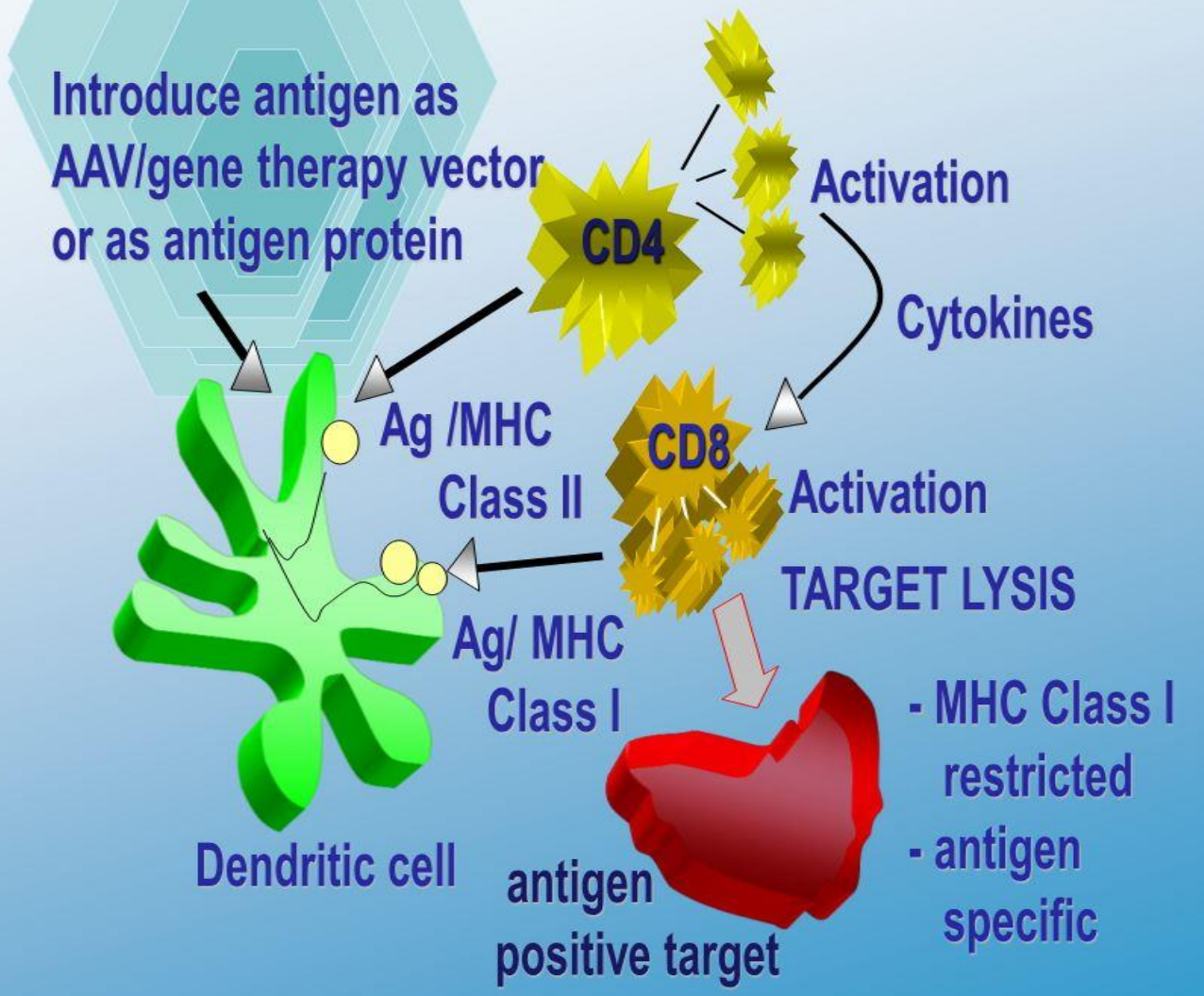


Figure 13: Analysis of the aortic lumen by high resolution ultrasound (HRUS). HRUS was used to measure the cross sectional area of the thoracic region of the aortas in 8-10 animals from each animal group. Shown is a quantification of the cross-sectional area for the abdominal/thoracic region of the aorta. Note that both CMVpr-hIL10- and LOX1pr-hIL10-HCD-treated animals had a significantly larger cross sectional area than the AAV/Neo-HCD-treated animals, indicating significant efficacy. However, CMVpr-hIL10-HCD- and LOX1pr-hIL10-HCD-treated animals were statistically similar to each other.

Zhu H, Cao M, Chriva-Internati M, **Hermonat PL.** (2014) Comparison of efficacy of human interleukin 10, expressed from the disease-specific LOX1 or constitutive cytomegalovirus promoters, against atherosclerosis in mice using adeno-associated virus 2/8 delivery. *in press* PLoS ONE

4) Immuno-Gene Therapy for Cancer Treatment

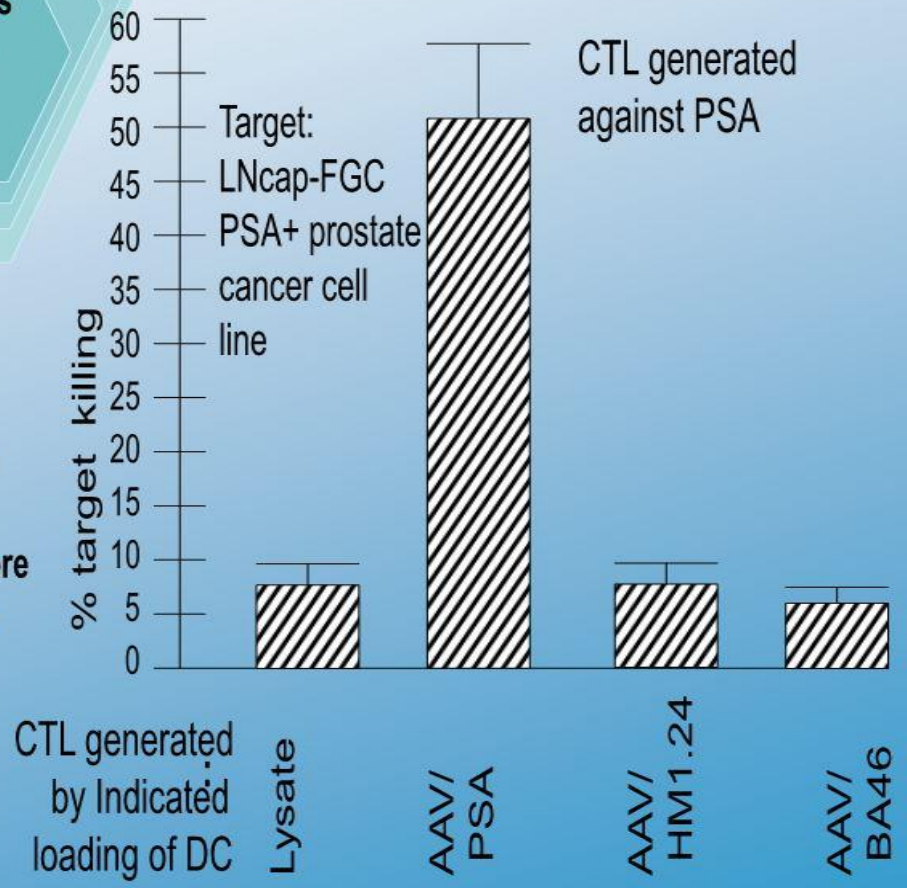
Generation of antigen-specific CD8+ cytotoxic T lymphocytes Is the best hope for curing cancer



Antigen-specific cytotoxic T lymphocytes (CTL) are generated by AAV/antigen transduced DC.

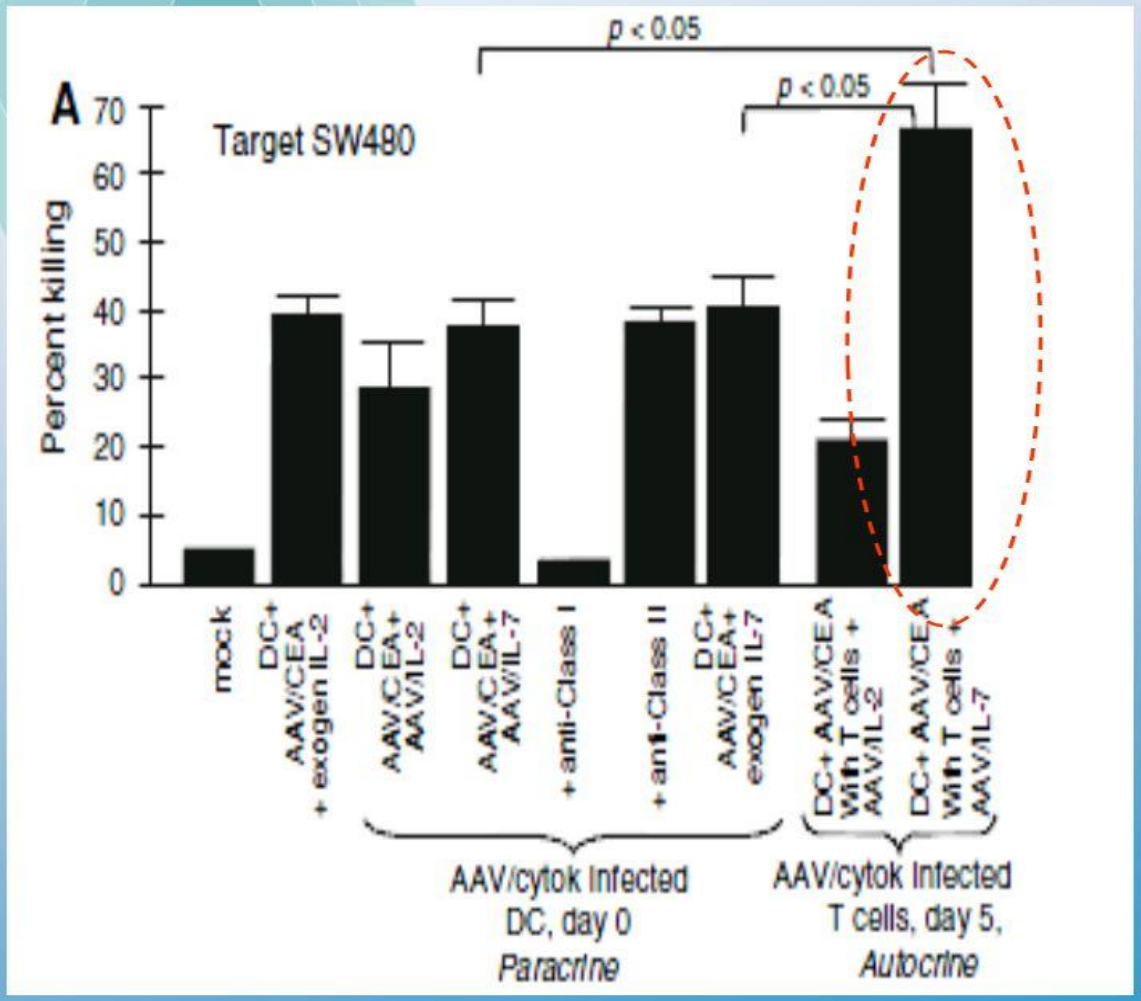
CTL generated are specific to the antigen gene loaded into DC

Our overall hypothesis is that the classic antigen specific, HLA Class I-restricted, CD8+ CTL will be a major weapon In defeating cancer. However, the mono -clonal CTL approach will not cure CA. The CTL must be polyclonal (multiple responders) and likely be against more than one tumor antigen, thus preventing the CA from circumventing CTL killing.



Additional anti-cancer immuno-gene therapy approaches:

Cytokine genes into T cells and or DC has significant benefit
IL-7 gene delivery into T cells generates
CTL populations with highest killing abilities.



Some of our papers on AAV- immuno-gene therapy

1) You CX, Shi M, Liu Y, Cao M, Luo RC, **Hermonat PL**. (2012) AAV2/IL-12 gene delivery into dendritic cells (DC) enhances CTL stimulation above other IL-12 applications: evidence for IL-12 intracrine activity in DC. Oncoimmunology 1: 847-855

2) You CX, Liu Y, Shi M, Cao M, Luo R-C, **Hermonat PL.** (2010) Comparison of AAV/IL-7 autocrine (T cell) versus paracrine (DC) gene delivery for enhancing CTL stimulation and function. Cancer Imm & Immunotherapy. 59: 779-787.

3) Zhang, D. Liu Y, Shi M, You CX, Cao M, Luo R-C, **Hermonat, PL.** (2010) Autocrine, not paracrine, interferon-gamma gene delivery enhances *ex vivo* antigen-specific cytotoxic T lymphocyte stimulation and killing. J. Biomed. Biotechnol. 2010: 270985

4) You, C., Liu, Y., Luo, R., You, H. Hermonat, P.L., and Mahadevan, M. (2007) Immunotherapy using cytotoxic T lymphocytes against prostate specific membrane antigen for prostate cancer. Book chapter in Cancer and Gene Therapy, Research Signpost, Kerala, India, pg 155-168.

5) Mahadevan M, Liu Y, You C, et al: Generation of robust cytotoxic T lymphocytes against prostate specific antigen by transduction of dendritic cells using protein and recombinant adeno-associated virus. Cancer Immunol Immunother 56:1615-24, 2007

6) Prasad, C.K., Liu, Y., You, C., Luo, R., Mehta, J.L. and **Hermonat, P.L.** (2007) Generation, comparison of cytotoxic T lymphocyte stimulation against Her2/neu by rAAV and protein antigen loading of dendritic cells. Book chapter in Cancer and Gene Therapy, Research Signpost, Kerala, India, Hermonat, Paul L. Editor pp 17-28.

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5) AAV transduction of keratinocytes for Recombinant skin / tissue engineering.

Not only are differentiating keratinocytes a natural host tissue for AAV in which it is an autonomous parvovirus, but the skin is an excellent target for gene therapy. The skin could be a factory for any protein which is secreted. Additionally, as the skin is exposed it can be monitored and manipulated much more readily than gene therapy through other sites.

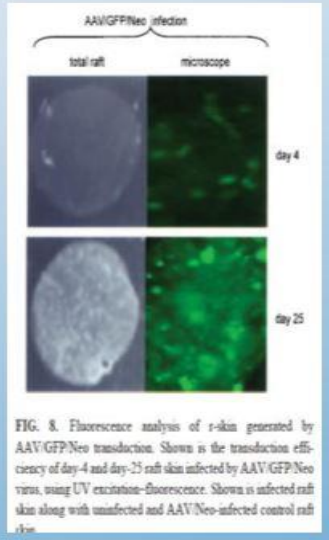
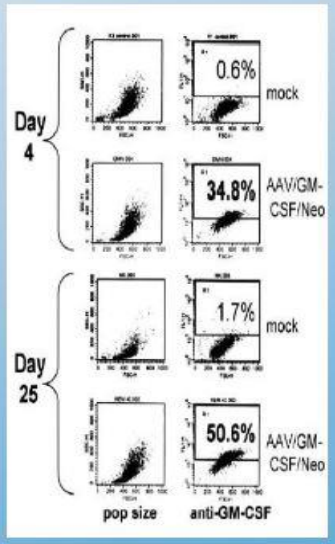


FIG. 8. Fluorescence analysis of r-skin generated by AAV/GFP/Neo transduction. Shown is the transduction efficiency of day-4 and day-25 raft skin infected by AAV/GFP/Neo virus, using UV excitation–fluorescence. Shown is infected raft skin along with uninfected and AAV/Neo-infected control raft



Papers on AAV and keratinocytes / skin:

1) Agrawal, N., You, H., Liu, Y., Chiriva-Internati, M., Grizzi, F., Prasad, C.K., Mehta, J.L., and **Hermonat, P.L.** (2004) Generation of recombinant skin *in vitro* by adeno-associated virus type 2 vector transduction. Tissue Engineering 2004, 120, 1707-1715.

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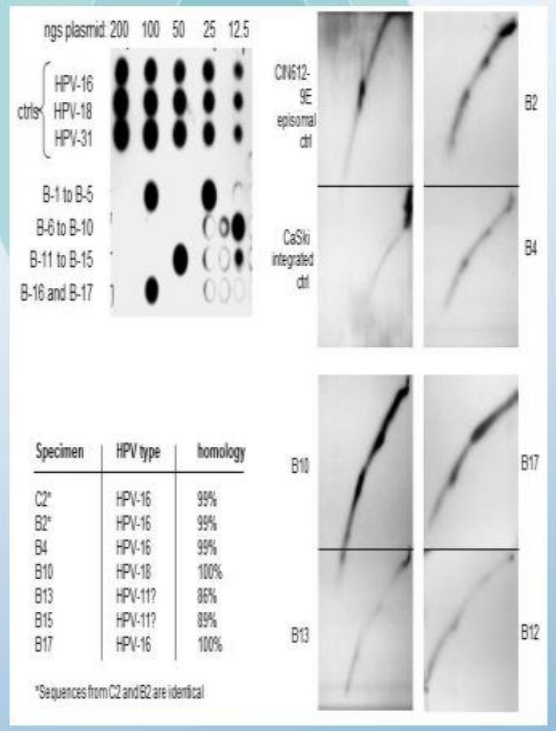
6) Other viruses: HPV is present in breast cancers and miscarriages



Specimen	HPV type	homology
C2*	HPV-16	99%
B2*	HPV-16	99%
B4	HPV-16	99%
B10	HPV-18	100%
B13	HPV-11?	86%
B15	HPV-11?	89%
B17	HPV-16	100%

*Sequences from C2 and B2 are identical

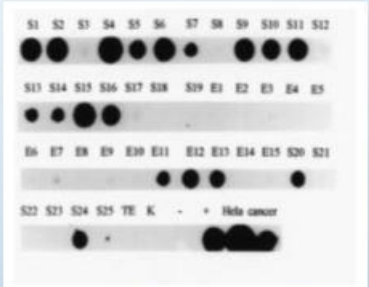


Fig. 2. Autoradiograph of dot blot hybridization for HPV E6 PCR products. A representative dot blot hybridization analysis of PCR products from spontaneous (indicated by S prefix) and elective (E prefix) is shown. Note that samples S1, S2, S4-7, S9-11, S13-16, S20, S24, and E11-13 were HPV positive. Negative

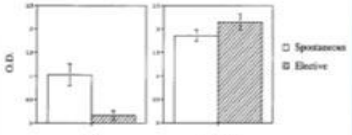


Fig. 3. Semi-quantitative analysis of the HPV and β-globin dot blots. These graphs show the means of the optical density values obtained by densitometric scanning of the HPV and β-globin dot blots. Error bars indicate the standard error of the mean for each sample group. The O.D. mean of the HPV PCR amplified DNA from spontaneous material was 1.028 ± 0.234 (standard mean error) ($n = 25$). The O.D. mean of the HPV PCR product from elective specimens was 0.168 ± 0.99 ($n = 15$). The O.D. mean of β-globin products from spontaneous material was 1.86 ± 0.122 ($n = 25$), and 2.15 ± 0.167 ($n = 15$) from elective products.

Human papillomavirus (HPV) is present at such high levels in some breast cancers that PCR amplification is not needed to observe it, only a Southern blot.

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