The episomal vectors

Requisiti di un vettore ideale:

- minima invasività
- selettività del bersaglio
- assenza di immunogenicità
- elevata capacità di clonaggio
- stabilità nel tempo
- corretta ploidia (N di copie)
- manipolabilità
- dimensioni ridotte

Non-viral vectors: by non-viral vectors the therapeutic genes are delivered to target cells using non-viral carriers such as synthetic polymers, liposomes or polyamines.

Episomal plasmid

Historical view

Attempts to construct non-viral episomal vectors for mammalian cells date back to the early 1980s, immediately after autonomously replicating sequences (ARSs) were described in yeast (Stinchcomb et al., 1980).

Non viral episomal vectors would replicate as an autonomous unit and would not require any exogenous trans-acting protein.

ARSs were isolated from the yeast genome and found to promote episomal replication when inserted into a plasmid in yeast.

When restriction-digested mammalian DNA was inserted into yeast plasmids numerous ARSs were found to promote plasmid replication in yeast.

However, transfection of such vectors into mammalian cells never resulted in replicating plasmids and in most cases the such constructs was either lost or integrated into the genome.

Subsequent sequence analyses of various mapped mammalian origins of replication failed to reveal sequence homologies but rather a number of **structural characteristics**, such as **long AT-rich regions**, **CpG islands**, **bent DNA**, and the presence of **scaffold/ matrix attached region** (S/MAR) sequences were identified.

It is believed that mammalian origins are not only determined by the DNA sequence itself but also by epigenetic factors, such as chromatin structure, gene expression, and even global nuclear architecture

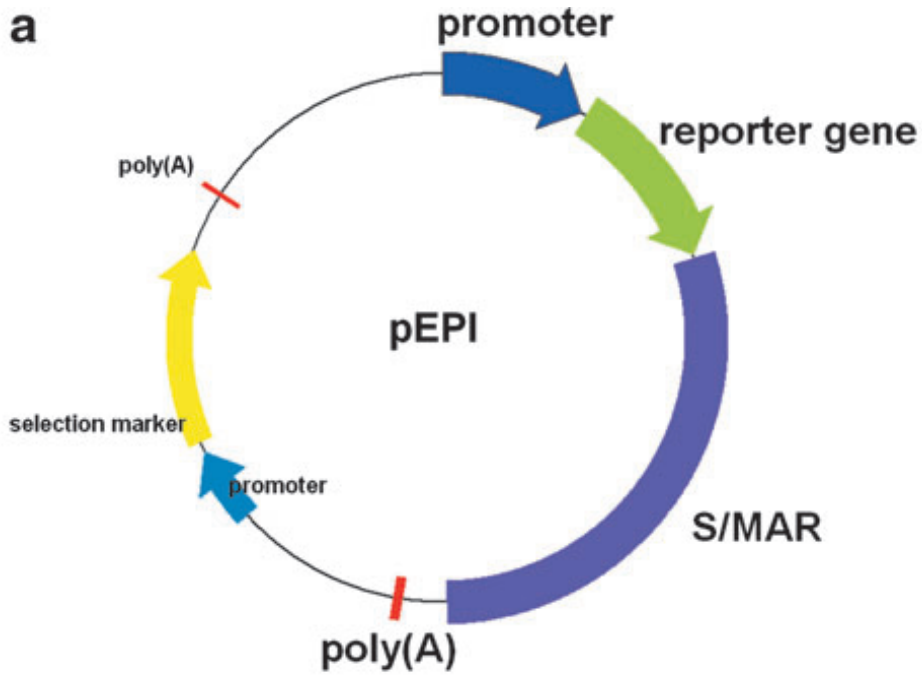
pEPI vectors

Our knowledge of the replication process is too limited to allow the rational design of vectors behaving as endogenous replication origins.
However, it is believed that origins bind to a sub-nuclear structure, the nuclear matrix or skeleton, at the onset of the S phase by an interaction of S/MAR sequences, which are often observed to be associated with mapped origins of replication.

S/MAR sequences do not share high sequence homologies but are rather characterized by their structural features. Often they contain **stretches with more than 70% AT-rich sequences, DNA-unwinding elements, and show binding sites for transcription factors and topoisomerase II.**

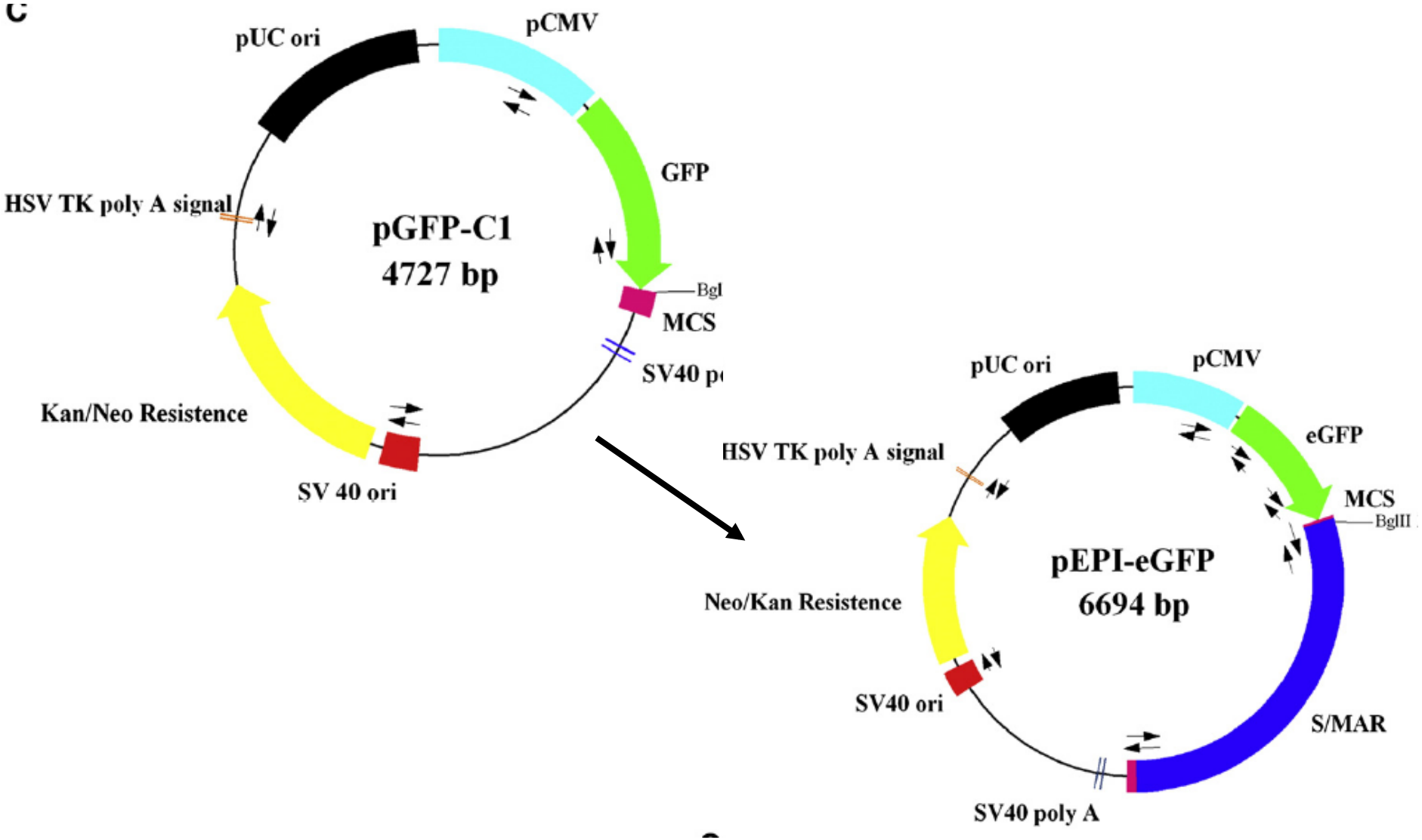
The insertion of an S/MAR sequence into a plasmid led to the first episomal vector for mammalian cells ➔ **pEPI - plasmid EPIsome**

pEPI: the first vector that replicates autonomously in mammalian cells

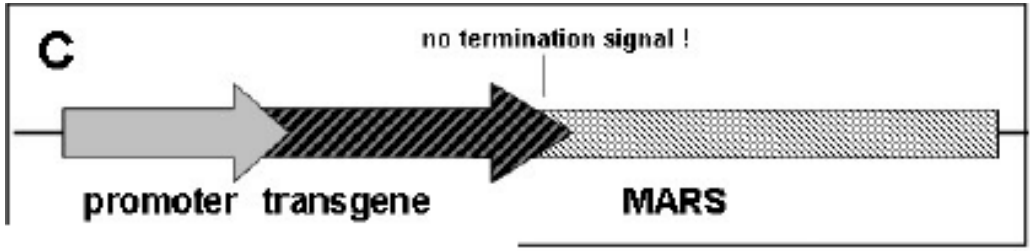


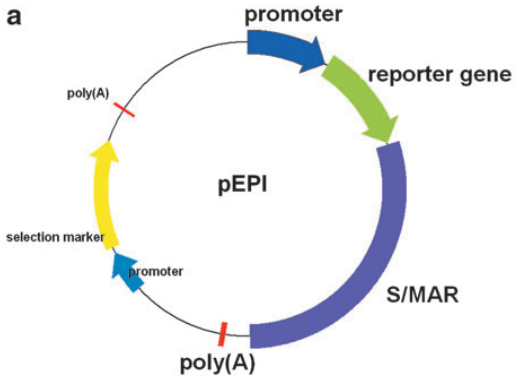
In this construct the sequence encoding the SV40 large T-antigen was replaced by a strong S/MAR sequence derived from the human interferon (IFN)-b gene cluster.

pEPI-eGFP assembly

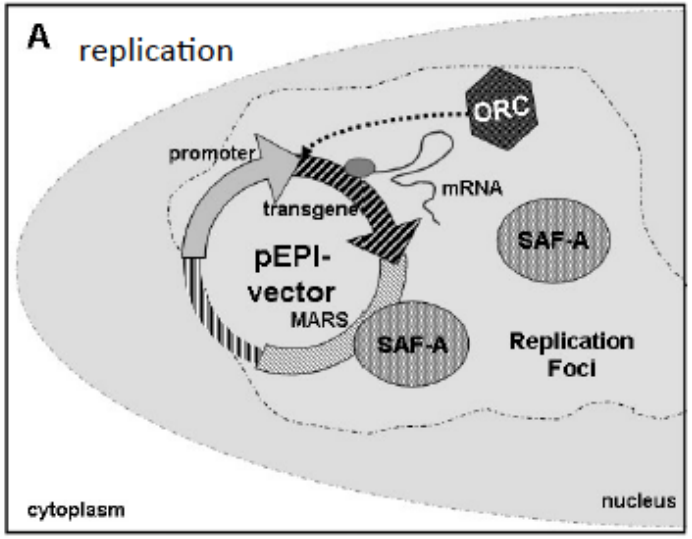


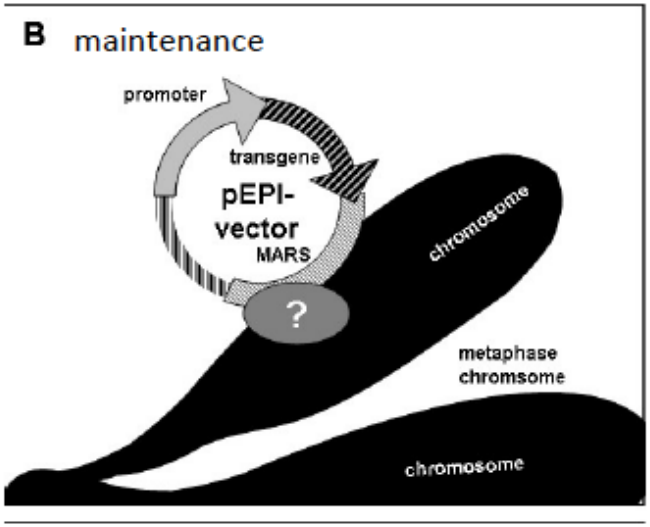
Replication and maintenance





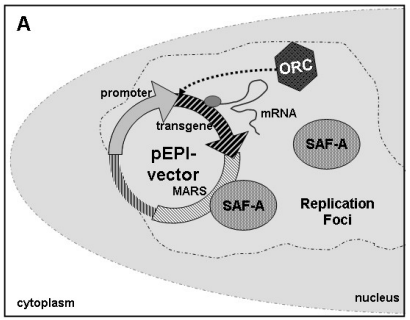
pEPI episome strictly depends o transcription throughout the S/MAR

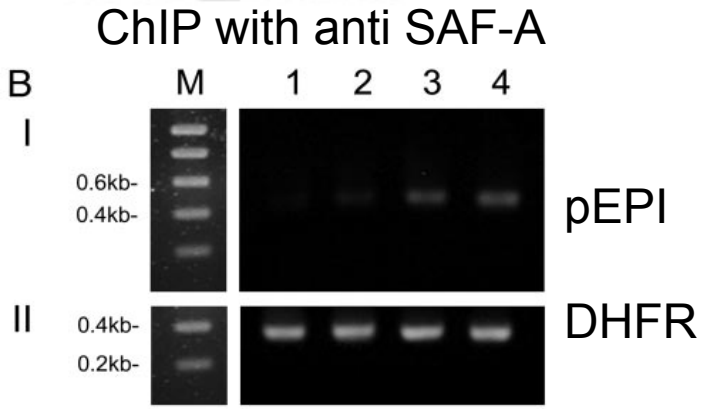




Critical for replication is the binding with SAF-A protein

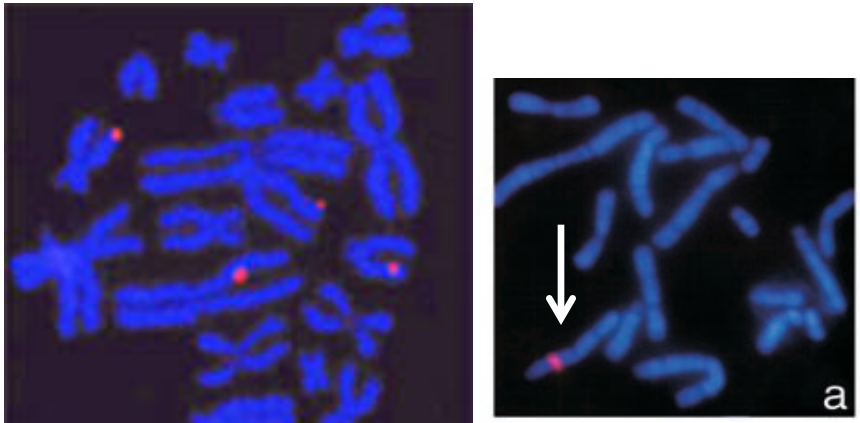
SAF-A binding to pEPI was demonstrated by ChIP





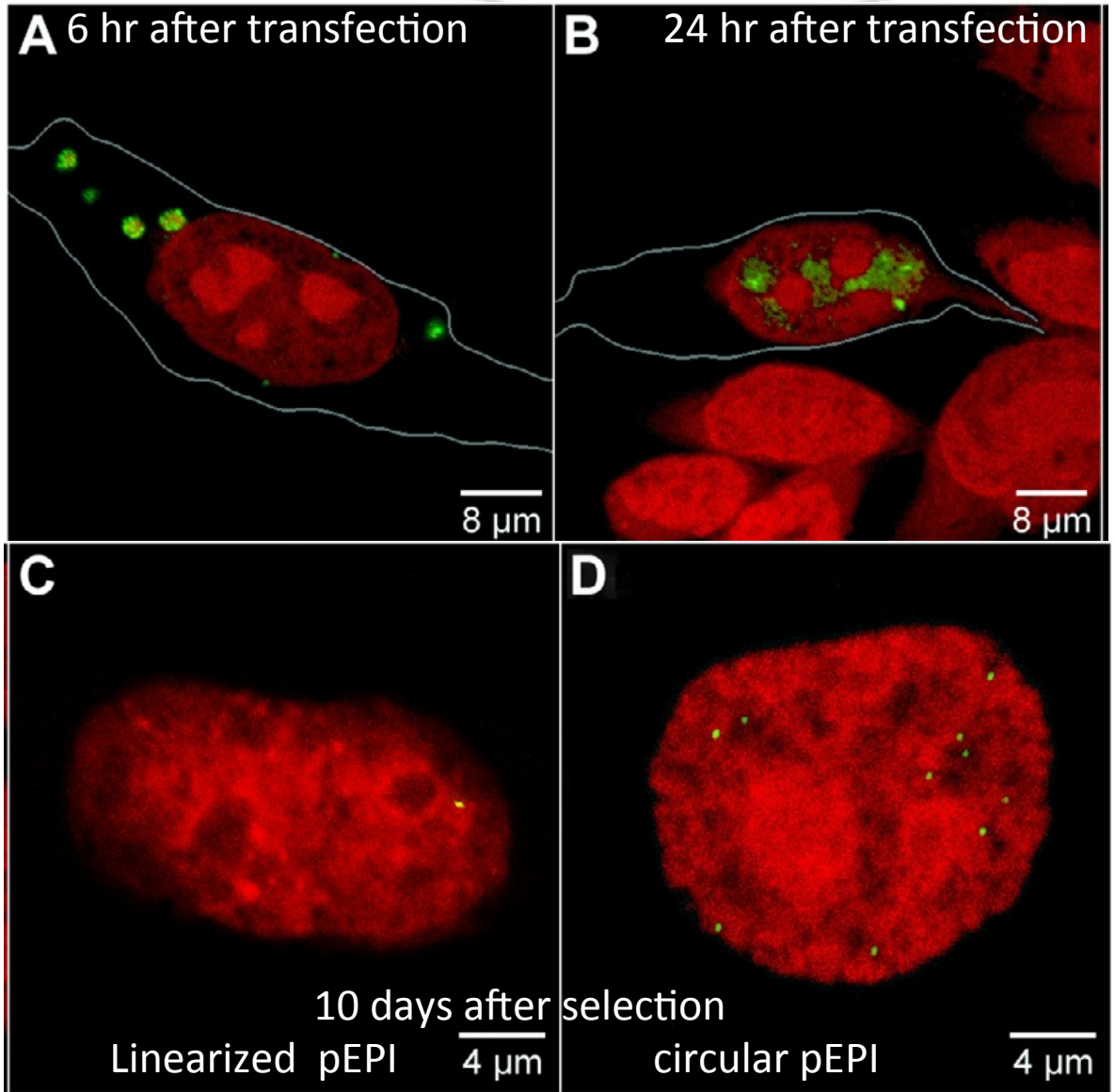
Chromatin immunoprecipitation of S/MAR from pEPI or DHFR. Samples: 1 and 2 plasmids without S/MAR; 3 and 4 pEPI-eGFP; DHFR internal control

SAF-A is believed to promote pEPI association with the host chromosome



pEPI molecules appear as small dots associated with the host chromosomes

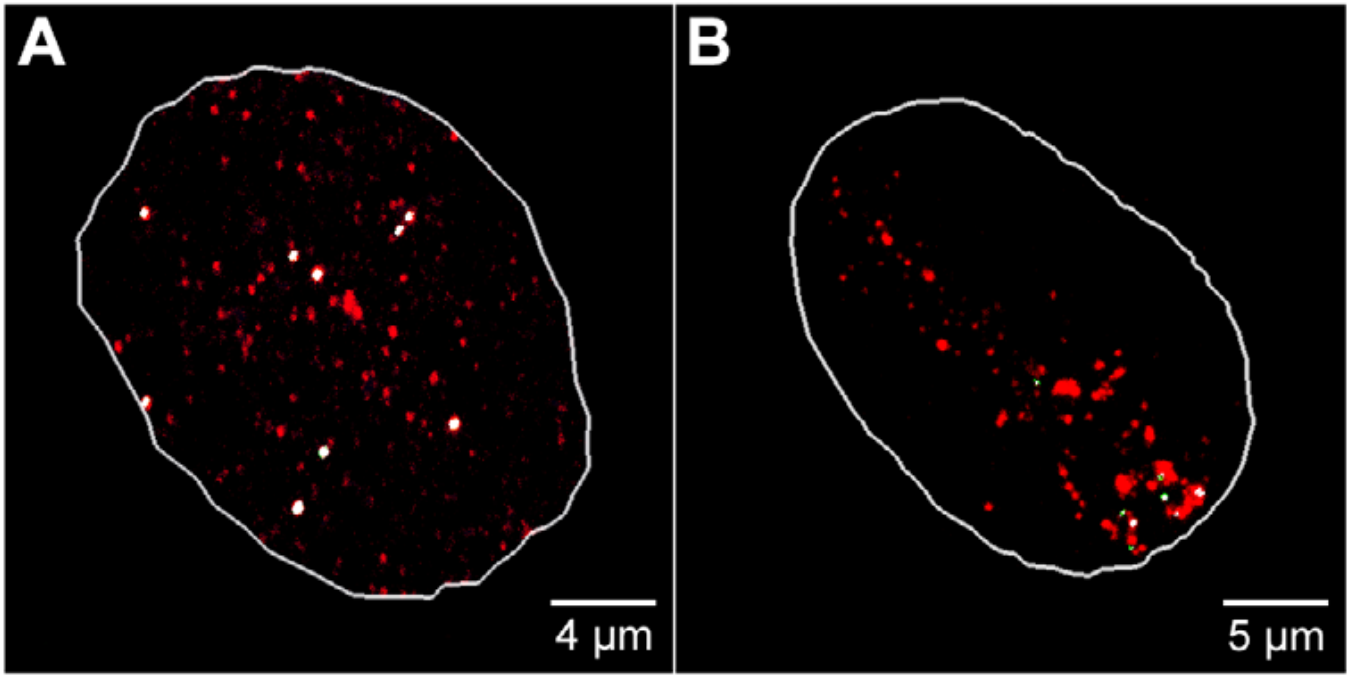
Replication and maintenance



The presence of single spot per cell suggests an integration event whereas, multiple spots indicate non-integrated episomes

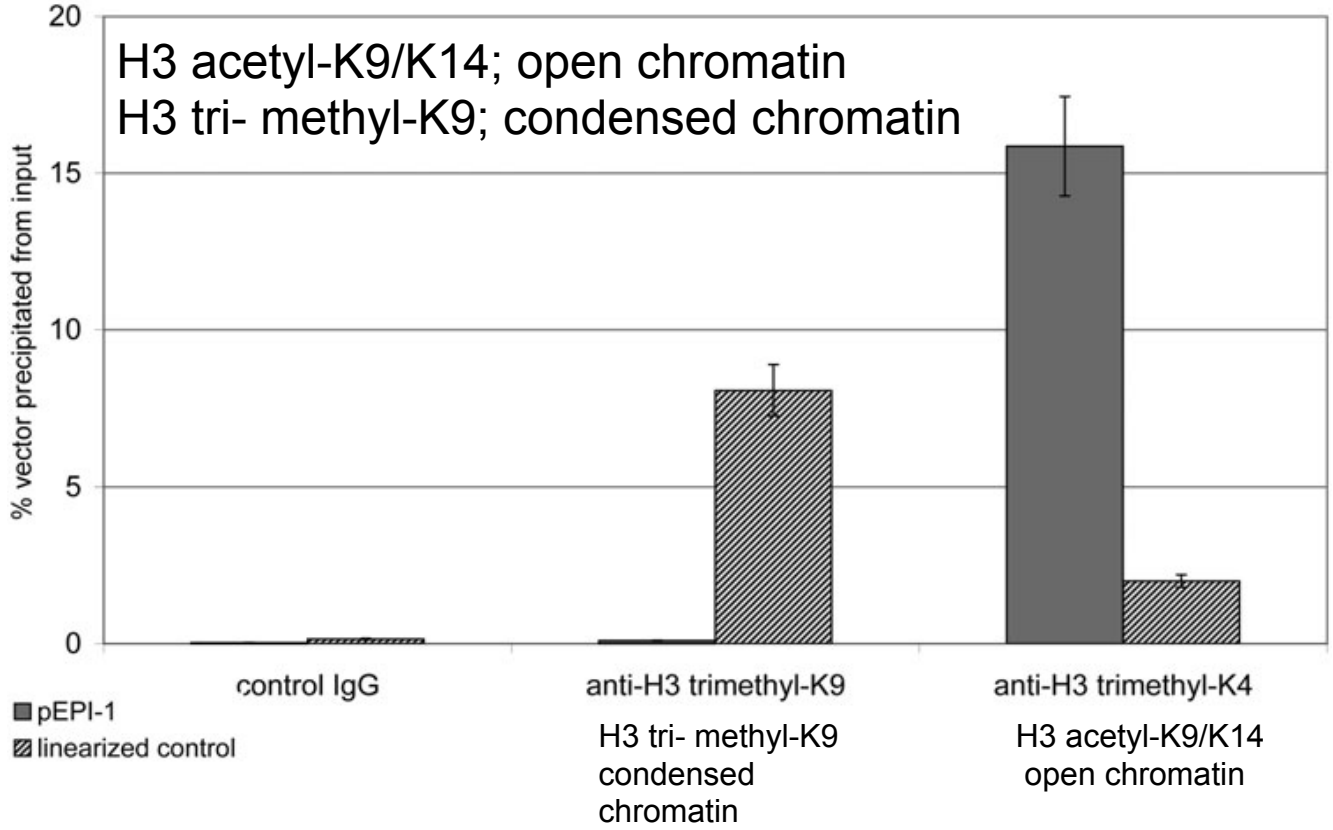
pEPI co-localize with early replication foci

The co-localization of the episome and replication foci was analysed using BrdU pulse labelling and subsequent FISH with a pEPI probe.



Replication foci labelled during early S-phase by incorporation of BrdU (1 h). Nuclei were fixed and newly synthesized DNA was visualized by anti-BrdU antibody (red). The episome (green) was visualized by pEPI FISH. Co-localization was highlighted in white colour.

pEPI assumes an open chromatin configuration

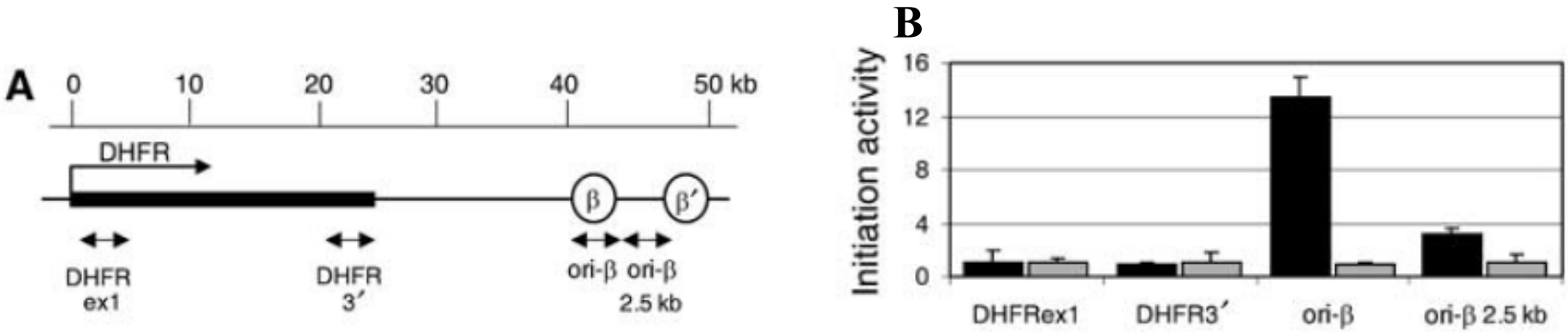


In almost all cases, pEPI is associated with histone modifications typical of active chromatin, with an accumulation of histone 3 methylated at Lys-4 (H3K4me1,me3) in the S/MAR element.

Ori mapping

The origin of replication is mapped by measuring the relative abundance of nascent DNA strands. Nascent DNA strands are revealed by PCR. As a negative control, DNA is shared in order to destroy the nascent ssDNA.

Ori mapping to a known chromosomal loci, the DHFR (dihydrofolate reductase). DNA is extracted from replicating cells. Nascent ssDNA is detected as selective amplification fragments that differ between un-sheared and sheared DNA samples



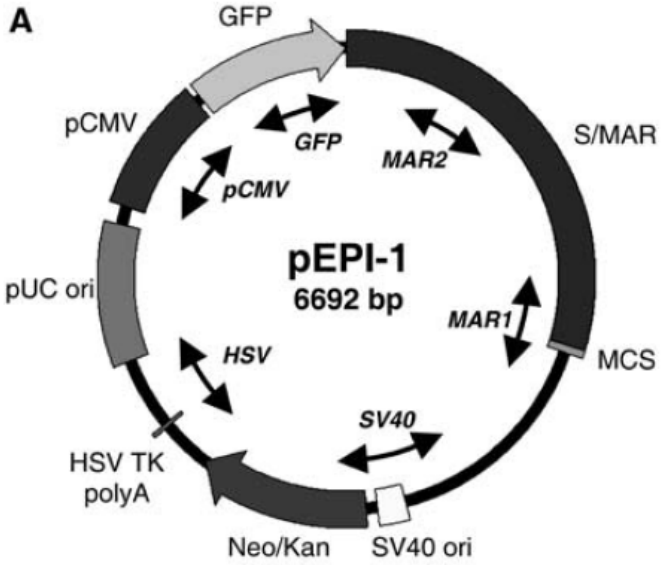
A) Genomic organization of the DHFR locus; double-head arrows indicate the regions amplified by PCR; B) ori mapping by PCR. Grey bars: shared DNA; black bar un-sheared DNA.

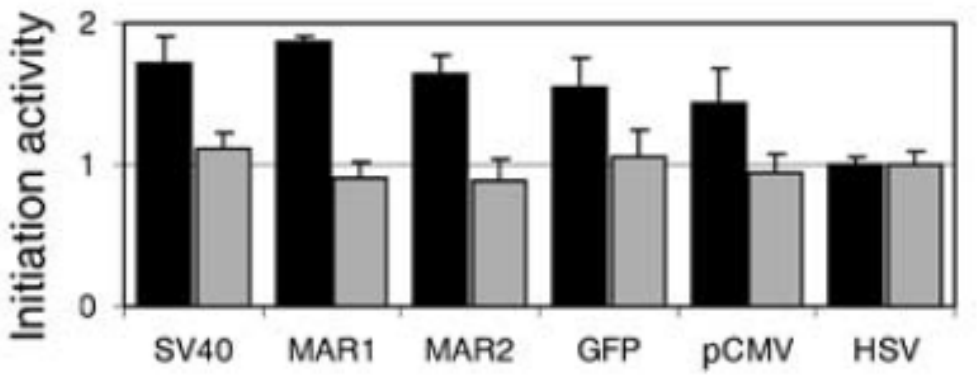
ori-β and ori-β' are the preferred sites for initiation of DNA replication (ori)

Ori mapping fails to reveal discrete points of replication.

The origin of replication is mapped by measuring the relative abundance of nascent DNA strands.

Ori mapping at pEPI plasmid





Nascent DNA strands are revealed by PCR. As a negative control, DNA is shared in order to destroy the nascent ssDNAs.

Grey bars: shared DNA; black bar un-sheared DNA

The pEPI plasmid does not exhibit significant difference between shared and un-sheared DNAs along the entire length of the molecule. This result suggests that **no preferential sites of DNA replication exists.**

How can you demonstrate that pEPI is an episome?

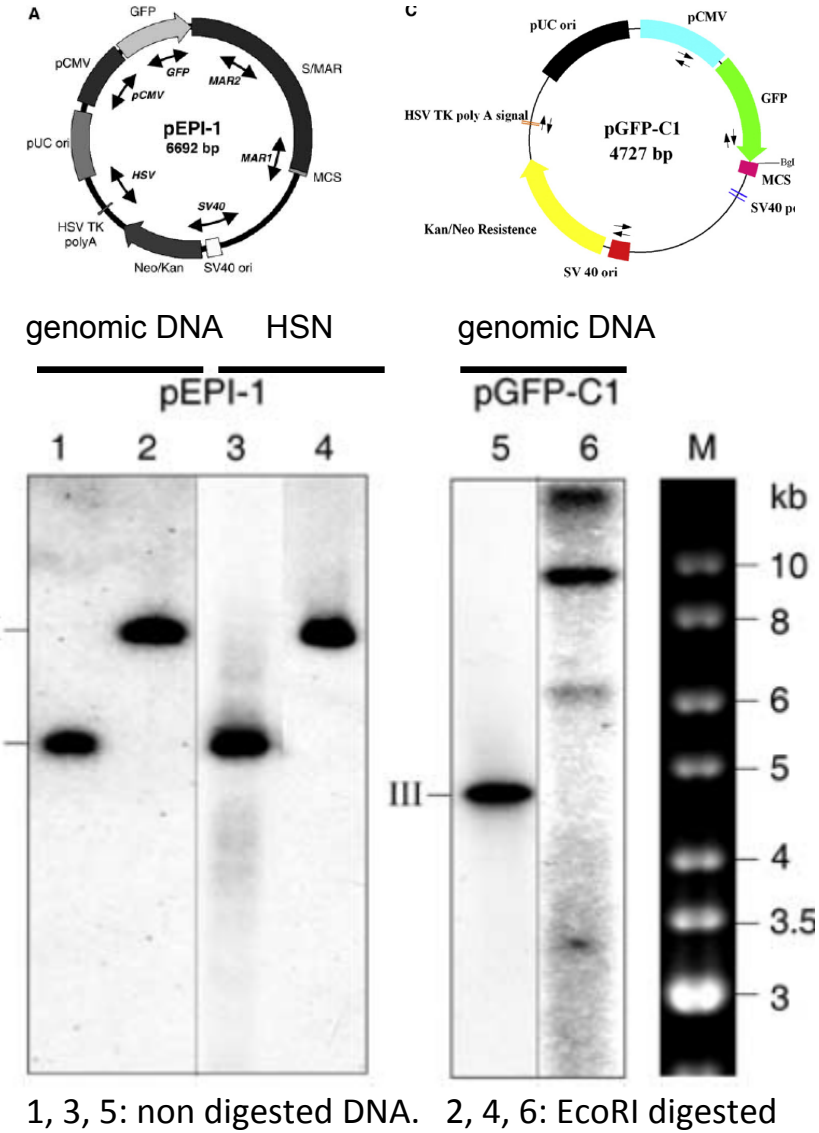
pEPI episome in mammalian cells
Southern Blots

DNA samples can be prepared by genomic DNA extraction or Hirt supernatant (HSN).

HSN allows isolation of low molecular weight DNAs (the non integrated plasmids). It is based on a controlled partial - lysis of the cells which allows diffusion of only small DNA molecules in the supernatants.

Restriction enzyme digestion with a single cut enzyme of a circular episome yields one band.

Restriction enzyme digestion with a single cut enzyme of an integrated episome yields multiple bands.

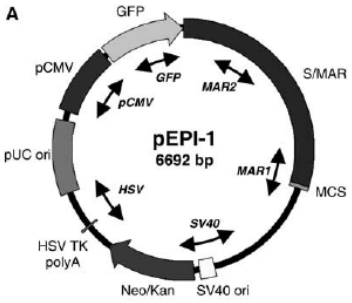


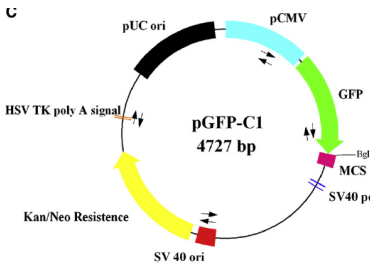
1, 3, 5: non digested DNA. 2, 4, 6: EcoRI digested

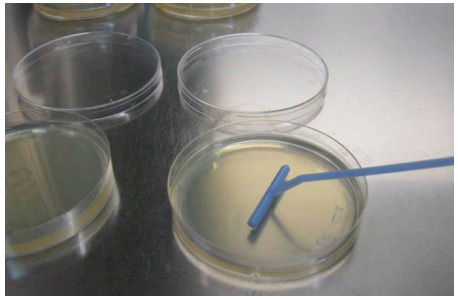
pEPI episome in mammalian cells
E. coli rescue

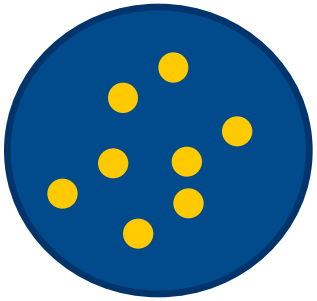
Hirt supernatant (SN)
E. coli rescue

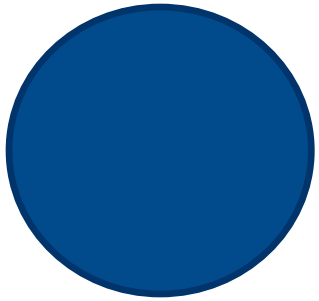
E. coli transformation
kanR selection











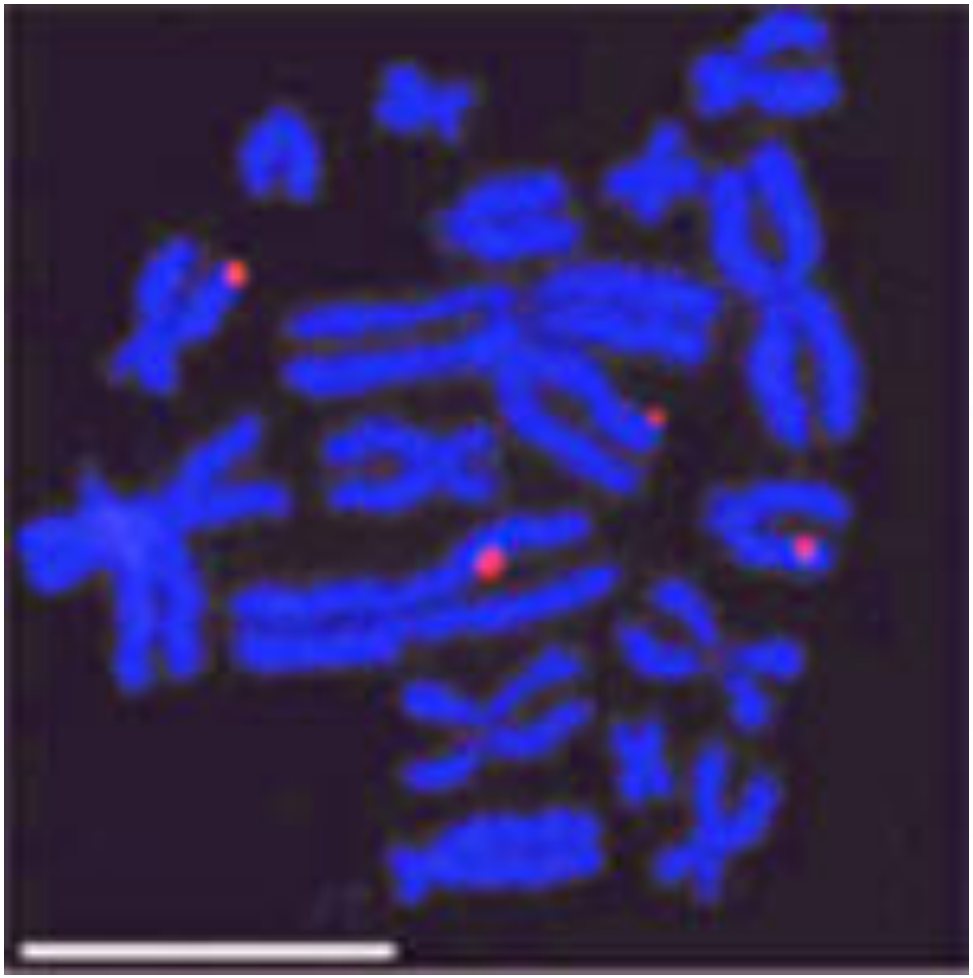
Plasmid preparation
Restriction analysis

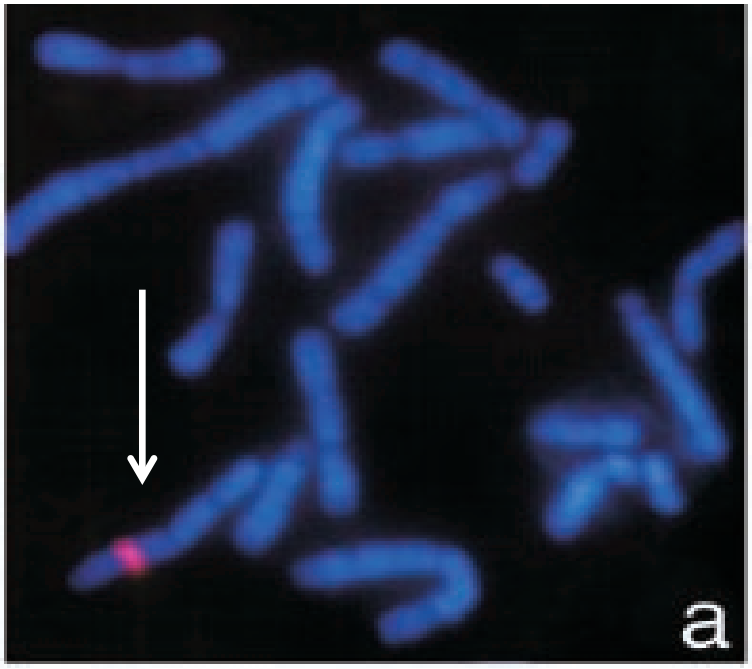
KanR colonies
Plasmid preparation
Restriction analysis

no colonies

pEPI episome in mammalian cells

FISH



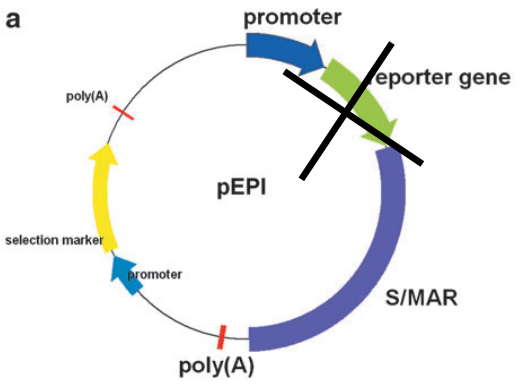


The next generation vectors based on S/MAR

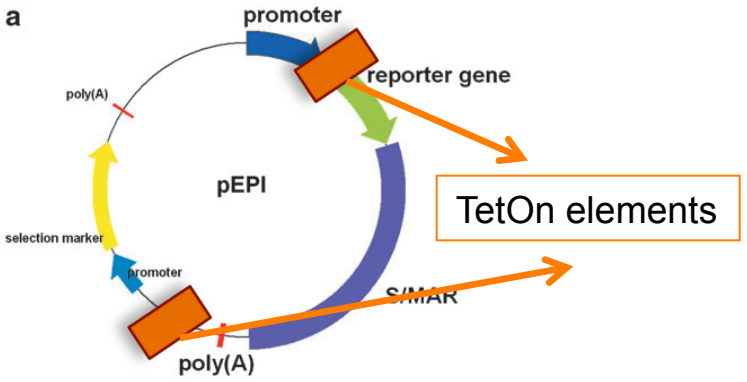
pEPI - TetON

pEPI episome strictly depends o transcription throughout the S/MAR

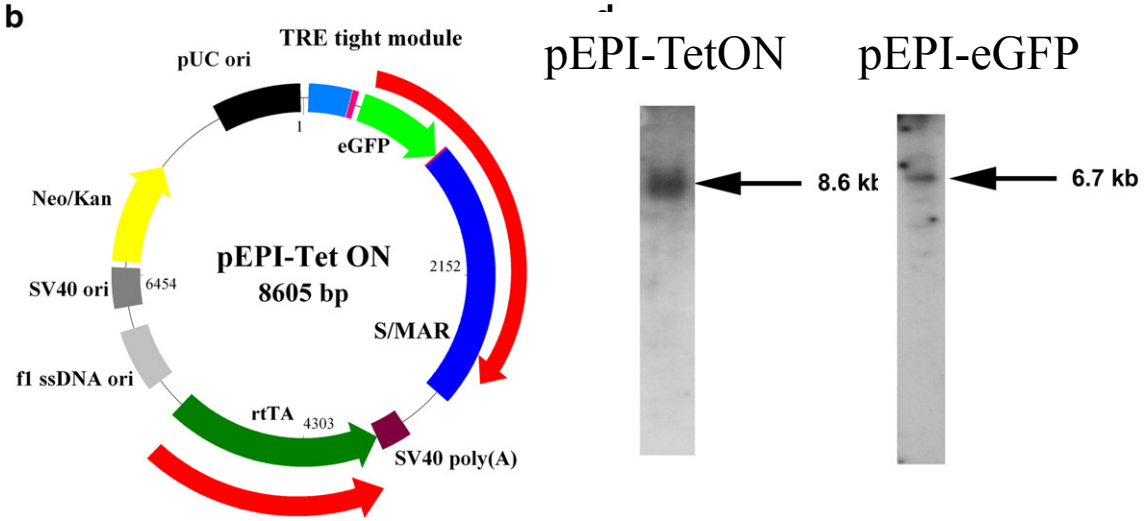
Whenever transcription of the transgene running into the S/MAR sequence was abrogated, that is, by deletion of the transgene, deletion of the promoter, or insertion of a termination signal between the transgene and S/MAR, the plasmids either integrated into the host genome or became lost from the cells.

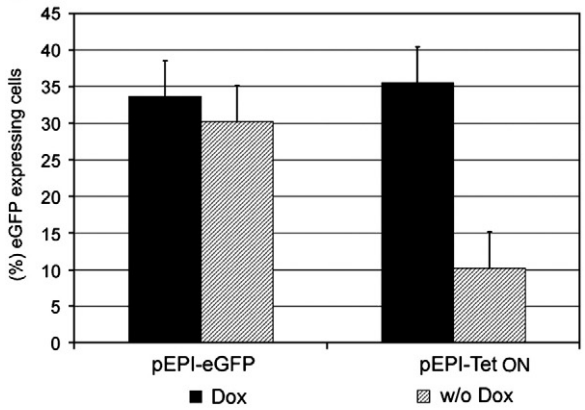


The transgene - S/MAR cassette was placed under the control of a tetracycline-responsive promoter (TetON). **Stably transfected cells were established in the presence of doxycycline**.
Afterwards, removing doxycycline resulted in a continuous loss of vector molecules from the cells. It was shown that this inducible vector system also functions *in vivo*.



pEPI - TetON: the episome is lost in medium w/o Doxy





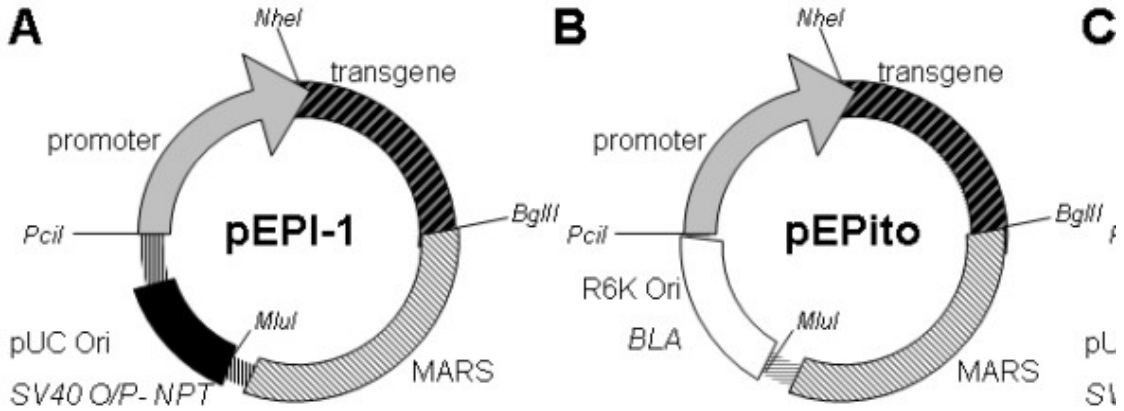
Flow cytometric analyses of pEPI–Tet ON transfected cells (72h post – transfection)

pEPI – TetON was constructed by inserting the TRE tight module upstream the eGFP gene. The reverse trans-activator cassette (rtTA) was inserted downstream of the S/MAR sequence with transcription running contrariwise to the transcription that is driven by the inducible promoter.
Transcripts from rtTA and eGFP are indicated as arrows (SV40, simian virus 40; ori, origin of replication; eGFP, enhanced green fluorescent protein; S/MAR, scaffold/matrix attachment region; Neo/Kan, neomycin/ kanamycin; TRE, tetracycline responsive element).

Southern analyses of DNA isolated from clones transfected either with pEPI–eGFP or pEPI–Tet ON. BglII digested DNA isolated from individual clones of cells transfected with pEPI–eGFP or pEPI–Tet ON demonstrating the episomal state of the vectors. The results of all Southern analyses were identical; only one example is shown.

The pEPito

pEPito is not only reduced in size but it also displays increased establishment in vitro, up to 6-fold over pEPI-1, as well as increased and stable transgene expression in vivo, up to 32 days after hydrodynamic injection into MF-1 mice.

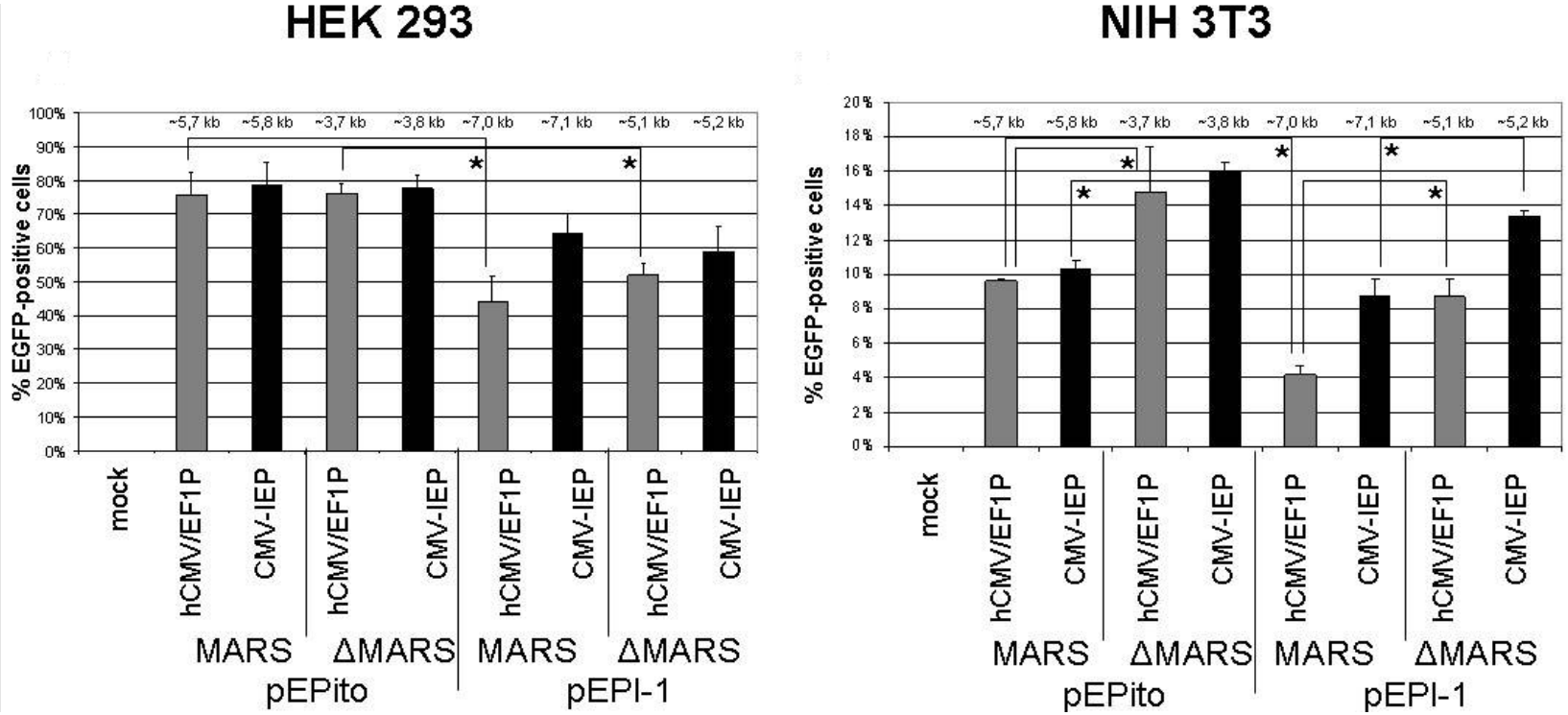


The CMV promoter of pEPI-eGFP was sonstituited with the enhancer element and the elongation factor-1 promoter (hCMV/EF1P).
The CpG content of the pEPI backbone was reduced by 60% of that in pEPI-1.
These modifications achieved significantly increased and prolonged transgene expression *in vitro* and *in vivo*.

E

# plasmid	backbone	promoter	transgene	size	CpGs
1 pEPI-1-[CMV-IEP]-[EGFP-IRES-BSD]	pEPI-1	[CMV-IEP]	[EGFP-IRES-BSD]	7127 bp	324
2 pEPI-1-[CMV-IEP]-[EGFP-IRES-BSD]-ΔMARS	pEPI-1-ΔMARS	[CMV-IEP]	[EGFP-IRES-BSD]	5166 bp	325
3 pEPI-1-[hCMV/EF1P]-[EGFP-IRES-BSD]	pEPI-1	[hCMV/EF1P]	[EGFP-IRES-BSD]	7033 bp	312
4 pEPI-1-[hCMV/EF1P]-[EGFP-IRES-BSD]-ΔMARS	pEPI-1-ΔMARS	[hCMV/EF1P]	[EGFP-IRES-BSD]	5072 bp	313
5 pEPito-[CMV-IEP]-[EGFP-IRES-BSD]	pEPito	[CMV-IEP]	[EGFP-IRES-BSD]	5776 bp	155
6 pEPito-[CMV-IEP]-[EGFP-IRES-BSD]-ΔMARS	pEPito-ΔMARS	[CMV-IEP]	[EGFP-IRES-BSD]	3815 bp	156
7 pEPito-[hCMV/EF1P]-[EGFP-IRES-BSD]	pEPito	[hCMV/EF1P]	[EGFP-IRES-BSD]	5680 bp	142
8 pEPito-[hCMV/EF1P]-[EGFP-IRES-BSD]-ΔMARS	pEPito-ΔMARS	[hCMV/EF1P]	[EGFP-IRES-BSD]	3719 bp	143

The pEPito

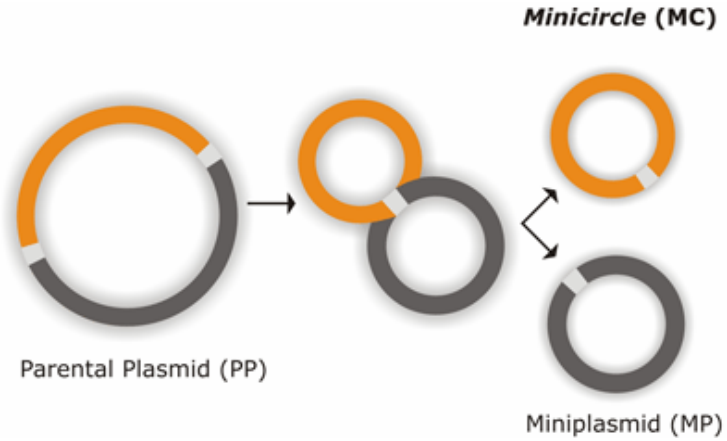


efficienza di trasfezione di vettori pEPI e pEPIto (con e senza sequenza s/MAR) in HEK293 e NIH3T3

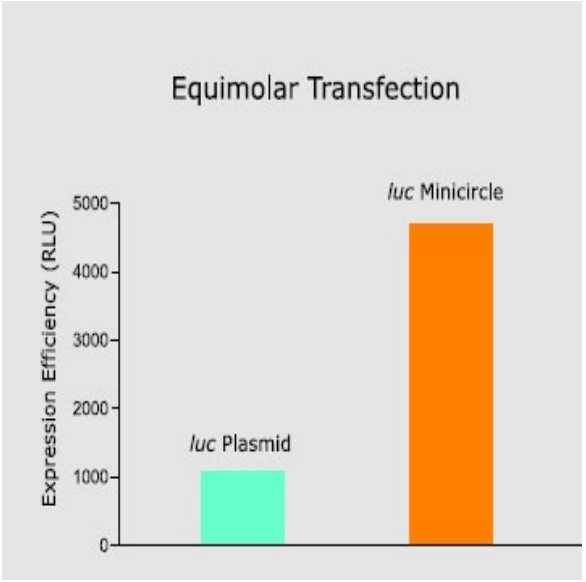
S/MAR based minicircles

Increased and prolonged transgene expression in vitro and in vivo has been reported for ‘‘minicircles,’’ which lack any residual elements for bacterial propagation and may therefore avoid immune responses of the host organism.

All methods to produce minicircles were based on the use of recombinases such as l integrase, Cre recombinase, Flp recombinase and fC31 recombinase.
They are also synthetically produced and commercially available



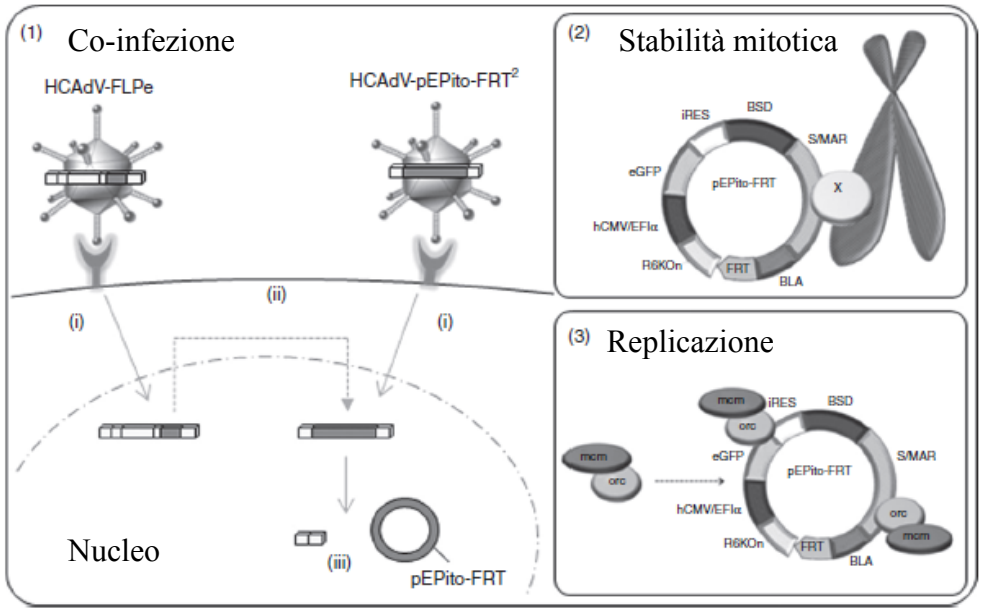
www.**plasmidfactory**.com

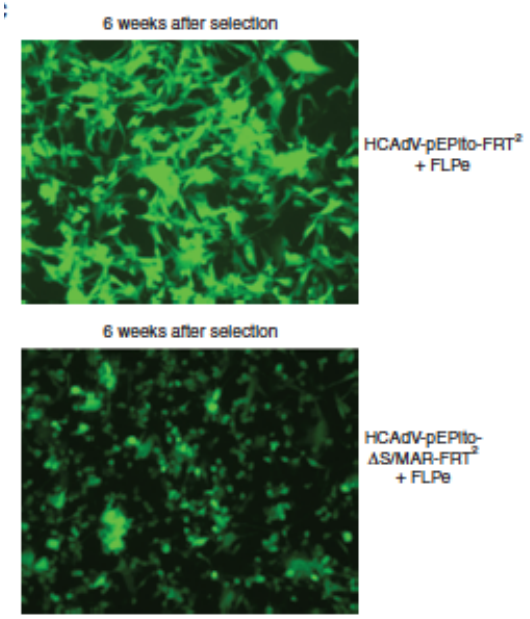


Similar to the original pEPI, S/MAR minicircles are mitotically stable but show improved transgene expression and establishment *in vitro* and *in vivo*.

A Novel Adenoviral Hybrid-vector

This hybrid-vector system synergizes high-capacity adenoviral vectors (HCAdV) for efficient delivery and the scaffold/matrix attachment region (S/MAR) – based pEPito plasmid replicon for episomal persistence





The efficacy of Adenoviral Hybrid-vector was demonstrated *in vitro* (U87 cells) and *in vivo* (liver of C57BI/6 immunocompetent mice) by PCR and plasmid rescue.

pEPI plasmids *in vivo*

Genetically modified pigs produced with a nonviral episomal vector (*Manzini 2006*)

Genetic modification of cells and animals is an invaluable tool for biotechnology and biomedicine. Currently, integrating vectors are used for this purpose. These vectors, however, may lead to insertional mutagenesis and variable transgene expression and can undergo silencing. Scaffold-matrix attachment region-based vectors are non-viral expression systems that replicate autonomously in mammalian cells, thereby making possible safe and reliable genetic modification of higher eukaryotic cells and organisms.

In this study, **genetically modified pig fetuses were produced with the scaffold-matrix attachment region-based vector pEPI, delivered to embryos by the sperm-mediated gene transfer** method. **The pEPI vector was detected in 12 of 18 fetuses** in the different tissues analysed and was shown to be retained as an episome. **The reporter gene encoded by the pEPI vector was expressed in 9 of 12 genetically modified fetuses**. In positive animals, all tissues analysed expressed the reporter gene; moreover in these tissues, the positive cells were on the average 79%.

The high percentage of EGFP-expressing cells and the absence of mosaicism have important implications for biotechnological and biomedical applications. These results are an important step forward in animal transgenesis and can provide the basis for the future development of germ-line gene therapy.

Genetically modified pigs produced with a nonviral episomal vector (*Manzini 2006*)

eGFP detection in transgenic fetuses

Eighteen fetuses were harvested from two sows on day 70 of pregnancy

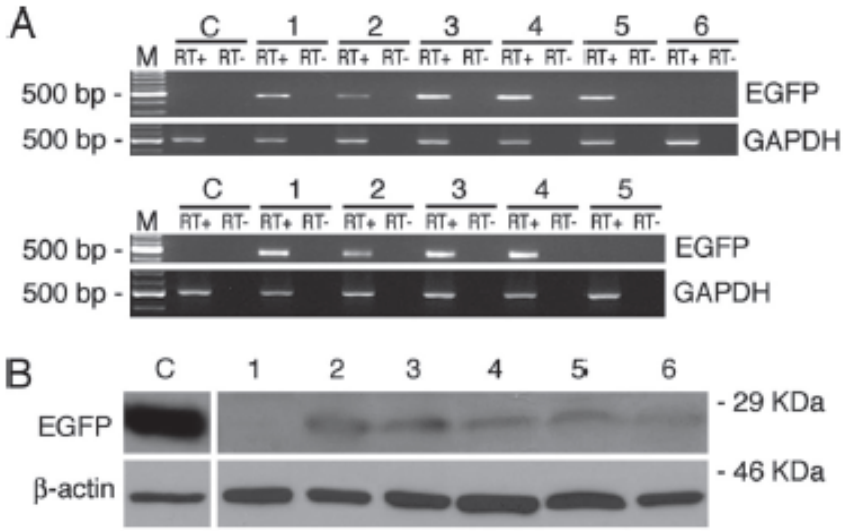
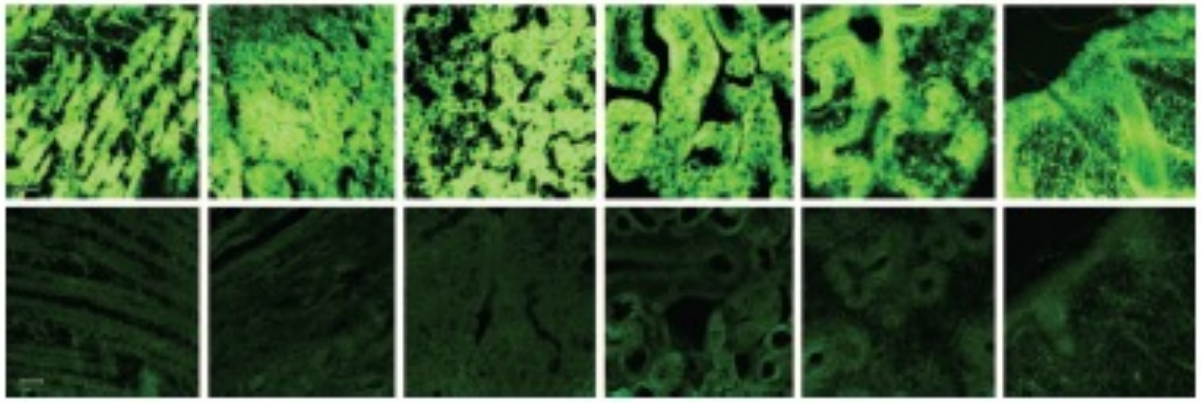


Table 1. Efficiency of SMGT method to produce genetically modified pig fetuses by using the nonviral episomal plasmid pEPI-EGFP

Fetus	Tissues analyzed, no.	DNA		RNA	Protein
		Total	Extrachromosomal		
M	1	–	–	–	–
C	1	–	–	–	–
1	3	+	+	+	+
2	5	+	+	+	+
3	4	+	+	+	+
4	3	–	–	–	–
5	5	–	–	–	–
6	5	+	+	+	+
7	3	–	–	–	–
8	4	+	+	+	+
9	4	+	+	+	+
10	4	+	+	+	+
11	4	+	+	+	+
12	3	+	+	+	+
13	2	+	+	–	–
14	2	+	+	–	–
15	3	+	+	–	–
16	2	–	–	–	–
17	2	–	–	–	–
18	2	–	–	–	–
Total, %	62	12/18 (67)	12/18 (67)	9/12 (75)	9/12 (75)

+ and – indicate the presence or absence of EGFP sequence or gene product. Total DNA extracted from nuclei prepared from different tissues was analyzed by PCR using three set of primers specific for EGFP, pCMV, and Neo/Kan sequences. Extrachromosomal DNA extracted by the Hirt method was also analyzed by PCR, Southern blotting, and plasmid rescue. RNA and proteins extracted from tissue biopsies were subjected to RT-PCR and Western blotting, respectively. Tissue sections were subjected to confocal microscopy. C, tissue samples from negative control fetus. M, tissue samples from the mother of one offspring, as negative control.

skeletal muscle heart liver kidney lung skin



Genetically modified pigs produced with a nonviral episomal vector (*Manzini 2006*)

Conclusions

- We concentrated our analyses on skeletal muscle, heart, liver, kidney and lung because these tissues are expected to be the main targets for therapeutic approaches. We have also studied transgene expression at the protein level in skin so as to have expression data on tissues derived from all three embryonic layers.

- pEPI-eGFP DNA was found in 43 tissues of 12 of the 18 fetuses generated, and its episomal status was demonstrated by PCR, Southern blot, and plasmid rescue analyses of Hirt extracts.

- The episomal state of the plasmid in the fetuses generated by SMGT shows that the presence of S/MAR DNA sequences, which ensures episomality of the plasmid in cultured cells, also prevents integration of the plasmid into the genome of sperm cells and subsequently in the cells of the individuals generated by that sperm.

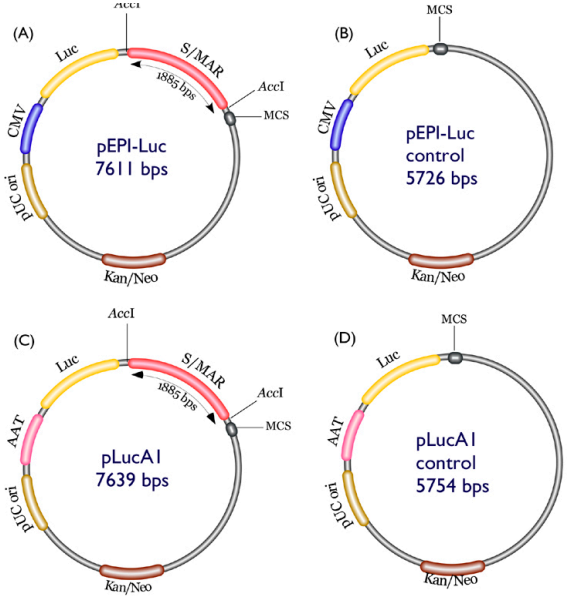
- In this study we did not investigate the germ-line transmission of pEPI vector, and at this stage we cannot rule out the possibility that this vector type will not be properly transmitted to the next generation.

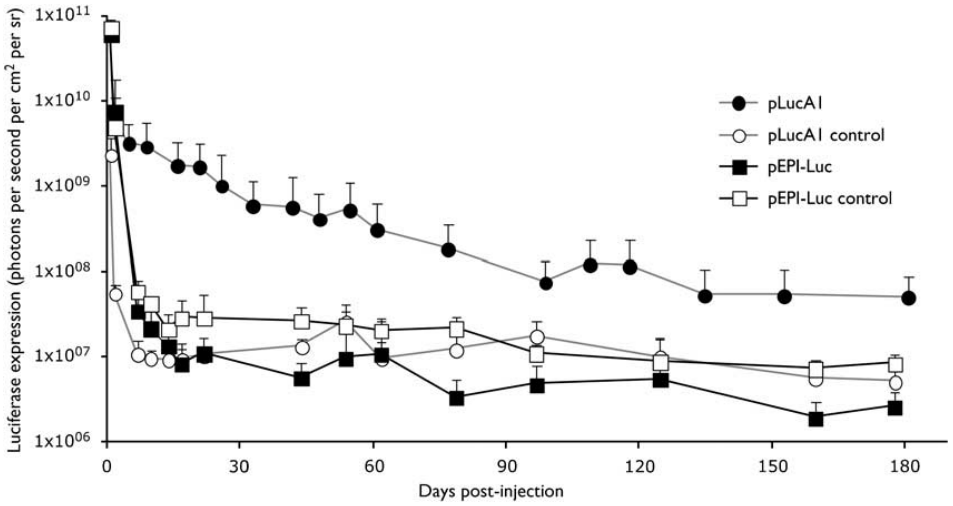
Non-viral S/MAR vectors replicate episomally *in vivo* when provided with a selective advantage *(Wong 2011)*

Previous work (Argyros 2008)

A prototype plasmid vector, containing a scaffold matrix attachment region (S/MAR) domain and the luciferase reporter gene, showed transgene expression for at least 6 months following a single administration to MF1 mice.

Following partial hepatectomy of the animals, however, no detectable vector replication was found.





Non-viral S/MAR vectors replicate episomally *in vivo* when provided with a selective advantage *(Wong 2011)*

in vivo selection strategy:

specific induction of cell death by the **Fas antigen**, a transmembrane liver protein that **induces apoptosis on engagement with** its physiological ligand**, FasL**.
This pathway can be activated in vivo by administration of an agonistic Fas antibody**, Jo2.**
Jo2 induces cell death
The **inhibition of Jo2-mediated cell death** can in turn be achieved **by overexpression of Bcl-2.**

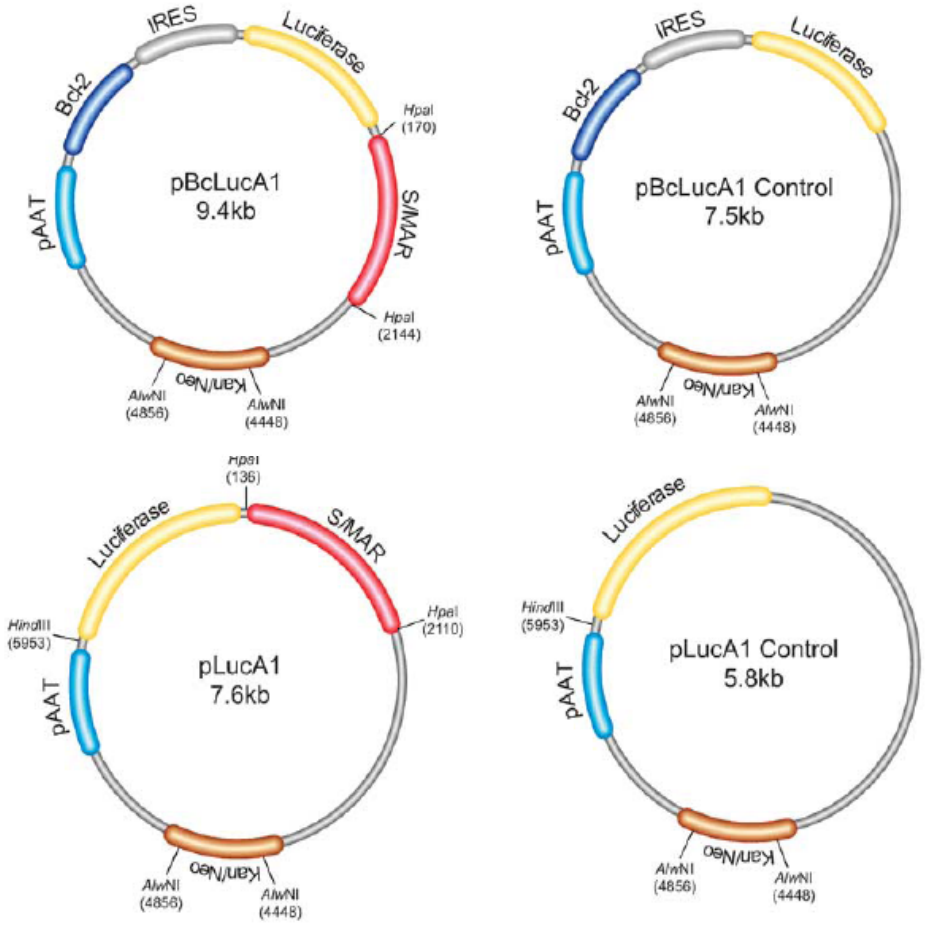
↓

Bcl-2 expression confers resistance against apoptosis-mediated by the Fas-activating antibody Jo2

Liver cells transfected with an S/MAR-Bcl2 plasmid have a survival advantage over non-transfected cells when animals are treated with Jo2

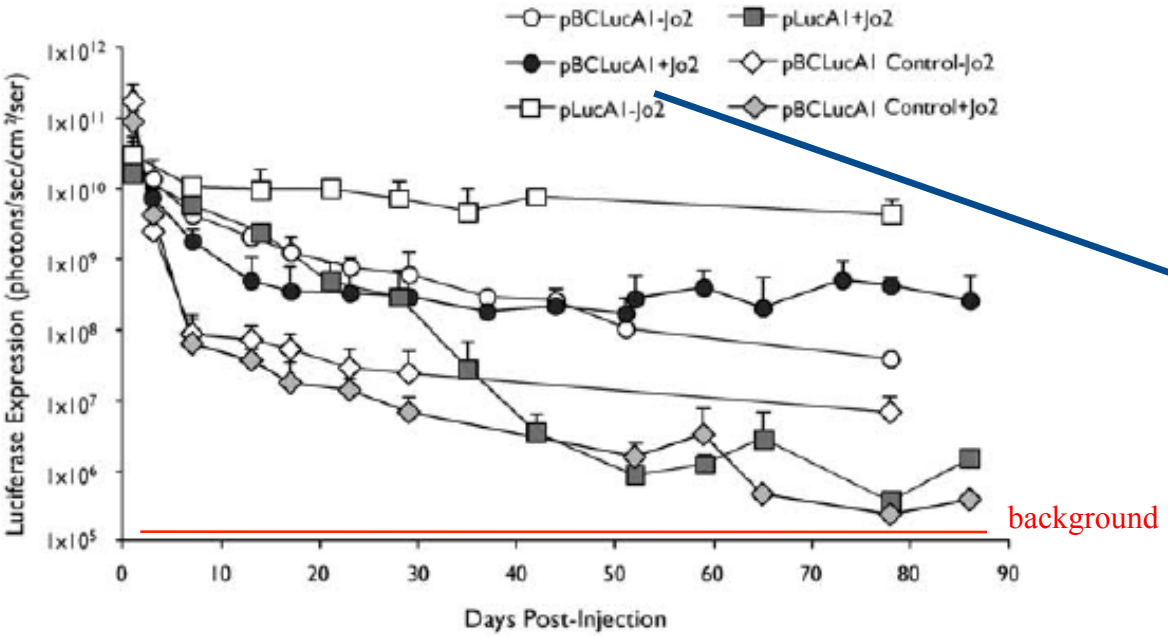
Non-viral S/MAR vectors replicate episomally *in vivo* when provided with a selective advantage *(Wong 2011)*

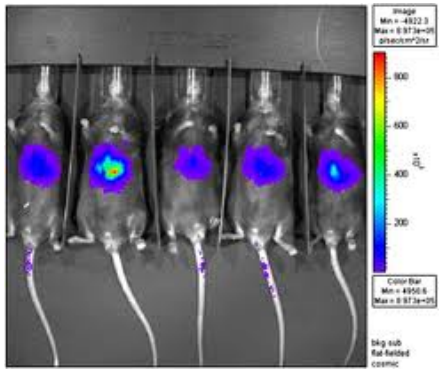
pBcLucA1 and control plasmids



Non-viral S/MAR vectors replicate episomally *in vivo* when provided with a selective advantage *(Wong 2011)*

in vivo luciferase detection





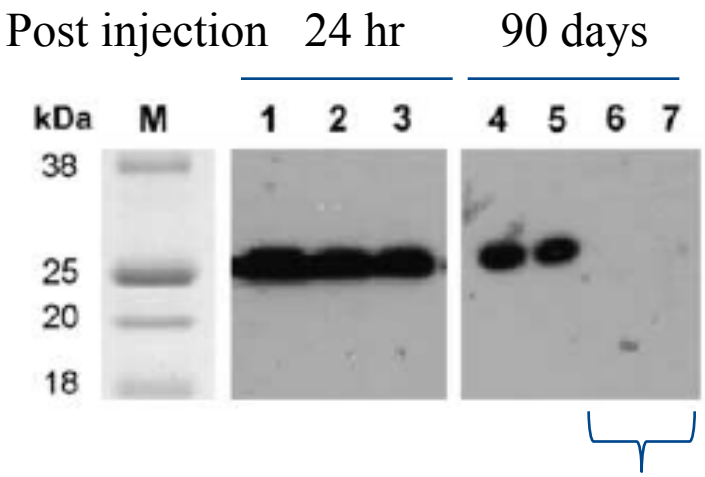
The figure illustrates the longitudinal study of the mice, treated with and without twice-weekly doses of 0.12 mg/kg Jo2 for up to 3 months, as quantified using Xenogen Living Image software.

Luciferase levels continue to fall in animals receiving Jo2, with the exception of the pBcLucA1+Jo2 group. The background level of light emission on non-treated animals is $3x10^5$ photons s1cm2 sr1. Mean ± s.e.m. (n¼3) for each time point is shown.

Non-viral S/MAR vectors replicate episomally *in vivo* when provided with a selective advantage *(Wong 2011)*

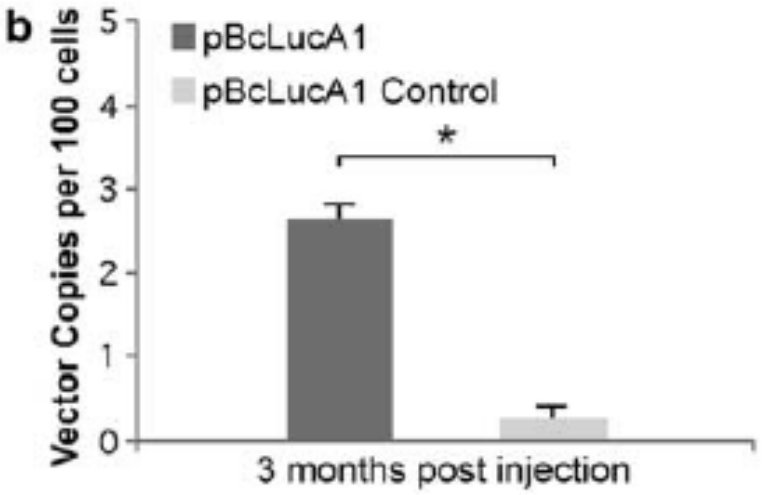
Sustained level of Bcl-2 expression from pBcLucA1 but not with the non-S/MAR control

Western analysis for Bcl2

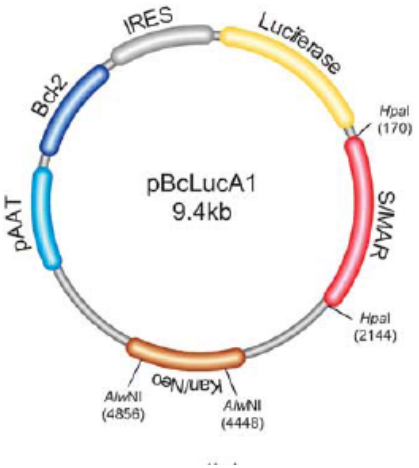


pBcLuc Control (D S/MAR)

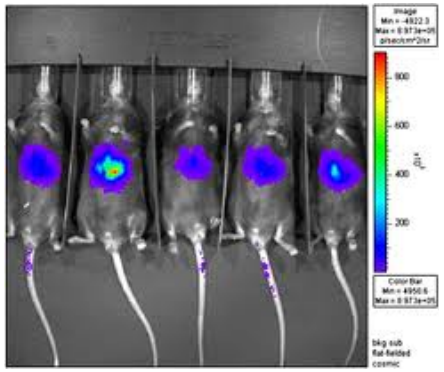
Copy number



Non-viral S/MAR vectors replicate episomally *in vivo* when provided with a selective advantage *(Wong 2011)*



conclusion



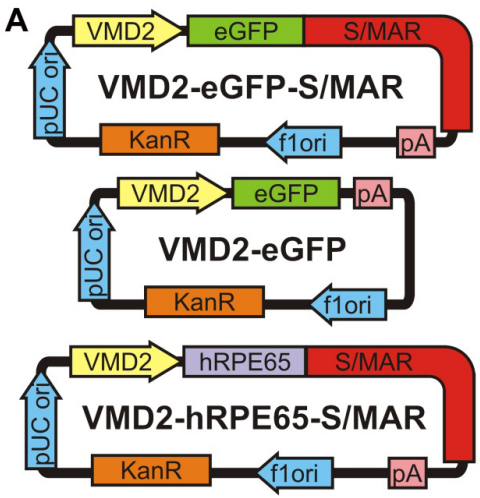
Following hydrodynamic delivery to the livers of mice and frequent Jo2 administrations (twice a week), Bcl-luciferase S/MAR plasmid is indeed capable of providing sustained luciferase reporter gene expression for over 3 months **demonstrating that the plasmid replicates as an episomal entity *in vivo***

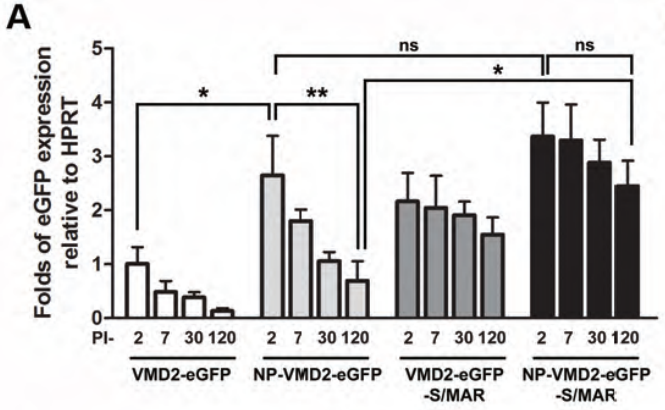
Episomal maintenance of S/MAR-containing non-viral vectors for RPE-based diseases (Koirala 2014)

Currently, AAV vectors are in phase I/IIa clinical trials for the treatment of Leber's Congenital Amaurosis (LCA), a rare inherited retinal degenerative disease characterised by severe loss of vision at birth.

DNA nanoparticles compacted with polyethylene glycol and polylysine and uncompacted plasmid DNA have significantly higher genetic capacity than AAV and they are safe when delivered to the eye.

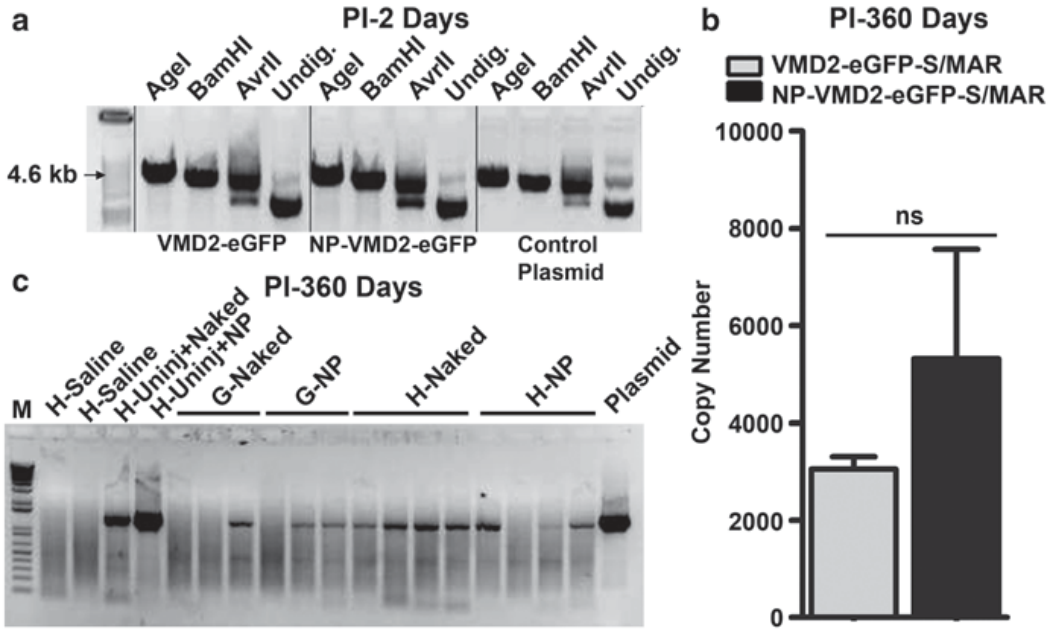
DNA nanoparticles and naked DNA carrying SMARs can efficiently transduce the RPE (Retinal Pigment Epithelium) and mediate long term gene expression and rescue in the rpe65-/- model without detectable toxicity.





VMD2: vitelliform macular dystrophy 2, RPE specific promoter

Episomal maintenance of S/MAR-containing non-viral vectors for RPE-based diseases (Koirala 2014)



At 2, 30 and 60 days, we detected a substantial number of colonies from each group, indicating the presence of intact vectors in the samples. However, at 360 days, we only observed a single colony on the plate for NP-VMD2-eGFP-S/MAR and none for naked VMD2-eGFP-S/MAR. At 360 days, the eGFP expression cassette is episomal, but amplification of expression cassette from genomic DNA preps indicates the possibility that some of the vectors may have integrated into the genome.

Episomal maintenance of S/MAR-containing non-viral vectors for RPE-based diseases (Koirala 2014)

conclusion

- Compacted DNA NPs and naked DNA carrying S/MARs can efficiently transduce the RPE and mediate long-term gene expression and rescue in the rpe65−/− model without detectable toxicity.

- The vector DNA continues to be detected in the nucleus up to PI-180 days (last timepoint tested) and that intact plasmid and expression cassette can be isolated up to PI-360 days.

Da consultare

Hagedorn C et al “Scaffold/Matrix Attached Region-Based Nonviral Episomal Vectors” Human Gene Therapy 2011, 22: 915-923