SYNOPSIS OF THE WORK

Haemophilia A is an X-linked blood clotting disorder that affects 1 in 5000 males worldwide. This severe disorder is a result of the deficiency of clotting factor VIII (FVIII), the protein that is expressed in hepatocytes and then secreted in blood. Haemophilia A has been classified as severe moderate and mild depending on FVIII activity in blood. Since, as little as 5% of normal plasma level of FVIII activity is sufficient to overcome the severity of the disease, haemophilia A has long been considered as an attractive target for gene therapy. But, the large size of FVIII cDNA is a major obstacle for gene therapy of the disease with normal retroviral and adeno-associated viral vectors. However, in the active form of FVIII, its largest domain, B-domain, is not present. Therefore B-domain-deleted FVIII cDNA is commonly used in most of the gene therapy approaches.

We have therefore developed an episomal EBV-BAC based vector for transfer of FVIII cDNA into human B-cells. Haematopoietic cells could be ideal targets for gene therapy of haemophilia A by virtue of their immediate access to the bloodstream. EBV is a human B-lymphotropic herpes virus that has been estimated to be asymptptomatically carried by more than 90% of adult worldwide human population. After infection, this large double stranded DNA virus (~172 kb) circularizes through its Terminal Repeat (TR) and exits as an extra chromosomal element, i.e. episome. For its episomal maintenance, replication by using latent origin, ori P requires a single viral trans-activator, Epstein Barr
Nuclear Antigen 1 (EBNA1). Therefore an EBV based vector can be engineered to carry large DNA insert. When EBV switches from latent mode to lytic mode, it uses another replication origin, the lytic origin, called ori lyt and with the help of lytic specific EBV proteins it generates a linear concatamer of EBV genome. Viral genome is then packaged into infectious viral particle after cleavage at Terminal Repeat (TR), the viral packaging signal.

The EBV-BAC vector developed for this study carries the Bacterial Artificial Chromosome (BAC) backbone, which enables cloning of large DNA insert (>150 kb) in \textit{E. coli}. For replication in human cells the vector includes the latent viral replication origin, \textit{ori P} and its only viral transactivator \textit{EBNA1}. Presence of the lytic replication origin, \textit{ori lyt} and, the viral packaging signal, \textit{TR} in the EBV-BAC allows production of infectious virions from a helper cell line, B95-8. A human full-length FVIII cDNA (7.2 kb) was used for initial cloning. Using overlapping PCR, a B-domain deleted FVIII cDNA (4.6 kb), more commonly used in gene therapy research, was also constructed. We then subcloned the B-domain deleted FVIII cDNA under the control of RSV 3' LTR in the EBV-BAC vector. Infectious recombinant EBV-BAC was then produced from a helper cell-line that can supplement all the lytic EBV proteins in trans. The infectious virions were subsequently used to infect various human B-lymphoblastoid cell-lines Raji, DG-75, BJAB and BL-41. Ectopic expression of FVIII from the infected cells was then studied. In the present study we have been able to demonstrate the efficacy of this EBV-BAC vector to transfer B-domain deleted as well as full length FVIII cDNA into human lymphoblastoid
cells. The ectopic expression of the therapeutic gene was studied by RT-PCR, Northern blot and immunostaining of human B-cell lines using anti-FVIII antibody CLB-CAg A. Functional activity of FVIII secreted in culture medium from the cells carrying the vector was confirmed by using a chromogenic assay, COATEST VIII: C/4. Low-level expression of FVIII in the order of 5–8 ng FVIII/C/ml were observed in the cells stably transduced with full-length FVIII, while cells with the B-domain-deleted version expressed 8–16 ng FVIII/C/ml. We have also modified our vector one step further by incorporating von Willebrand Factor (vWF) expression cassette in the same vector backbone carrying B domain deleted FVIII. In another vector we have introduced a fluorescent marker, DsRed2 instead of vWF. The co-expression of vWF has shown marked augmentation of functional activity of FVIII up to 18–30 ng/ml of culture medium. The expression of DsRed2 has enabled us to study the efficiency of infection using fluorescent microscope and fluorescent activated cell sorter (FACS). Transduction efficiencies were observed to be in the range of 20-35% in different B cell lines. We have already published a portion of the work in *Journal of Gene Medicine* (2004) 6, 760-768.

The Herpes Simplex Virus *thymidine kinase* (HSV-tk) gene, a well-known suicide gene was also incorporated in the vector. The product of HSV-tk has the potency to convert nontoxic compound ‘pro-drug”, acyclovir or gancyclovir into their toxic phosphorylated derivative. Therefore this suicide gene has been used in cancer gene therapy to selectively kill malignant cells. In the present study we have shown the efficacy of our EBV-BAC based vector
to transfer HSV-tk in different B lymphoblastoid cell lines rendering them
susceptible to gancyclovir at a concentration of 25 μM, a non-toxic dose under
standard condition. These results, not only suggests the potency of EBV-BAC
vector in anti cancer therapy, but also shows that selective elimination of vector
transduced cells is quite feasible. In other words, from ethical perspective the
vector is safe enough at least in vitro.

Life long persistence and expression of therapeutic gene is an essential
criteria for gene therapy of inherited disorders. Retroviral and lentiviral vectors,
the most commonly used vectors ensure this by integration into the host
chromosome. But, it might be disadvantageous as non-specific integration of
vector could lead to activation (or silencing) of some cellular genes forever. In
this regard, episomal vectors are safe enough as they don’t integrate with the
host chromosome. Among all the episomal vectors this novel EBV-BAC vector
has the unique capacity to carry very large insert (~140 kb) and the presence of
<tk gene, has opened the scope of elimination of all the vector carrying cells, if
required for safety reasons. Most of the gene therapy vectors are enable to
transfer the therapeutic gene in the target cells, but sustained expression of the
gene is still one of the major challenges of gene therapy. In the current study we
observed a moderate-term expression of FVIII for 9–12 weeks in the transduced
cells regardless of selection pressure. This reduction of FVIII level in culture
medium with time in absence of selection could be better explained by episomal
loss. In absence of selection, the number of DsRed2 expressing cells also
declined, but interestingly, if selection is reapplied, they started to predominate gradually.

Therefore, in the present study we demonstrate the potential of episomal EBV-based vector to transfer and express FVIII as well as vWF from human B-lymphoblastoid cells lines and expression of FVIII in those cells followed by its secretion was shown to be more efficient when coexpressed with vWF. This system could therefore be effective not only for haemophilia A and von Willebrand disorder but for other haematopoietic disorders such as thalassemia, haemophilia B, chronic granulomous disease (CGD) etc.