IDENTIFICATION OF NOVEL, NATURALLY CpG-FREE, HUMAN AND MURINE PROMOTERS FOR NON-VIRAL GENE THERAPY

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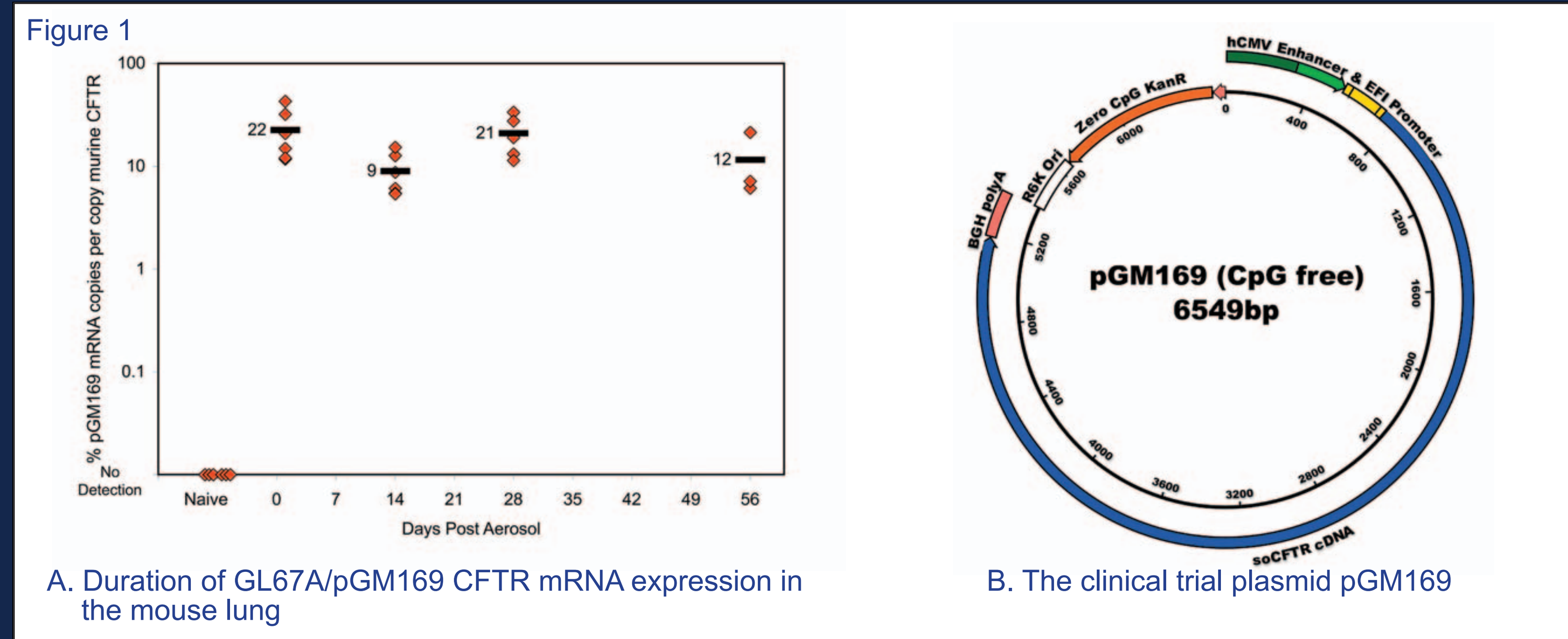
Overview

- The UK Cystic Fibrosis Gene Therapy Consortium are undertaking clinical trials of a gene therapy for Cystic Fibrosis (CF) in 2008 and 2009
- Studies are planned for aerosol delivery of single and multiple doses of Genzyme Lipid 67 (GL67A) complexed with a CpG-free[1,2] plasmid DNA vector
- The plasmid for these clinical studies was selected using the following screening process:-
  1. Duration and levels of expression in the mouse lung
  2. Inflammatory response following delivery of GL67A/pDNA to the mouse lung
- These studies indicated that CpG-free plasmids exhibited the lowest levels of inflammatory cytokines in the mouse lung[3] (Hyde et al., 2008. Nature Biotechnology, 25, 549-551)
- Combinations of two CpG-free enhancers and two different CpG-free promoters (Table 1) were assessed for the duration of expression they could provide in-vivo
- Table 1. Existing CpG-free enhancers and CpG-free promoters

Enhancer	Abbr	Promoter	Abbr
Murine Cytomegalovirus immediate early enhancer	mC	CpG-depleted human elongation 1 alpha promoter (InvivoGen)	EFI
Human Cytomegalovirus immediate early enhancer	hC	CpG-depleted human CMV promoter (Genzyme)	GZB

- Plasmids containing the human CMV enhancer and EFI promoter (hCEFI) expressed luciferase or CFTR mRNA for at least 56 days following aerosol delivery of GL67A/pDNA to the mouse lung
- Based on these results, the clinical trial plasmid pGM169 was selected (Figure 1)



A. Duration of GL67A/pGM169 CFTR mRNA expression in the mouse lung

B. The clinical trial plasmid pGM169

Limited choice of CpG-free promoters

- Persistent expression is possible with a CpG-free promoter
- There is a shortage of defined CpG-free promoters for other applications and other organs
- More CpG-free promoters need to be identified if CpG-free pDNA technology is to develop

1. Utilizing CpG-free plasmid technology developed by Cayla-InvivoGen (Toulouse, France)
2. pGM169 manufactured by VGX Pharmaceuticals (Houston, TX, USA)
3. Hyde SC, Pringle IA, Abdullah S, Lawton AE, et al (2008). Nature Biotechnology, 26, 549-551
4. Schmid et al (2006). Nucleic Acids Research, 34, D82-85

Aims of this study

1. Identify novel CpG-free human or mouse promoters in Genbank
2. Demonstrate that these promoters can function in a gene therapy model

Identification of novel CpG-free promoters

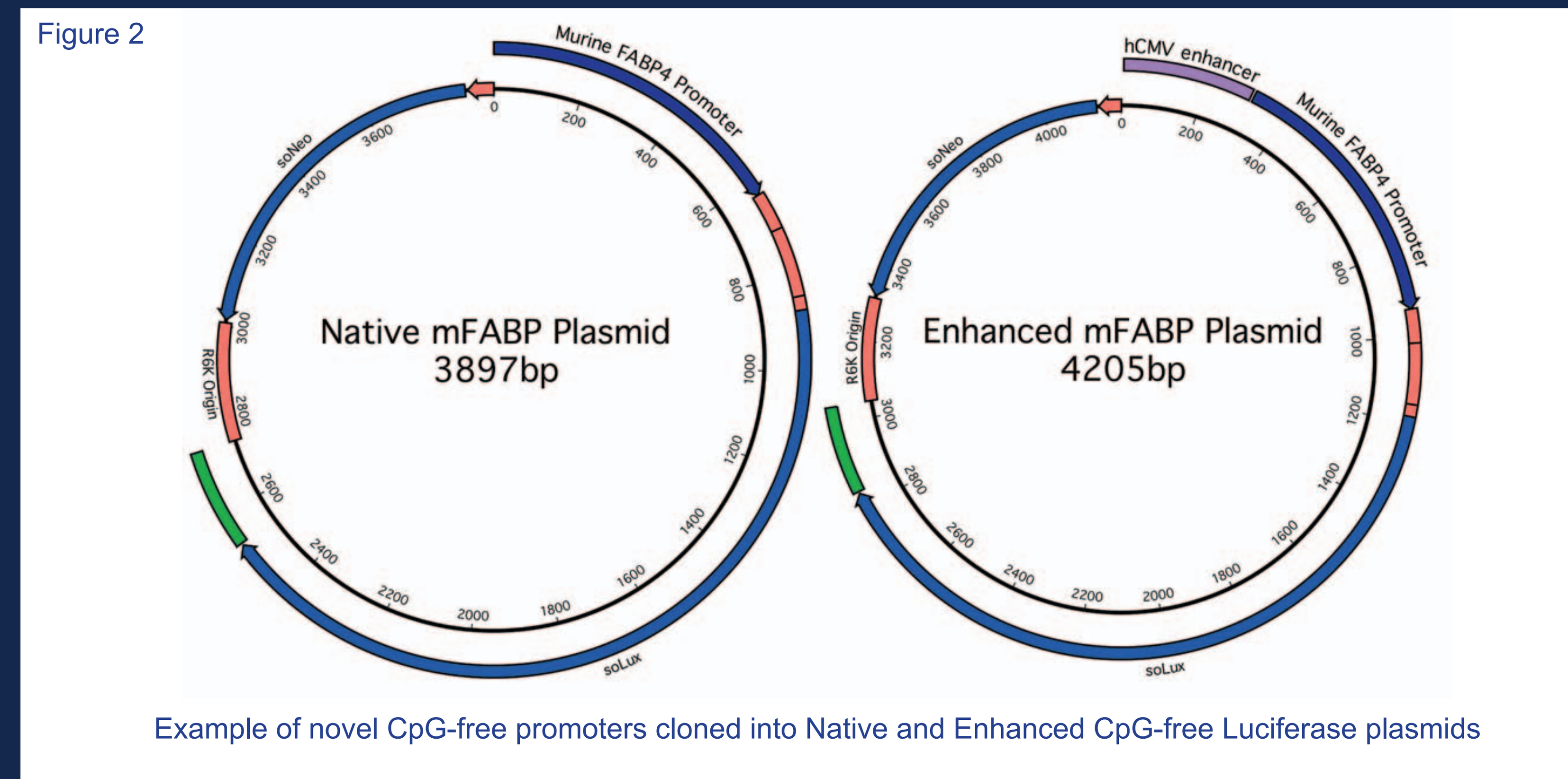
1. The Eukaryotic Promoter Database[4] (http://www.epd.isb-sib.ch) was used to identify a basic list of human and mouse promoters that did not contain CpGs in the region around the transcription start site (-49 to +10)
2. This screen yielded 183 potential human promoters and 68 potential mouse promoters
3. Extended promoter sequences (-1000 to +200) were downloaded from Genbank
4. Sequences screened for presence of CpGs and any promoters with >500 bp free of CpGs selected for cloning (Table 2)

Table 2. Six potential novel CpG-free promoters identified from screening

Species	Gene	Abbr	-5' to +3' CpG-free
Human	Thyroid stimulating hormone-ß	hTSHB	-750 to +350
Human	Apolipoprotein A	hAPO2A	-513 to +26
Human	Carboxypeptidase B1	hCBOX	-630 to +16
Human	Regenerating islet-derived 1ß	hREG1B	-625 to +70
Human	Tryptophan 2,3 dioxygenase	hTDOX	-670 to +36
Mouse	Fatty acid binding protein 4	mFABP	-623 to +52

Cloning novel promoters into CpG-free luciferase expressing backbones

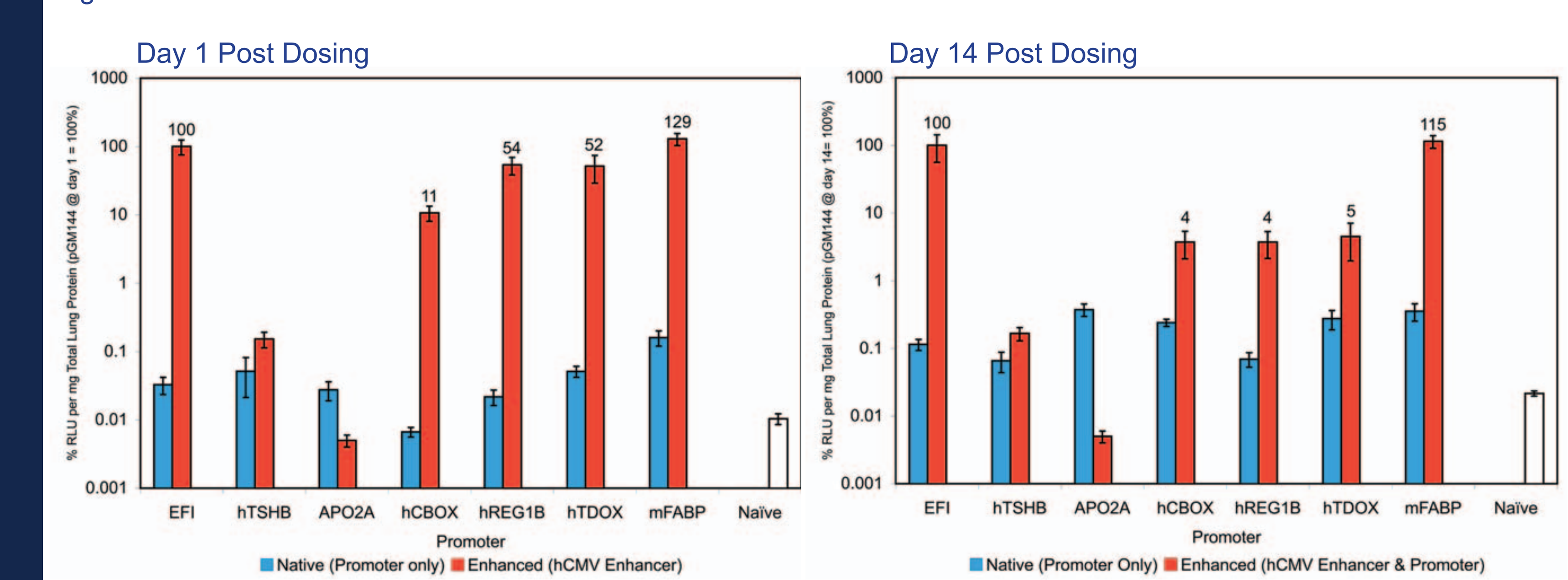
1. Genomic BAC or PAC clones containing the promoter sequences (Table 2) purchased (Invitrogen)
2. BAC/PAC DNA purified by Qiagen Large Construct Purification Kit
3. Promoter sequences amplified by PCR and cloned into pTOPO vectors (Invitrogen)
4. Cloned promoters sequenced to confirm Genbank sequence data and CpG-free status
5. Promoters cloned into CpG-free plasmid backbones containing the luciferase gene and no enhancer (Native plasmids)
6. Promoters cloned into CpG-free plasmid backbones containing the luciferase gene and the human CMV enhancer (Enhanced plasmids)



Example of novel CpG-free promoters cloned into Native and Enhanced CpG-free Luciferase plasmids

In vivo testing of novel CpG-free promoters in mouse lung gene transfer model

1. Native and enhanced plasmids purified by Qiagen Endotoxin Free Mega kit
2. Plasmids complexed with GL67A (80 µg/100 µl)
3. Female BALB/c mice (aged 6-8 weeks) anaesthetised with methoxyflurane
4. 100 µl GL67A/pDNA administered intranasally (n=12 per plasmid)
5. Mouse lungs harvested for Luciferase activity at day 1 and day 14 post dosing
6. Mice dosed with positive control clinical plasmid (hCEFI) and native version without enhancer as controls (EFI)



CpG-free promoters can drive robust expression in the mouse lung

- At d1 and d14 post dosing the native plasmids resulted in background levels of activity
- At d1, enhanced hREG1B, hTDOX & mFABP had similar levels of expression to hCEFI (Mann Whitney U, P>0.05)
- At d14, there was still significant expression from enhanced hCBOX, hREG1B and hTDOX
- Enhanced mFABP still matched the activity from hCEFI at day 14 (P>0.05)
- Very few promoters drive significant levels of expression at d14 in the mouse lung
- Further studies are planned to assess the activity of these novel promoters in other mouse organs

CONCLUSIONS

- CpG-free promoters exist in the human and mouse genomes
- These can be obtained and cloned into CpG-free plasmids
- Enhancer required to drive expression in murine lung
- Three of the enhanced versions had equal activity to hCEFI at day 1
- Enhanced mFABP had equal levels of expression to hCEFI at day 14
- Novel CpG-free promoters available for testing in other models