Towards a dermal papilla specific expression system in mice

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Master's Thesis, Second Term 2013, 30 credits
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Abstract

Stem cells hold great promise for regenerative medicine. In order to realize the potential of stem cell-based therapies, it is highly required to understand the basic mechanisms of stem cell regulation and transform such knowledge into medical applications. However, understanding stem cell regulations is still challenging due to technical difficulties in studying stem cell biology. The hair follicle is a continuously regenerating mini-organ, in which keratinocyte and melanocyte stem cells cooperate to achieve periodical cycles of pigmented hair formation. The mouse hair follicle provides an ideal model by which to investigate molecular basis of stem cell regulation as defective molecular regulation results in abnormal hair formation, a phenotype easily recognized by appearance. It has been widely appreciated that the regulation of these stem cells is mediated through the epithelial-mesenchymal interactions where a cluster of mesenchymal cells, known as the dermal papilla, plays a key role. Here we aim to establish a system manipulating genes in dermal papilla to identify genes involved in the stem cell regulation. To this end, the purpose of this study is to identify a promoter element permitting stable and specific dermal papilla transgene expression in mice. In this report, by integrating BAC transgenesis and transposon-meditated gene delivery systems, we establish a simple pipeline to identify a gene expression element enabling dermal papilla-specific genetic modifications.

1. Introduction

The field of stem cell biology is of considerable interest to modern medicine. Stem cells have the potential to revolutionize the field as a whole by opening up avenues in regenerative medicine allowing the repair of tissue injuries beyond the capabilities of the body's spontaneous regeneration. However, there is need for a better understanding of the underlying mechanisms regulating differentiation and proliferation in order to develop safe and efficient therapies.

1.1 The hair follicle as a model to study the molecular basis of hair regeneration

During mouse embryogenesis epithelial cells invaginate into the skin to form the hair follice[1]. As the invagination begins, mesenchymal cells located below the epithelial cells form a specialized cell cluster, known as a dermal condensate. The condensate eventually becomes enveloped by the forming follicular matrix and the condensate cells differentiate into the dermal papilla[1]. Once specified, these dermal papilla cells play a major role in controlling hair follicle morphogenesis as well as hair cycling by secreting various signaling factors[3]. As the hair follicle matures, two discrete populations of stem cells, which are responsible for producing follicular keratinocytes and melanocytes in subsequent hair cycles, emerge in the bulge[2], a compartment in the upper part of the outer root sheet. Keratinocyte stem cells located in the bulge migrate to the follicular center inside the matrix and form the hair shaft[1, 2]. Hair pigmentation is supplied by a cluster of melanocyte stem cells located above the dermal papilla[2].

Once the hair follicle morphogenesis is completed, hair regenerative cycling is initiated[3]. The hair cycle is the periodical formation of hair in the hair follicle whereby old hairs are shed and replaced by younger ones. After the formation of the first hair during the follicle morphogenesis, the hair cycle starts from the regression phase, a process called catagen, whereby the lower parts of the follicular matrix undergo apoptosis resulting in shedding of the old hair. Subsequently, hair follicles enter the resting phase, a stage called telogen, where they are in a quiescent stage. At the end of the telogen phase the hair follicle re-enters the growing phase, called anagen. During anagen, various growth factors are secreted form the dermal papilla to stimulate proliferation and differentiation of keratinocytes and melanocyte stem cells, resulting in formation of new pigmented hairs. After this anagen phase is complete another catagen will follow and the hair coat will be continuously renewed[3].

Integration of a functional genomics approach and subsequent phenotypical assessment of hair formation and regeneration provides a way to identify physiologically relevant genes involved in regulation of the hair follicle stem cells. Defects in coat formation, regeneration or pigmentation are all phenotypes which are readily assessed simply by inspecting the hair coat making the hair follicle a potentially very powerful tool for rapid functional screening of multiple genes affecting stem cell regulation.

1.2 The dermal papilla plays a central role in coordinating stem cell activation and hair cycling.

The dermal papilla cells in the mouse hair follicle are known to directly influence the morphology and formation the hair coat. It has been shown that dermal papilla cells are required in the follicle in order to initiate hair formation[4] and that the number of cells present directly influences the size and shape of the hair. The central role of the dermal papilla cells in hair follicle regulation has been recently exemplified in the reports using the *Corin* expression system[5], a system which allows a high degree of transgene expression in the dermal papilla, that ablation of β-catenin expression in the dermal papilla results in the loss of hair regenerative functions in mice[6]. Dermal papilla β-catenin has also been shown to effect coat pigmentation and its absence leads to abnormal pigmentation patterns[7]. Whereas the *Corin* promoter provides a valuable tool to explore molecular basis of dermal papilla regulation using a transgenesis approach, it is known that its promoter activity is not detectable during early stage of dermal papilla development. Therefore, it has been highly anticipated to identify an alternative promoter element that allows the expression of transgene in all stages of dermal papilla development.

Given the crucial involvement of the dermal papilla in the generation and pigmentation of the mouse hair coat it is an attractive target for genetic manipulation due to changes having a negative impact on the keratinocyte or melanocyte stem cell regulatory pathways would also give rise to an easily observable phenotype in the formation of abnormal hair coating and/or pigmentation. This would provide an advantageous model allowing identification of important molecules involved in keratinocyte and/or melanocyte stem cell regulation simply through observing the hair coat abnormalities arising in genetically modified animals.

1.3 BAC transgenesis as a tool to establish dermal papilla specific transgene expression

In order to use the dermal papilla for stem cell research it is necessary to establish a stable dermal papilla specific expression system. This would serve to prevent interfering expression of transgene constructs in tissues surrounding the hair follicle or elsewhere in the mouse body as is observed using the *Corin*[5] system discussed above. Such an expression system would require a dermal papilla specific promoter but to this date reports on such promoters have been limited. In an attempt to identify a suitable candidate for such a specific promoter, a microarray analysis had been previously conducted in our lab (data not shown). One such candidate that we have identified is Prokr2, whose expression was confirmed to be confined to the dermal papilla throughout development. Therefore the Prokr2 gene represents a subject of further study to determine the effectiveness of this regulatory element in driving dermal papilla specific expression of transgene constructs.

BAC transgenesis has previously been reported as an efficient tool to generate transgenic cell lines[8]. The approach utilizes a BAC construct carrying the gene of interest as well as ~100k base pairs of the upstream and downstream elements. BAC transgenesis carries several advantages over traditional plasmid transgenesis as it contains many of the regulatory elements present in the proximity of the gene of interest[8]. This assists in avoiding transcriptional silencing of the transgenic construct as well as allowing reporter gene expression which more closely resembles the normal expression patterns. Although BAC engineering can be a labor intensive process due to the size of the BAC making its purification and handling difficult, there are tools such as the RedET recombination system available to modify the BAC as desired.

1.4 Recombineering as a tool to sequence specifically modify BAC DNA.

To enable easy BAC modification the RedET recombineering system has been recently established. RedET recombination or recombineering allows insertion of fragments up to several thousand base pairs in length into a target site[8]. The recombination is based on homologous recombination and carried out using recombinases derived from phage lambda. These recombinases recognize homologous sequences flanking the insert and the corresponding sequences at the target site. They then catalyze a recombination reaction between the two and in doing so site specifically integrates the insert into the BAC vector. This allows for insertion in a sequence specific manner at virtually any location on a BAC simply through equipping the desired insert with flanking DNA sequences.

1.5 Transposon-gene delivery of Bacterial artificial chromosomes

The sheer size of a bacterial artificial chromosome makes its intact integration into the chromosome almost impossible without a system mediating the integration. Transposable elements have previously been shown to allow the integration of very large DNA constructs into the human chromosome using PiggyBac (PB) or Sleeping Beauty (SB) transposon mediated insertion[8]. The process consists of introducing two transposon Inverted terminal repeat (ITR) sequences in the BAC backbone through the recombineering process outlined above. ITRs are transposase recognition sequences and once recognized the transposase cleaves the DNA at the outside end of the sequence and proceeds to integrate the DNA contained in between them at a new site. This allows integration of very large ITR carrying constructs inside cells expressing a compatible transposase which is easily accomplished through cotransfection or coelectroporation of an appropriate construct. The transposase targets the ITR sequences of the insert, cleaves it and then mediate its integration into the chromosome.

1.6 Aim

This paper aims at identifying a specific promoter element permitting stable and dermal papilla specific transgene expression in mice. To this end the paper reports the generation of a BAC vector, using RedET recombination, containing a functional GFP-reporter gene in direct succession of the start codon of the second exon of the *Prokr2* gene. The vector has been harboring transposable elements consisting of the SB transposase Inverted Terminal Repeat (ITR) sequences integrated into the backbone of the vector. The paper also demonstrates evidence that these elements are functional by inserting the BAC vector into Human Embryonic Kidney (HEK) 293 cells lines as well as present data comparing insertion efficiencies between the PB and SB transposases.

2. Materials and methods

2.1 Cloning and ligation

All cloning was conducted by digesting vectors and insert with the corresponding enzymes for 1h at optimum temperature followed by gel purification of desired fragments. All restriction enzymes used were supplied by New England biolabs. Gel purification was carried out using Fastgene gel purification kits. Ligation was carried out for 20 minutes at room temperature using Mighty Mix DNA ligation kit (Takara Bio) ligation mixtures. Transformation of TOP10 *Escherichia coli* cells was performed by thawing vials of competent *Escherichia coli* TOP10 (Invitrogen) on ice followed by the addition of 1ul of DNA solutions. The mixture was incubated on ice for 10 minutes followed by incubation at 42 degrees. After 45 seconds 1ml of SOC media was added to

the vial. Cells were selected on LB agar plates containing the appropriate antibiotics until colonies were large enough to allow harvesting. The insertions were verified by colony PCR. Purification of plasmids was carried out using either plasmid miniprep kits (Fastgene) or plasmid midi prep kits (QIAGEN) using high copy number protocols.

2.2 Construction of vector *pSL-Prokr5-EGFP-Pgk-neo-Prokr3*

DNA fragment containing *eGFP-BGHpA* was amplified by PCR from a *pROSA26-mT/mG* vector as a template using eGFP ApaI and eGFP SalI primers. The resulting PCR fragment was digested with ApaI and SalI and subcloned into *pBS-FRT-pgk-neo-FRT* vector. The vector backbone was later changed to that of *pSL-1180* vector. Genomic fragments containing ~500bp of sequence homologous to the *Prokr2* gene were amplified by PCR using *Prokr2* SalI and *Prokr2* ApaI primers as well as Prokr2 SacII and *Prokr2* HindIII. The PCR products were digested with their corresponding enzymes and the DNA fragments were subcloned into *pSL-eGFP-BGHpA-FRT-PGK-neo-FRT* vector resulting in vector *pSL-Prokr5-EGFP-Pgk-neo-Prokr3*. Detailed vector maps and primer sequences are presented in the supplementary material.

2.3 Verification of eGFP fluorescence

Prokr5-EGFP fragment from vector *pSL-Prokr5-EGFP-Pgk-neo-Prokr3* was cloned into vector *pBK-CMV* using SalI and KpnI. The vector was transfected into HEK293 cell using FuGENE6 reagent (Invitrogen). Cells stocks frozen in -80˚C were thawed in a water bath followed by addition of the cell suspension into 10ml DMEM-glutamax medium (Gibco), supplemented with 10% FCS, 1x Anti-Anti (Gibco) and 1mM sodium pyruvate, to wash out the stock media. The cells were centrifuged for 5 min at 1200rpm and the media was removed before resuspension in the DMEM-glutamax, media supplemented as above, and cell counting. Cells were counted using a Buchner chamber and ~50000cells/well were added to 24 well plates. The cells were grown until ~80% confluence and the media was replaced followed by the addition of the of FuGENE 6 reagent mixture. The mixture was prepared by mixing 200ng of vector and 1,8ul of FuGENE 6 reagent and diluting to 20ul using opti-MEME (Gibco) media. The mixture was incubated at room temperature for 15 min before addition to the culture. GFP fluorescence was detected by fluorescence microscopy after 24 hours.

2.4 BAC engineering using RedET recombination

BAC engineering was performed using the RedET recombination (Gene Bridges) kit.

Escherichia coli strains carrying Prokr2 BAC, provided by CHORI BACPAC resource center (Children's Hospital Oakland Research Institute, USA), were grown overnight in 10ml of LB medium supplemented with chloramphenicol. 100ul of the overnight culture was used to inoculate 10ml LB medium supplemented with chloramphenicol and the culture was grown for 2 hours. 1,4ml of the culture was harvested by centrifugation and the cells were washed 3 times with ice cold water before resuspension into 50ul of the same ice cold water. 1ul of RedET plasmid solution was added and the suspension was moved to a 1mm electroporation cuvette (Bio-Rad). The cells were electroporated at 1350V 10μF and 600Ω followed by the immediate addition of 1ml of SOC media. The resulting cell suspension was incubated at 30 degrees for 70 minutes before plating onto LB agar supplemented with chloramphenicol and tetracycline. The plates were incubated at 30˚C for two days. A single tetracycline resistant colony was picked and grown overnight at 30 ˚C in 10ml LB media supplemented with chloramphenicol and tetracycline. 100ul of the overnight culture was used to inoculate 10ml LB supplemented with chloramphenicol and tetracycline and the culture was grown for 3 hours at 30 ˚C followed by addition of arabinose to a concentration of 0.4% and incubation at 37 ˚C for 1h. The cells were induced for 1h after which 1.4 ml of the culture was harvested by centrifugation, washed 3 times with ice cold water and resuspended in 50ul of the same water. 1ul of DNA solution containing *Prokr5-EGFP-Pgk-neo-Prokr3* fragment which was obtained from vector *pSL-Prokr5-EGFP-Pgk-neo-Prokr3* by digestion with SalI and BsiWI was added to the cell suspension followed by electroporation using 1mm cuvettes (Bio-Rad) at 1350V 10μF and 600Ω. 1ml of SOC media was added immediately after electroporation and the cells were incubated at 37 ˚C for 90 minutes followed by plating onto LB agar supplemented with chloramphenicol and kanamycin. The plates were incubated at 37 ˚C for two days. Successful recombination was verified by colony PCR using primers Prokr2 5' Scr together with GFP genotyping to verify the 5' integration and Prokr2 3' Scr together with Neomycin genotyping to verify the 3' end. Recombination of PB and SB ITR was carried out by retransforming the RedET plasmid and carrying out recombination as described above. The inserts carrying PB and sleeping beauty ITRs were isolated from vector *pR6K-Hygro-Amp-PB* for PB and *pR6K-Hygro-Spec-SB* for sleeping beauty by digestion with PacI and AscI followed by the addition of 1u of Shrimp alkaline phosphatase and incubation at 37 ˚C for 1h. The resulting selectable markers with flanking ITRs were purified using PCR cleanup kit. After the 90 minute incubation at 37 ˚C the cells were plated onto LB agar supplemented with chloramphenicol, kanamycin and ampicillin for cells recombinated with PB ITRs and

chloramphenicol, kanamycin and spectinomycin for cells recombinated with SB ITRs. The plates were incubated at 37 ˚C for 2 days. To purify the BAC a single colony carrying either ampicillin or spectinomycin resistance was grown overnight in 500ml of LB media supplemented with antibiotics as above. The BAC was prepared using QIAGEN plasmid midiprep kit according to low copy number plasmid protocols. The constructs were designated *Prokr2-PB* for the BAC carrying PB ITRs and *Prokr2-SB* for the corresponding BAC carrying SB ITRs. Primer sequences, detailed PCR protocols and vector maps are presented in supplementary material.

2.5 Sequencing

Sequencing was performed using M13 forward and reverse primers by DNA sequencing Core, Life Science Research Center, Gifu University according to sequencers specifications.

2.6 Verification of Neomycin selection strategy by transfection of vector pMCS5-PB and pMCS5-SB

PB and SB ITR carrying fragments from section 2.3 were cloned into vector *pMCS5* between PacI and AscI sites. The resulting vectors were designated *pMCS5-PB* for the vector carrying PB ITRs and *pMCS5-SB* for the corresponding vector carrying SB ITRs. The vectors were transfected into HEK293 cells as described above with the exception of 200ng of transposase gene coding vector *pCMV-hyPBase* for PB or *pC-SB100XN-pA* for Sleeping Beauty being cotransfected with 600ng of DNA *pMCS5-pB* or *pMCS5-SB* together with a FuGene reagent volume of 3ul/ug DNA. The mixture was also diluted to 100ul as compared to 20 using opti-MEME (Gibco) media. 24 hours after transfection the growth media was removed and the cells were dissociated through addition of 200ul of Tryple express (Gibco) trypsin containing media directly into the well. The cells were incubated for 5 min at 37 degrees to allow for complete dissociation before harvest. Harvested cells were washed with 10ml DMEM-glutamax media, supplemented as above, before centrifugation for 5 min at 1200 rpm. The cells were then resuspended in 4ml of the same media and plated onto 6cm dishes. Geneticin (Gibco) was added the following day to a final concentration of 1mg/ml and the plates were incubated for 2 weeks with weekly media changes.

2.7 Transfection of vector Prokr2-PB and Prokr2-SB into HEK293 cells

BAC transfection was carried out as described in 2.5 using 600ng of BAC DNA. After

neomycin selection the media was removed and 4ml of PBS buffer (pH 7.2) was added and surviving cells were picked up using pipette and transferred to a 96 well plate containing 30ul of Tryple express (Gibco) trypsin containing media and incubated for 5 minutes at 37 degrees before addition of an equal volume of DMEM-glutamax media, supplemented as above, in order to inactivate the trypsin. The dissociated cells were moved to 48 well plates and 200ul of glutamax media was added. The cells were left to grow until ~80% confluence before genomic DNA extraction.

2.8 Preparation of genomic DNA from transfected 293 cells

Genomic DNA was harvested from HEK293 cells using lysis buffer consisting of 10 mM Tris pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween 20 and 50 ug/ml Proteinase K. The growth media was removed and the lysis buffer was added directly to the wells before incubation at 37 degrees for 5 minutes. The lysate was harvested and incubated for 1h at 55 ˚C followed by 45 min at 95 ˚C to inactivate proteinase K.

2.9 Genotyping of genomic DNA from HEK293 cells transfected with either Prokr2-PB or Prokr2-SB

Genomic DNA preparations from section 2.8 were used for genotyping PCR at a ratio of 1ul to 20ul of PCR reaction mixture. PCRs were conducted to amplify amplicons of ~450bp directly upstream of the *eGFP* reporter gene and ~400bp directly downstream of the Neomycin resistance cassette using primers Prokr2 5' SalI and Prokr2 5' ApaI primers and Prokr2 3' SacII and Prokr2 3' HindIII primers. A ~800bp amplicon consisting of ~350bp *eGFP* gene sequence as well as ~450bp upstream sequence was amplified using GFP genotyping and Prokr2 SalI primers. Internal control reactions were conducted by amplifying a ~800bp fragment from the *AAVS1* site of the human genome using AAVS15 and AAVS13 primers. Detailed PCR protocols and primer sequences are presented in supplementary material.

2.9 Staining of HEK293 cell cultures

HEK293 cell staining was performed by washing the cells twice using 4ml of PBS media followed by the addition of 3ml 4% Paraformaldehyde 5min incubation a 37 degrees followed by 2 times washing with 4ml PBS and the addition of 1ml Meyer hematoxylin solution. The mixture was incubated for 5 min at 37 ˚C before removal of the hematoxylin solution.

3. Results

3.1 Construction of recombineering constructs

In this study, we modified a bacterial artificial chromosome (BAC) vector (RP23-33N15) containing a genomic region of the *Prokr2* gene to generate a *Prokr2* promoter reporter construct. To this end, we constructed a plasmid vector containing an enhanced green fluorescent protein (eGFP) reporter cassette, in which DNA fragments encoding an eGFP gene followed by a polyadenylation sequence and a selectable marker were assembled. This cassette was further flanked by ~450bp sequences homologous to the *Prokr2* gene to allow insertion of the eGFP reporter cassette directly after the initiating methionine (ATG) in the second exon of *Prokr2* using BAC recombineering technology (see Fig 1). As a selection maker, a *FRT-PGK-gb2-neo-FRT* cassette was designed to allow neomycin/kanamycin selection in prokaryotic and eukaryotic cells. A schematic of the fragment is presented in Fig 1. Detailed procedures for the vector construction are described in Materials and Methods.

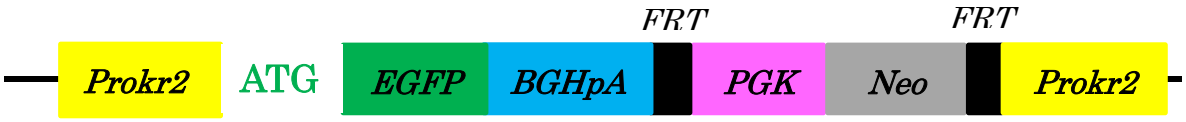


Fig 1: Schematic drawing of fragment used for recombineering. The reporter gene (GFP) is followed by a *BGHpA* polyadenylation sequence and a selectable marker, surrounded by FRT elements, under the control of a *PGK* promoter. GFP and the selectable marker are flanked by homologous sequences to the *Prokr2* gene.

3.2 Verification of eGFP fluorescence

In order to verify the integrity of the *eGFP* reporter gene fragment, the eGFP gene was subcloned into a mammalian expression vector, *pBK-CMV* to examine eGFP fluorescence in HEK293 cells. The *pBK-CMV* vector contains the *SV40* promoter element which allows for efficient expression in human cell lines. It was confirmed by the results that the *eGFP* gene was functional and that the cloning in front of the *Prokr2* start codon was successful and in frame (see Fig 2). The DNA sequence of the *eGFP* gene including the initiation codon was further confirmed by direct DNA sequencing.

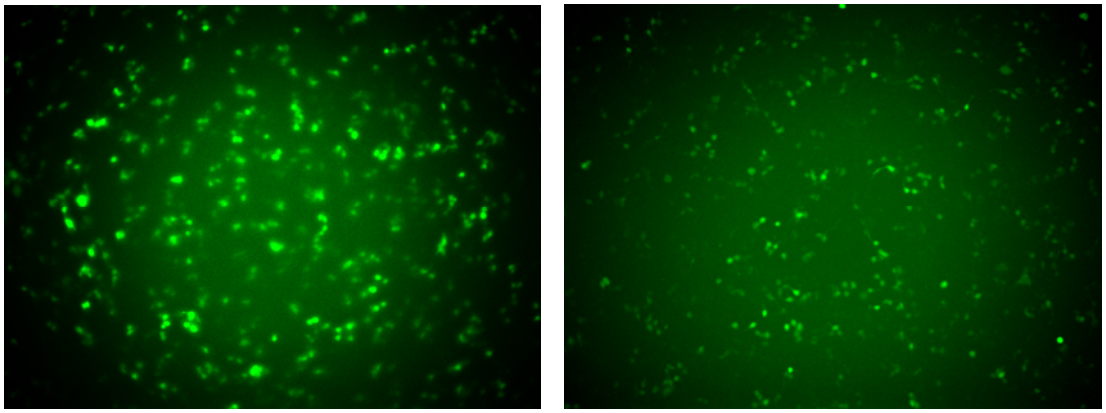


Fig 2 GFP fluorescence, detected after 24 hours, of HEK293 cells transfected with *pBK-CMV-GFP* (left) and p*BK-CMV-Prokr2-GFP* (right). Bright spots represent individual HEK293 cells expressing the GFP reporter gene.

3.3 Construction of BAC vector

Following the functional verification of the *eGFP* reporter gene, the reporter cassette was integrated in the BAC vector harboring *Prokr2* genomic sequence using recombineering (see Fig 3)

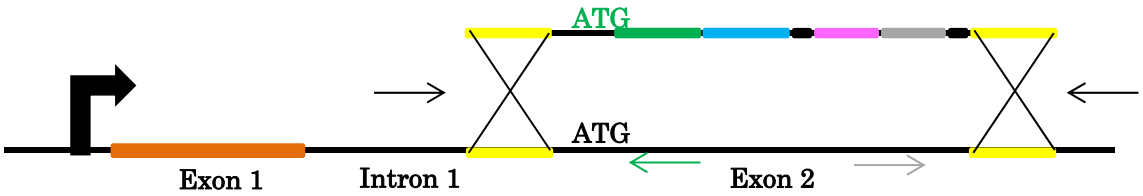


Fig 3: Schematic of the recombination between the *Prokr5-EGFP-Pgk-neo-Prokr3* and the Prokr2 gene region of the BAC vector. The recombination inserts the fragment in between the homologous regions in the second exon whereby the ATG of the eGFP replaces the ATG of *Prokr2* as to avoid frame shifts. The primers used for colony PCR verification are marked as arrows with green representing GFP genotyping and grey representing Neomycin genotyping primers. Prokr2 5' Scr and Prokr2 3' Scr are black and located outside the recombinated fragments in the 5' and 3' direction respectively.

As shown in Fig. 4, the insertion of eGFP reporter cassette was verified by colony PCR. The expected band lengths for a recombination which took place at the intended site were ~800 for the 5' end and ~1400 for the 3' end. Based on the results we concluded that 1 transformant had the correct insertion of the *eGFP-BGHpA-FRT-PGK-neo-FRT*

fragment as indicated by the presence of bands of the expected size in the wells loaded with reactions amplifying the 3' and 5' end of the fragment. Three transformants were found to carry the neomycin resistance cassette fragment while lacking the reporter gene insertion. The 4 transformants were found to contain no insert as no PCR product was detectable.

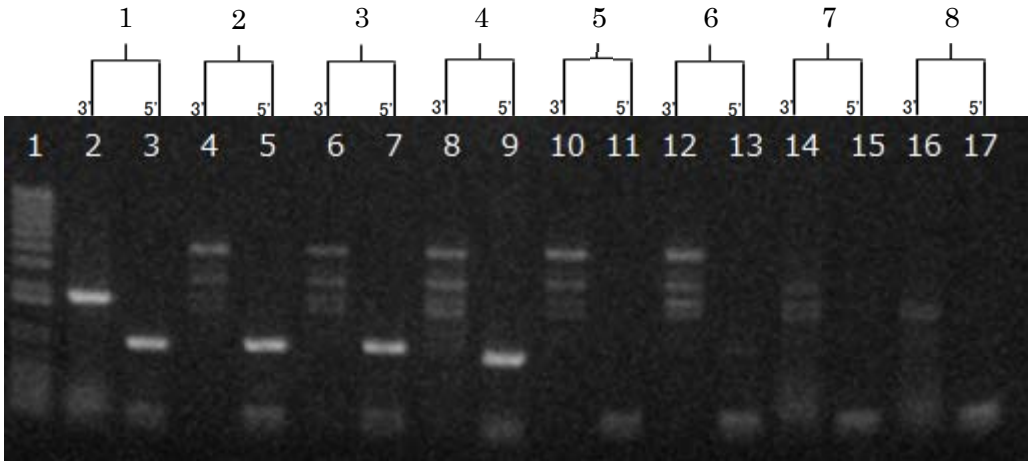


Fig 4: Colony PCR reactions of *Prokr2* BAC carrying Escherichia coli strain after recombination with *Prokr2-eGFP-bpA-NeoR-Prokr2* fragment. Samples loaded in the wells: 1, Ladder 1(supplementary material), 3' and 5' colony PCR reactions for transformants 1-8. The colony number is indicated above the forks and the location of the amplicon is shown at the end of the fork respectively.

To facilitate transposon-mediated BAC insertion, *Prokr2* BAC was further modified to insert a PB ITRs cassette into the BAC backbone. To facilitate stable recombination, the PB ITRs flanking an ampicillin resistance cassette was recombined into a *LoxP* site of the BAC using recombineering (*Prokr2-PB*). We also constructed a BAC construct harboring SB ITRs using the same approach with the exception of the flanked resistance cassette being spectinomycin (*Prokr2-SB*). A single colony carrying either ampicillin resistance or spectinomycin resistance was picked up and the BAC was purified for further functional validation.

3.4 Verification of neomycin selection strategy

Before integration of BAC constructs into the genome of HEK293 cells we verified if an element flanked by PB or SB ITRs was successfully integrated into the host genome. HEK 293 cells were transfected with *pMCS5-PB* (see Materials and Methods) along

with *pCMV-hyPBase* vector (expressing PB transposase). For SB ITRs validation, *pMCS5-PB* (see Materials and Methods) and *pC-SB100XN-pA* vectors (expressing SB transposase) were also transfected. The results of transfecting *pMCS5-PB* and *pMCS5-SB* and cotransfection with their corresponding transposases into HEK293 cells are shown in Fig 5.

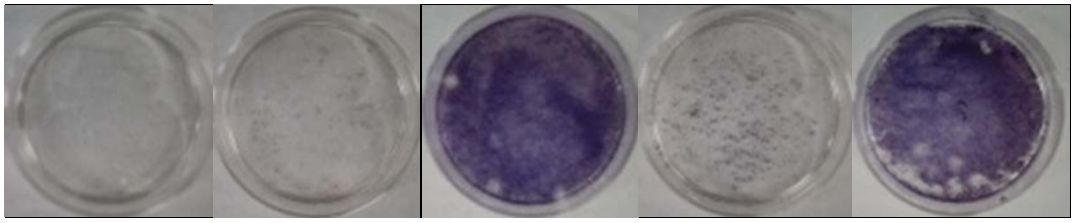


Fig 5: Hematoxylin stained HEK293 cells transfected with vector pMCS5-PB and pMCS5-SB as well as pCMV-hyPBase and pC-SB100XN-pA. On display from the left: negative control (media and lipofection reagent without added DNA) *pMCS5-SB* Without *pC-SB100XN-pA*, *pMCS5-SB* With *pC-SB100XN-pA*. *pMCS5-PB* without *pCMV-hyPBase pMCS5-PB* with *pCMV-hyPBase*.

It is evident from the staining patterns that the PB and SB transposases can mediate efficient integration of *pMCS5-PB* and *pMCS5-SB* into the genome of HEK293 cells based on the presence of a large number of Neomycin resistant cells on the plates into which *pMCS5-PB* and *pMCS5-SB* had been cotransfected with *pCMV-hyPBase* and *pC-SB100XN-pA* as compared to the negative control. We also found some neomycin resistant colonies growing on the plates in which *pMCS5-PB* and *pMCS5-SB* had been transfected without their corresponding transposases, indicating that both these vectors integrated randomly into the HEK293 cell genome. Although random integration was observed the number of neomycin resistant cells present was significantly higher on the plates where the vectors were cotransfected with transposase. Hence, this data strongly indicate robustness of transposon-mediated integration into host genome.

3.5 Cotransfections of BAC with PiggyBac and sleeping beauty transposase

To obtain stable transfectants carrying the *Prokr2-PB* or *Prokr2-SB* construct, the *Prokr2-PB* BAC vector together with *pCMV-hyPBase* or *Prokr2-SB* with *pC-SB100XN-pA* were transfected into HEK293 cells. Following neomycin selection, a total of 10 resistant colonies from those cells transfected with the *Prokr2-SB* and 50 colonies from the *Prokr2-PB* transfection were obtained, respectively. Contrary to our expectation, the *Prokr2-PB* vector displayed a detectable level of random integration as some neomycin resistant colonies appeared on the plate transfected with Prokr2-PB

without PB transposase (see supplemental data).

3.6 Verification of BAC integration into the genome of HEK293 cells

Out of 50 colonies isolated from the cells transfected with *Prokr2-PB* 10 colonies were chosen for further PCR screening. For Prokr2-SB transfections, the 10 colonies were also isolated. The screening was conducted by amplifying several sites in and around the reporter GFP gene in order to verify the integrity of the BAC insertion. The results can be seen in Fig 6, 7 and 8.

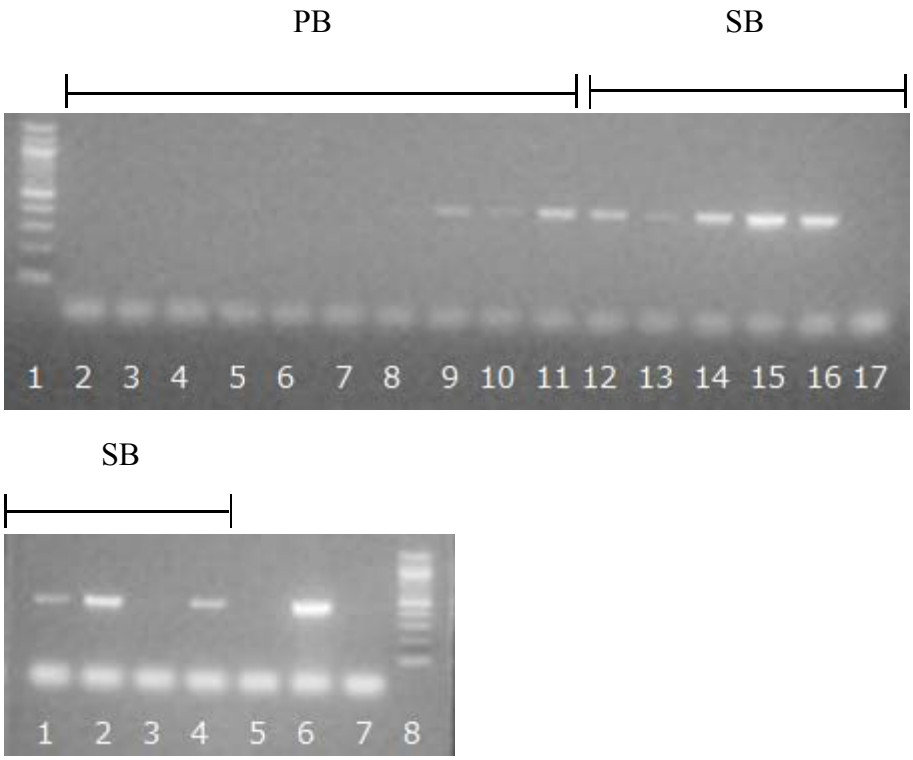


Fig 6: PCR on 20 colonies of which 10 were transfected with *Prokr2-PB* and the remaining 10 with *Prokr2-SB*. Amplified is a ~450bp amplicon upstream of the GFP inserted into *Prokr2-PB* and *Prokr2-SB*. Sample order: ladder 2 (supplementary material) *Prokr2-PB* transformed colonies 1-10, *Prokr2-SB* transformed colonies 1-6. Continued on second gel: *Prokr2-SB* transformed colonies colonies 7-10, negative control (human genomic DNA prepared from 293 cells), Positive control (50ng of *Prokr2-PB* DNA), Water, ladder 2

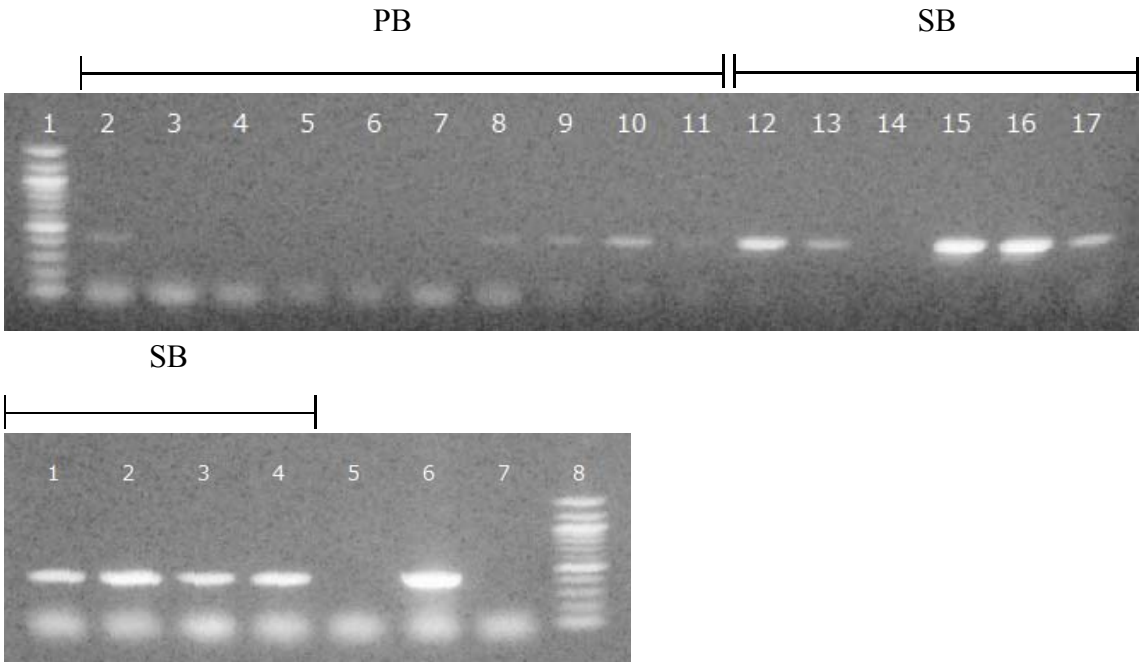
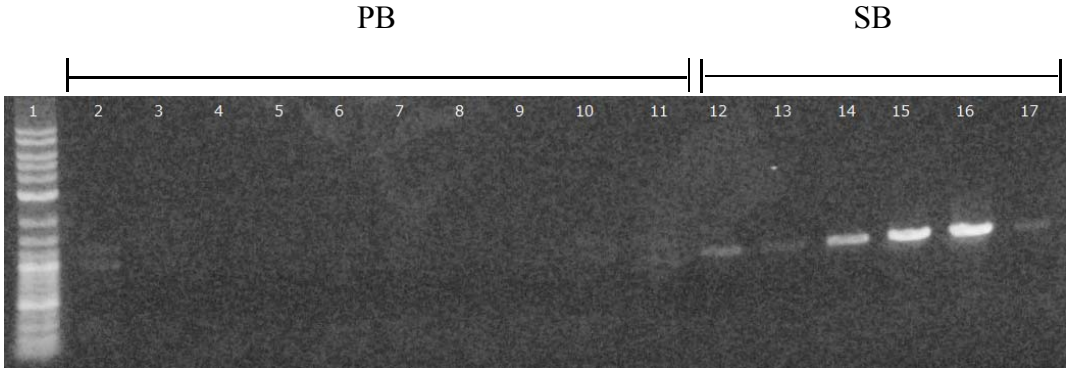


Fig 7: PCR on 20 colonies of which 10 were transfected with *Prokr2-PB* and the remaining 10 with *Prokr2-SB*. Amplified is a ~400bp amplicon downstream of the NeoR gene of *Prokr2-PB* and *Prokr2-SB*. Sample order: ladder 2 *Prokr2-PB* transformed colonies 1-10, *Prokr2-SB* transformed colonies 1-6. Continued on second gel: *Prokr2-SB* transformed colonies colonies 7-10, negative control (human genomic DNA prepared from HEK293 cells), Positive control (50ng of *Prokr2-PB* DNA), Water, ladder 2



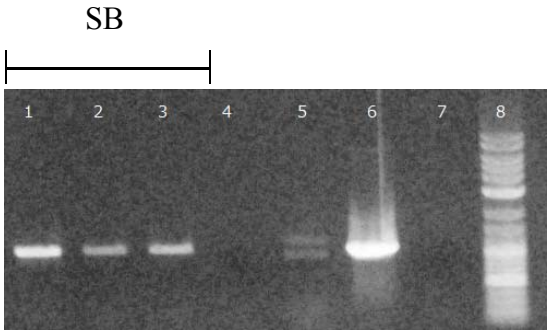


Fig 8: PCR on 20 colonies of which 10 were transfected with *Prokr2-PB* and the remaining 10 with *Prokr2-SB*. Amplified is a ~800bp fragment using a GFP reverse primer and a primer located ~500bp upstream of the GFP insertion of *Prokr2-PB* and *Prokr2-SB*. Sample order: ladder 3 (supplementary material) *Prokr2-PB* transformed colonies 1-10, *Prokr2-SB* transformed colonies 1-6. Continued on second gel: *Prokr2-SB* transformed colonies colonies 7-10, negative control (human genomic DNA prepared from 293 cells), Positive control (2,5ug of DNA), Water, ladder 3.

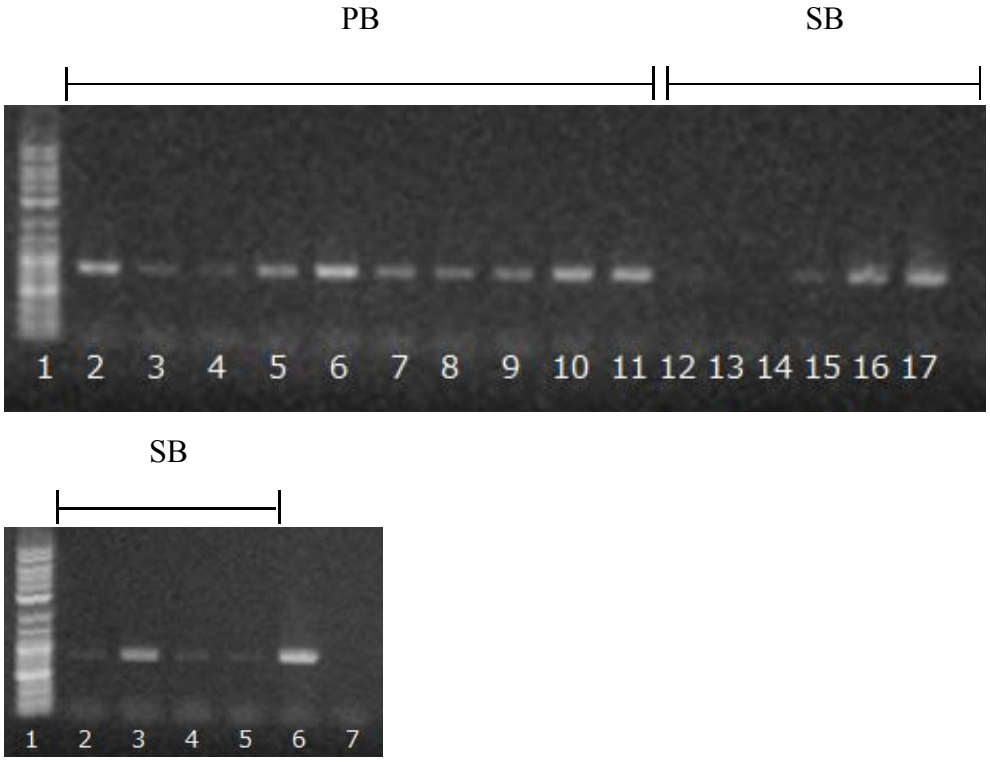


Fig 9: Internal control PCR of *Prokr2-PB* and *Prokr2-SB* transformed HEK293 cells amplifying a ~800bp amplicon located inside the *AAVS1* site on the human chromosome. Sample order: ladder 1, *Prokr2-PB* transformed colonies 1-10, *Prokr2-SB* transformed colonies 1-6. Continued on second gel: ladder 3, *Prokr2-SB* transformed

colonies colonies 7-10, Positive control (human genomic DNA prepared from HEK293 cells), water.

The internal control PCR confirmed that the absence of bands in some of the wells in Fig 6, 7 and 8 was not due to poor quality genomic DNA preparations or DNA degradation (see Fig 9).

The results in Fig 9 indicate that all but two genomic DNA preparations were intact enough to allow PCR amplification and we concluded that the absence of bands in Fig 6, 7 and 8 was representative of these BAC elements not having integrated in the genome. Based on this we found no intact integration of the region around the eGFP reporter gene on *Prokr2-PB* into the genome of HEK293 cells which is in agreement with the results in 3.5 suggesting that *Prokr2-PB* tends to be integrated randomly into the genome. In contrast, we found that at least 5 colonies exhibited successful integration of *Prokr2-SB* as demonstrated by PCR genotyping showing the expected size in well 11, 12, 16 on the first gel and 1 and 2 on the second gel in Figure 6, 7 and 8. In addition *Prokr2-SB* vector was successfully lipofected into mouse ES cells as indicated by the formation of neomycin resistant ES cells although successful integration has yet to be verified.

4. Discussion

This paper reports the generation of a BAC vector containing a reporter GFP gene inserted downstream of the start codon of the *Prokr2* gene through the use of a recombineering strategy using ~450bp of homologous sequences flanking the insert. Although sequences shorter than 50bp have been reported to be sufficient to successfully perform recombination[8], no transformants carrying the *eGFP-BGHpA-FRT-PGK-neo-FRT* insert at the intended location could be isolated in our hands (data not shown). Our such recombination experiments resulted in a significant amount of kanamycin resistant transformants in which the insert was not inserted in the intended location (data not shown) as verified by colony PCR. One possible explanation for this false positive colony formation may be due to minor contamination of intact *eGFP-BGHpA-FRT-PGK-neo-FRT* vector from which the *eGFP-BGHpA-FRT-PGK-neo-FRT* fragment was obtained by restriction enzyme digestion. This minor contamination would be negligible if recombination efficiency would be sufficiently high. We also observed fragmented integration where the clones seemed to carry only the kanamycin resistance cassette without the reporter gene as

verified by PCR (data not shown). We hypothesized that this was due to insufficient targeting by the recombinases and that increasing the sequence homology between the insert and the target site would improve recombination accuracy and therefore ~450bp of homologous sequences were cloned flanking the *eGFP-BGHpA-FRT-PGK-neo-FRT*, which allowed isolation of a transformant carrying the fragment at the intended location. We evaluated whether the BAC vector carrying the reporter construct as well as transposable elements were capable of integrating into the genome of HEK293 cells using either PB or SB transposase. We found that the BAC reporter carrying SB ITRs was successfully integrated by SB transposes, whereas that of PB ITRs showed only a limited number of integration. This is in sharp contrast with previous reports where PB transposase could mediate efficient insertion[8]. Although the reason for this discrepancy is not clear, it is suggested from our validation study that it is not be due to either defects in the ITR elements or the transposase as these were shown to be fully functional. In addition, we found that the *Prokr2-PB* construct also showed a higher random integration when compared to the SB vector. The tendency where the PB vector shows higher random integration was also noticed during transfections of *pMCS5-PB*, suggesting that the reason for the random integration is not related to the BAC. In contrast to the PB-mediated transposition, the SB transposase was found to be able to stably integrate the reporter gene construct, *eGFP-BGHpA-FRT-PGK-neo-FRT*, including the surrounding BAC elements as we found that 5 out of 10 colonies of HEK293 cells possessed the reporter construct, verified by PCR, indicating a ~50% integration frequency. We concluded from this data that the *Prokr2-SB* vector along with SB transposase is capable of integrating into the host genome, suggesting feasibility of this system to generate a transgenic mouse ES cell line harboring the a *eGFP* reporter gene under the control of the *Prokr2* promoter.

Based on genome-wide transcriptome analysis and subsequent *in situ* hybridization analysis, we found that endogenous Prokr2 is expressed specifically in the dermal papilla of mice (data not shown). Hence, the data obtained in this study is of considerable interest for future application of the Prokr2 reporter construct to develop a system where a transgene of interest is expressed specifically in the dermal papilla. Such a transgenesis system could greatly simplify the process of elucidating the regulatory mechanisms behind the regulation of hair follicle formation and the hair cycle by eliminating transgene expression in surrounding tissues. Future projects include verifying if the *Prokr2-SB* vector is able to stably integrate into mouse

embryonic stem (ES) cells followed by implantation of the transgenic cells into a pseudo-pregnant mouse, resulting in the birth of a transgenic mouse. This would allow determination of the *in vivo* expression patterns of the *Prokr2* gene by observation of the fluorescence originating from the reporter gene or histochemical analyses, a project which would constitute the final verification as to whether or not the expression of *Prokr2* truly is dermal papilla specific. If that is the case then the expression system presented here could become a valuable tool in order to elucidate the signaling pathways regulating the stem cells responsible for the formation of the hair follicle.

5. Acknowledgements

I would like to that Dr. Masatake Osawa for supervising this project, supplying the materials as well as providing valuable insight and council. I also thank Dr Dongsuu Lee for his advice on several issues and general guidance. Finally I would like to thank Mizuki Matsumoto for providing me with genomic DNA as well as pMCS5 plasmids and for assisting with technical difficulties.

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Smith3, Konstantinos Anastassiadis1,* and A. Francis Stewart 2, Nucleic Acids Research, Vol. 40: e150 (2012)

7. Supplementary material

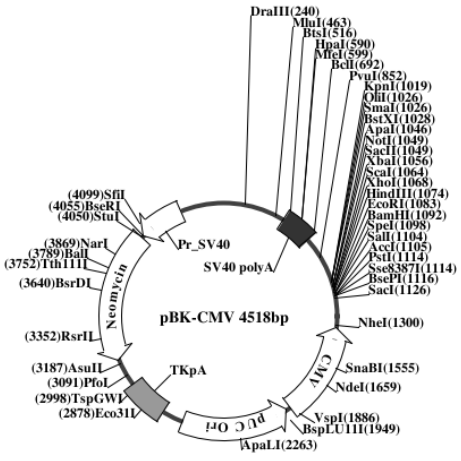
7.1 Sequence of eGFP-BGHpA gene

EGFP-BGHpA

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCC
ACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACC
GGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGAC
CACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGA
CGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGC
ATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCAT
GGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTC
GCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCA
GTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCA
CTCTCGGCATGGACGAGCTGTACAAGTAAAGCGGCCGCTCGAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTT
GTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTG
CATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAA
GACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGCATAGG

7.2 Plasmids used in this study

pBK-CMV



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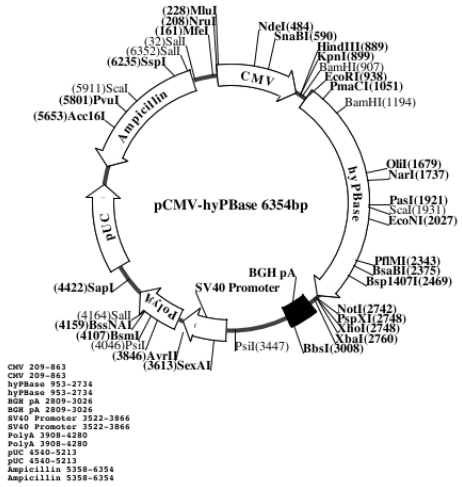
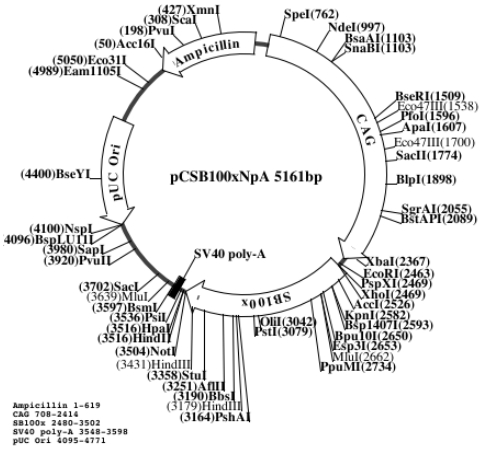
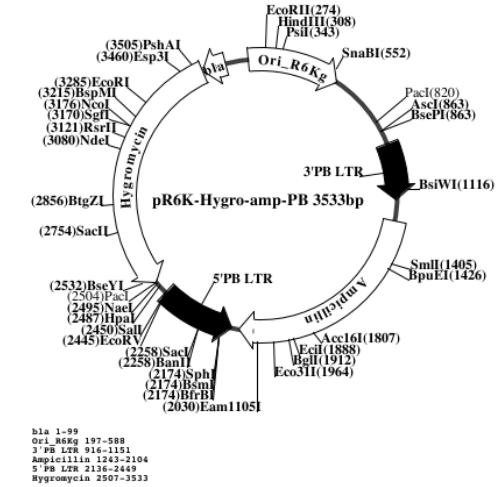


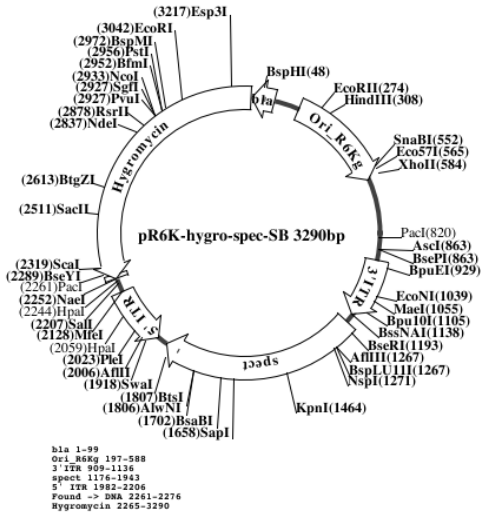
pC-SB100XN-pA



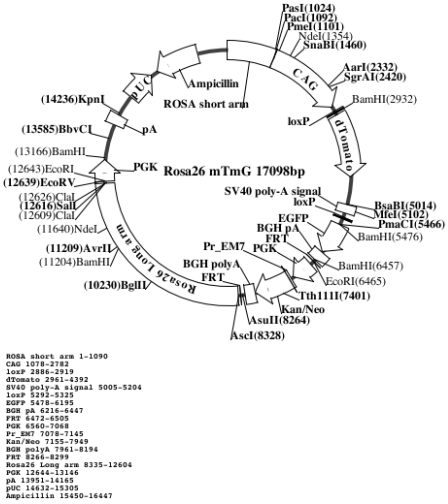
pR6K-Hyg-Amp-PB



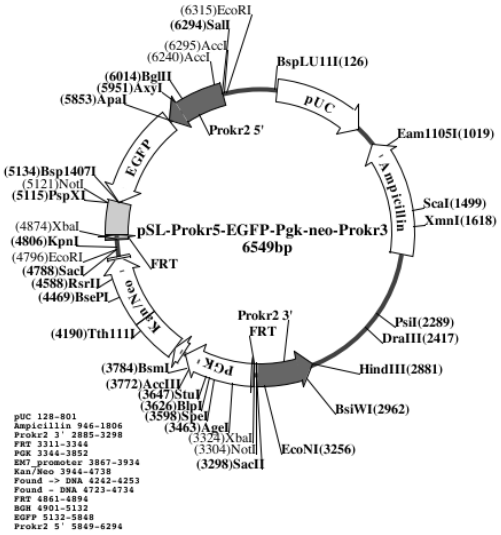
pR6K-Hygro-Spec-SB



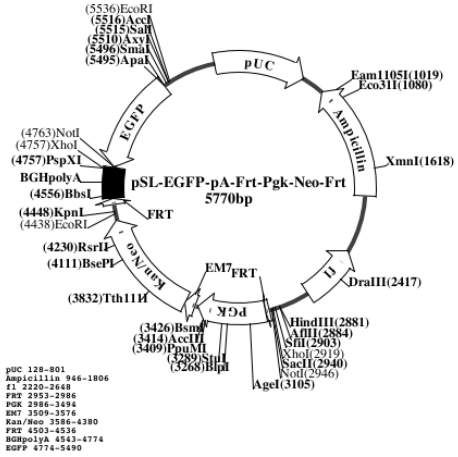
pROSA26-mT_mG



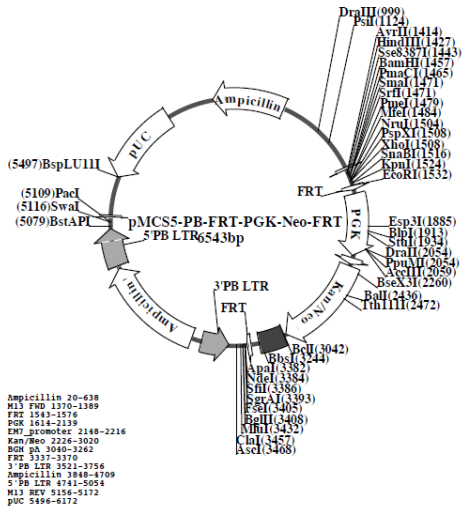
pSL-Prokr5-EGFP-Pgk-neo-Prokr3



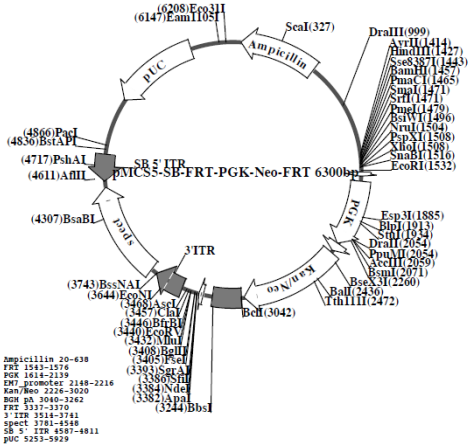
pBS-FRT-pgk-neo-FRT



pMCS-PB-FRT-Pgk-Neo-FRT



pMCS-SB-FRT-Pgk-Neo-FRT



7.3 Primers

eGFP primers

SalI (forward)

AGA GTC GAC TCC CCA GCA TGC CTG CTA TTG TCT TCC

ApaI (reverse)

TAC GGG CCC ACC ATG GTG AGC AAG GGC GAG GAG CTG

Prokr2 homologous 5' primers:

Prokr2 SalI (Forward)

ACC GTC GAC TCG AGA CAG GGT TTC TCT G

Prokr2 ApaI (Reverse)

TCT GGG CCC ATG CTA CAG GTG GGC

Prokr2 homologous 3' primers:

Prokr2 SacII (Forward)

AGT CCG CGG TTA AAC TAC AGTT TAT GGT G

Prokr2 HindIII (Reverse)

TCC AAG CTT TGT GAG AAC TGA AG TGA AC

Prokr2 Scr 5'

CAC TCC CAG CCA CCA ATA CAT TCC CGT CC

Prokr2 Scr 3'

TCA GAG GCT TCC AAC GTC CCC AAA GCT GG

GFP genotyping (reverse)

AAG TTC ATC TGC ACC ACC G

Neomycin genotyping (reverse)

AAG AAC TCG TCA AGA AGG CGA TAG AAG G

AAVS5

CCGGAACTCTGCCCTCTAACGCTGCCGTC

AAVS13

GGCCTCCTGGGATACCCCGAAGAGTGAG

7.4 PCR protocols

Colony PCR verification of recombination

Denaturing (30 sec)	Annealing (30 sec)	Elongation (1,5min)	Cycles/time
94			15 min
94	55	72	25
		72	7

Amplification of ~500bp amplicon upstream of eGFP gene from BAC constructs integrated into HEK293 cell genome

Denaturing (30 sec)	Annealing (30 sec)	Elongation (1,5min)	Cycles/time
94			15 min
94	66	72	35
		72	7

Polymerase used: HStaq (takara)

Amplification of ~500bp amplicon downstream of neomycin resistance cassette gene from BAC constructs integrated into HEK293 cell genome

Denaturing (30 sec)	Annealing (30 sec)	Elongation (1,5min)	Cycles/time
94			15 min
94	61	72	35
		72	7

Polymerase used: HStaq (Takara)

Amplification of ~800bp amplicon consisting of ~300bp of the eGFP gene and ~ 500bp of the upstream region from BAC constructs integrated into HEK293 cell genome

Denaturing (30 sec)	Annealing (30 sec)	Elongation (1,5min)	Cycles/time
94			15 min
94	55	72	35
		72	7

Polymerase used: HStaq (takara)

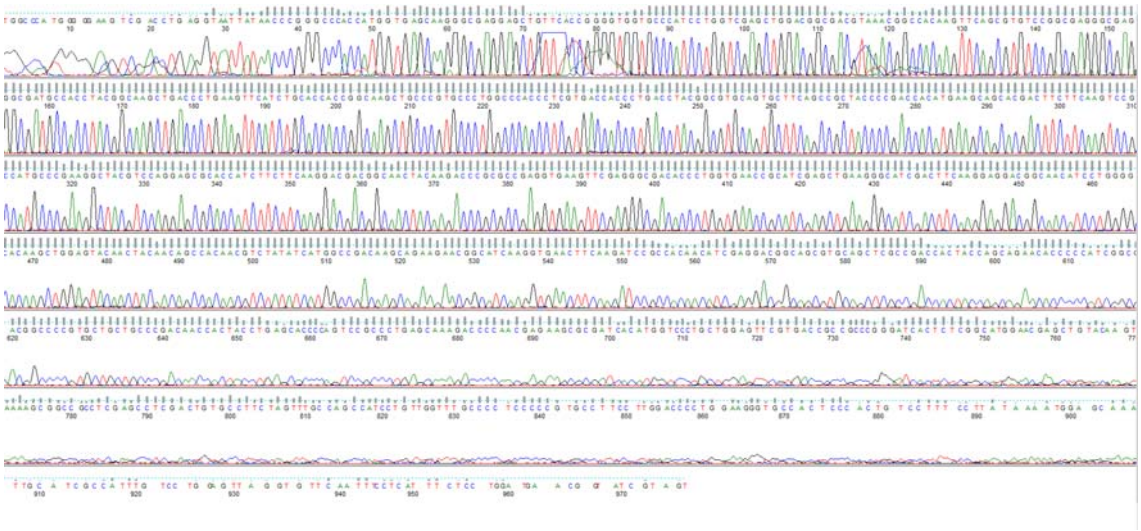
Internal control PCR on genomic DNA from Prokr2-PB and Prokr2-SB transfected 293 cells.

Denaturing (10 sec)	Annealing (30 sec)	Elongation (1min)	Cycles/time
98			2 min
98	65	68	2
98	63	68	2
98	61	68	2
98	59	68	2
98	57	68	2
98	55	68	20
		68	7min

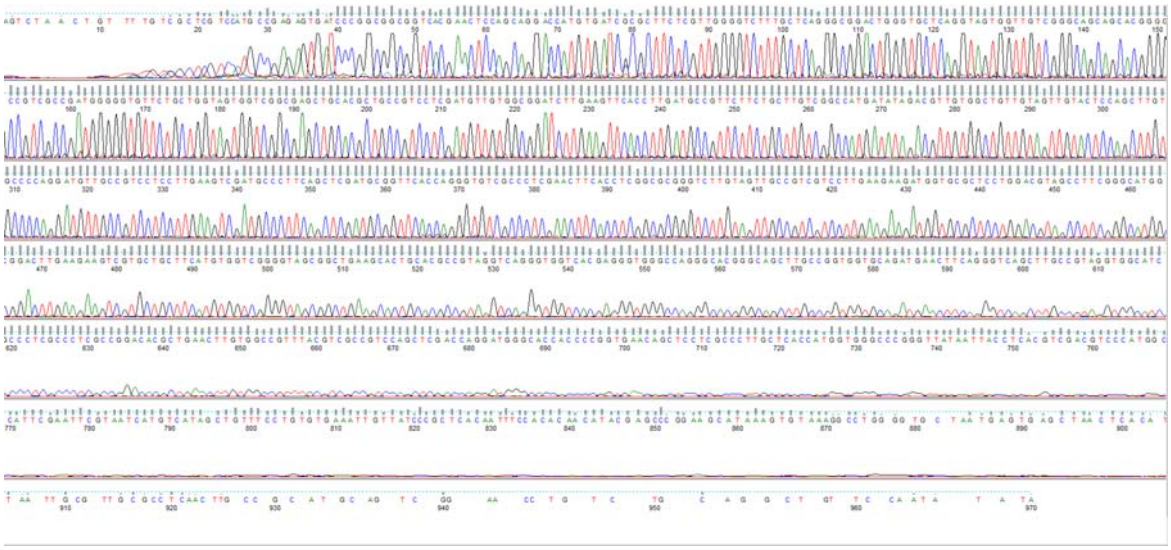
Polymerase used: KODFX (Toyobo)

7.5 Sequencing data from eGFP sequencing

Forward direction

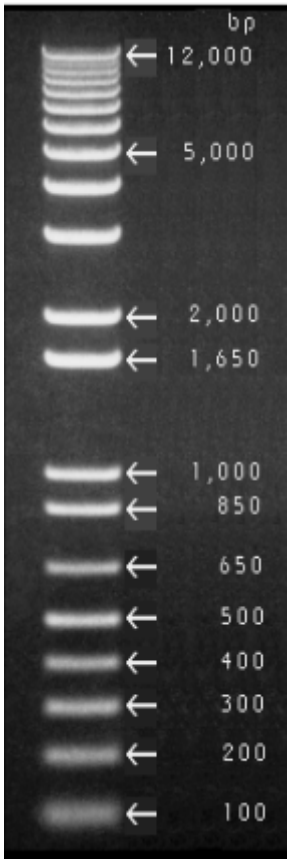


Reverse direction

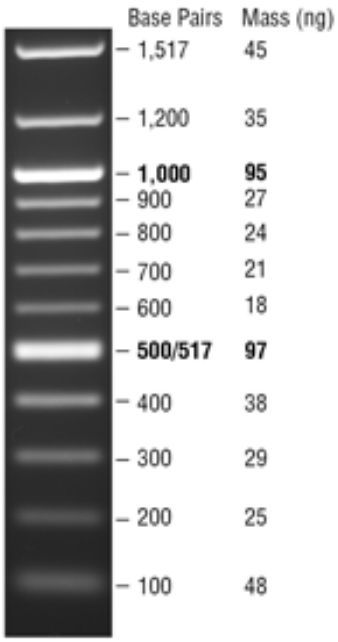


7.6 Ladders used in this study

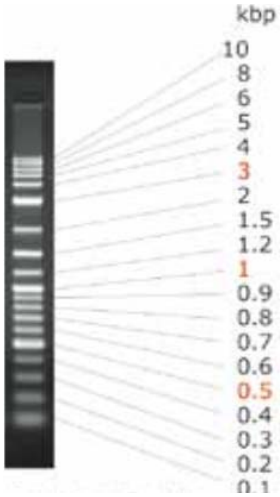
1 10787-018 1kb plus DNA ladder



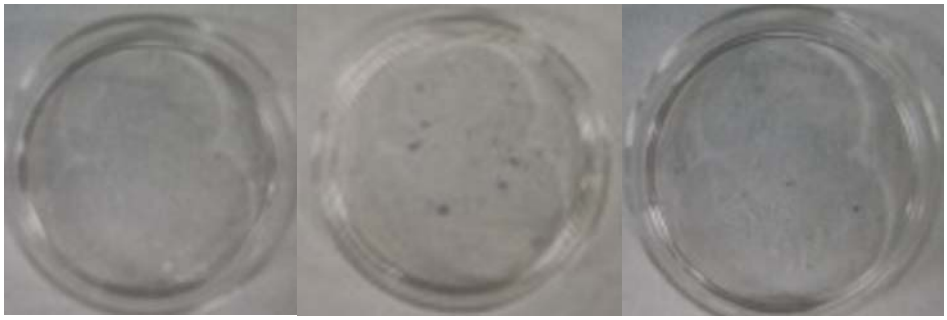
2 NEB N0467S Quickload 100bp ladder



3 Nakalai Tesque DNA ladder one



7.6 Assessment of random integration of Prokr2-PB and Prokr2-SB in HEK293 cells



Hematoxylin stained HEK293 cells Transfected with prokr2-PB and *Prokr2-SB* without *pCMV-hyPBase* and *pC-SB100XN-pA*. On display from the left: negative control (media and lipofection reagent without added DNA), *Prokr2-PB*, *Prokr2-SB*