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Translocation engineering in human somatic cells

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SUMMARY

Multiple studies confirm that gene fusions play critical roles in epithelial malignancies such as prostate cancers. However, the mechanisms behind their genesis, cell type specificities and exact phenotypic effects are still unclear. Fusions between TMPRSS2 (Transmembrane protease serine 2) and ETS (Erythroblast transformation specific) transcription factors family members are found in 36-78% prostate cancer samples. My project aimed to generate this clinically important translocation in a normal prostate epithelial cell line so that the exact cellular effects rendered by this translocation could be studied. The particular gene fusion that I was assigned to construct was the *TMPRSS2-ETV1* (ETS Translocation variant 1) translocation. The targeted cell-lines were karyotypically stable normal prostate epithelial cells, RWPE1. According to the literature the 4th exon of *ETV1* fuses with the 2nd exon of *TMPRSS2* after translocation.

The very first step of this gene targeting was to design primers for construct development. The construct contained the selectable marker gene that encodes neomycin phosphotransferase along with two *loxP* sites flanked by sequences identical to the targeted locus. I planned to place the *loxP* site in the intronic region of the targeted genes to artificially mediate translocation without disturbing the exons. The regions where I wanted to place the *loxP* site were precisely amplified from the target locus. After purifying bands of the appropriate molecular weight I did a series of recombination reactions to introduce the *TMPRSS2* and *ETV1* constructs into an adeno-associated viral vector (AAV). After obtaining the final construct, I introduced the recombinant AAV in the target cell line. After three weeks the cells were examined to score positive clones with a correct integrated sequence in the correct location of the genome. However, I found that the cells did not survive after infection with the *TMPRSS2* construct-carrying rAAV. The whole infection was repeated twice to rule out the possibilities of technical errors. The cells did not thrive to move on to the next step.

ABBREVIATIONS

AAV	Adeno-associated virus
*att*B	Bacterial *att*achment site
*att*L	Left *att*achment junction and
*att*P	Phage *att*achment site
*att*R	Right *att*achment junction
BPE	Bovine pituitary extract
BP	*att*B-*att*P recombination
Cre	Cyclic recombination protein
DMEM	Dulbecco's modified eagle medium
ES	Embryonic stem cells
ETS	Erythroblast Transformation Specific
ETV1	ETS translocation variant 1
FBS	Fetal bovine serum
FSPS	Forward screening primer site
HAs	Homology arms
HBSS	Hank's buffered salt solution
HEK	Human embryonic kidney cell
ICB	Intron containing breakpoint
LB	Luria-broth
lox P	Locus of x-over P1
LR	*att*L-*att*R recombination
PS	Penicillin–streptomycin
r- EGF	Recombinant epidermal growth factor
rAAV	Recombinant adeno-associated virus
RSPS	Right screening primer site
Rwpe1	Normal prostate epithelial cells
TAE	Tris-acetate-EDTA buffer
TMPRSS2	Transmembrane protease serine2
WT	Wild type

INTRODUCTION

Genetic rearrangements in solid tumors

Provisions of the reference human genome sequence at the turn of the millennium and the advent of large scale sequencing methods have enabled systematic surveying of cancer cells. The genetic alterations in different types of cancers include the full range of somatic mutations including single-nucleotide variants, insertions, deletions, copy number changes, translocations and other chromosomal rearrangements. This genetic analysis has revealed a multitude of mutated genes. Some of them are functionally well annotated but how most of these mutated genes affect cellular regulation to cause cancer is still not clear. In order to gain comprehensive idea about a gene status functional and phenotypic studies comparing mutant and wild-type alleles in relevant model systems is necessary. (Ali *et al*, 2009)

It was generally considered that chromosomal rearrangements resulting in gene fusions are quite common in leukemia, in contrast to epithelial malignancies like pulmonary, prostate or colorectal cancers (Wang *et al* , 2006). This concept was challenged recently by scientists (Tomlins *et al,* 2005 and Wang *et al*, 2006). They found gene fusions resulting in functional fused protein in a large fraction of epithelial malignancy cases. Their report suggests that fusions between TMPRSS2 (Transmembrane protease serine2) and ETS (Erythroblast Transformation Specific) transcription factors family members are frequently found in 36-78% prostate cancer samples (Tomlins *et al*, 2007) (Figure 1).

The *TMPRSS2* gene is located in the long arm of chromosome 21. This gene encodes a multi-domain protein that belongs to the serine protease family. Serine proteases are known to be involved in many physiological and pathological processes. This gene was demonstrated to be up-regulated by androgenic hormones in prostate cancer cells and down-regulated in androgen-independent prostate cancer tissue. The biological function of this gene is unknown. (Paoloni-Giacobino *et al*, 1997)

The ETS family is one of the largest families of transcription factors where members act as transcriptional repressors, or activators, or both. All ETS family members are identified by a highly conserved DNA binding domain, the ETS domain, which is a winged helix-turn-helix structure that binds to DNA sites. The gene encoding ETS Translocation Variant 1 (*ETV1*) is located in short arm of chromosome 7. The *ETV1* translocation can also be found in Ewing's sarcomas or neuroectodermal tumors. (Jeon *et al*, 1995)

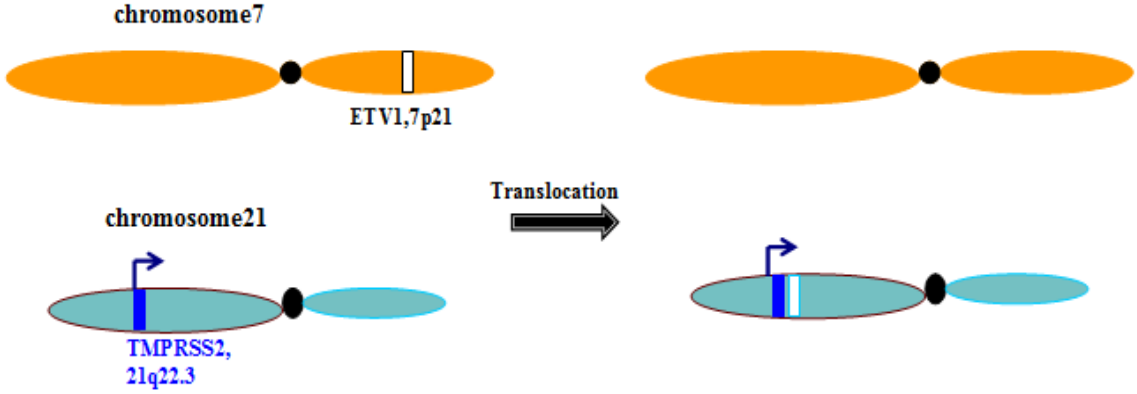

Figure 1: **Translocation of *TMPRSS2-ETV1*.** The *ETV1* gene is located in short arm of chromosome 7 and *TMPRSS2* is located in long arm of chromosome 21. The *TMPRSS2* gene is under the regulation of the androgen responsive promoter. *ETV1* comes under the control of the *TMPRSS2* promoter after translocation resulting in increased expression of the fused gene in prostate tissue.

Gene targeting by homologous recombination is the most direct and unambiguous approach to assess gene function through knock-in or knock-out of the genes in human somatic cells. This will play an increasingly important role to precisely dissect molecular details of translocation behind cancer development where rodent models do not adequately represent human biology. (Sedivy *et al*, 1999)

Gene knock-out or knock-in through homologous recombination can be efficiently accomplished in a variety of cell types, including those from bacteria, yeast, chickens and rodents (Bunz, 2002). However, the same methods are generally inefficient in human somatic cells (Sedivy *et al*, 1999). These impediments have led to widespread use of alternative methods like knockdowns through small interfering RNA and gene transfection technologies (Hannon, 2002). Though these approaches offer important information rapidly, the interpretation of such experiments can be difficult because of non-specific effects or incomplete inactivation of the gene product of interest (Jackson *et al*, 2003). These drawbacks can be avoided by site specific recombination where the genomic alleles are modified (Sedivy *et al*, 1999). Moreover, specific targeting can preserve the function of the endogenous gene, only affecting the level of its activity. This allows studies also of genes whose functions are essential. Such gene targeting can be performed in normal as well as cancer-derived human cell lines and can yield extremely valuable insights into gene function during tumor development (Yun et *al,* 2009). Currently, gene targeting in human cells is performed by insertion or deletion of exonic sequences using adeno-associated virus constructs (AAV).

Tools for translocation engineering

In order to artificially induce translocation in normal cells some genetic engineering tools are useful like Cre-*loxP* recombination system and multisite gateway cloning. The Cre protein is a site-specific DNA recombinase; it recognizes and binds to *loxP* sites. These *loxP* sites are 34 bp sequences. Depending on the orientation of the *loxP* sites, Cre mediated recombination results in deletion, inversion or translocation (see fig. 2). If the *loxP* sequences are placed in the way shown in figure 2, then Cre will mediate translocation. Cre always recombines two *lox P* sites. (Sauer *et al*, 1988)

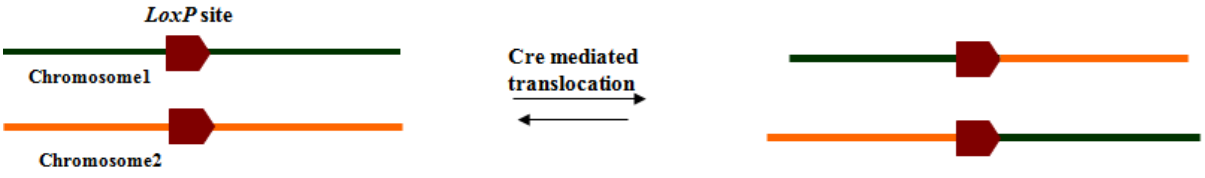

Figure 2: Cre mediated interchromosomal translocation. Chromosome 1 is represented by green and chromosome 2 by orange color. If the *loxP* sequences, denoted here as dark brown rectangles, are placed in the same orientation in the two different chromosomes then the Cre assisted recombination will result in reciprocal translocation.

The Multisite gateway cloning is a system by which multiple DNA fragments can be transferred into one or more vectors, maintaining the reading frame in correct order and orientation. It is based on the bacteriophage lambda site-specific *att* recombination system catalyzing the integration of lambda into the *E. coli* chromosome and its excision out of this

chromosome. This site specific recombination system is exploited to integrate the region of interest in gateway vectors. (Magnani *et al*, 2006). Another elegant beauty of this system is the negative selection gene *ccd*B which is incorporated into the destination vector such that it is lost upon correct integration. The presence of the *ccd*B gene permits negative selection of the donor and destination vectors in *E. coli* following recombination and transformation. The CcdB protein interferes with *E. coli* DNA gyrase (Bernard & Couturier, 1992) thereby inhibiting growth of most *E. coli* strains. When recombination occurs, *i.e.* between a destination vector and an entry clone or between a donor vector and an *att*B PCR product, the cloned gene replaces the *ccd*B gene. Cells that take up unreacted vectors or byproduct molecules retaining the *ccd*B gene will fail to grow. This allows high efficiency scoring of the positive clones (Magnani *et al*, 2006).

Vector delivery and integration in the human genome is a crucial step. The absolute frequency of homologous recombination in human somatic cells is two orders of magnitude lower than in murine embryonic stem (ES) cells (Sedivy *et al,* 1999). The rates of both vector delivery and site-specific integration have been significantly improved by the development of recombinant adeno-associated virus (rAAV) as a gene-targeting agent (Rago *et al*, 2007). All adeno-associated viruses (AAV) are replication-deficient single stranded DNA viruses that are unique in their ability to persist in human cells without causing any pathologic effect (Conlon, 2004). rAAV-based vectors are 25-fold more efficient in delivery and insert than comparable plasmid vectors (Rago, 2007).

Another factor that complicates the gene targeting is higher frequencies of nonhomologous recombination compared to homologous recombination in mammalian cells (Sedivy *et al*, 1999). In order to circumvent this problem the targeting vectors must provide a very powerful strategy to preferentially enrich for the desired homologous event suppressing random and nonhomologously recombined clones. One way is the application of promoter trap strategies, where a positively selectable marker, e.g. a neomycin resistance gene downstream of internal ribosome entry (IRES) site, is expressed only if correctly integrated at an appropriate distance from a usable promoter, as would be the case if correctly integrated in the desired locus of the genome.

To permit insertion of *loxP* sites at precise positions in the targeted genes, sequences adjacent to these positions are amplified and cloned next to *loxP*. In a second step, homologous recombination between these cloned sequences and the targeted gene will result in insertion of *loxP* at the desired position. The cloned sequences therefore are referred to as "homology arms" (HAs). To permit selection of the adeno-associated virus (AAV) vector carrying the construct, it contains a neomycin resistance gene surrounded by *loxP sites* adjacent to the homology arms (Topaloglu *et al,* 2005). Recombinant adeno-associated virus (rAAV) are then produced, and used to infect the target cell line. Following neomycin selection, clones with proper integrations are identified by PCR. The resistance gene is then removed using *Cre* recombinase.

Aim

The aim of my work was to generate the clinically important *TMPRSS2-ETV1* translocation in a normal prostate epithelial cell line by Cre-assisted recombination of two targeted loci, using a procedure developed in mouse models (Buchholz, 2000) and develop a human cell model by engineering this translocation in normal prostate epithelial cells, RWPE1. A comprehensive view of the phenotypic effect rendered by that translocation could be gained by comparing the wild type and genetically engineered cell line.

RESULTS

My goal was to artificially induce *TMPRSS2-ETV1* translocation in normal prostate cells. The fourth exon of the *ETV1* gene fuses with the second exon of the *TMPRSS2* gene after translocation in vivo (Tomlins *et al*, 2005), as shown schematically in figure 3. As *TMPRSS2* provides the promoter allowing expression of the fused gene, this gene needed to be targeted first in order to recreate that gene fusion artificially. I needed to place the *loxP* sites after second exon of *TMPRSS2* and before fourth exon of *ETV1*. These are the translocation breakpoint regions in the respective genes (Fig 3A). The targeting vectors were constructed by employing multisite gateway cloning system based on in vitro *att*-dependent site-specific recombination, shown in figure 4. The very first step to develop the targeting vector was to amplify sequences from the regions around the intended translocation region, called "homology arms" (HAs) since they would ultimately guide the targeting vector to be integrated in the correct genomic location via homologous recombination (Fig 3A-B). The amplified PCR products were cloned in pDONR vectors to make entry clones (Fig 4B-C), which were then combined together in a single adeno-associated recipient vector (pAAV) called destination vector (Fig 4D). In this second round of recombination the HA1-pbuoys-HA2 segment was recloned into the destination vector to produce the final targeting vector pAAV-*TMPRSS2* (Fig 3B). This was then introduced in the normal prostate RWPE1 cells. The selection cassette containing two *loxP* sites flanked by HAs then should be integrated into the chromosomal genes by homologous recombination (Fig 3B-C). After ensuring the correct integration, Cre mediated recombination step would excise the selection cassette from the genome (Fig 3C-D). The p*AAV-ETV1* vector would then be introduced into the same cells (Fig 3E) and its selection cassette removed by Cre. Cre-*loxP* mediated site directed recombination system would ultimately generate the fused gene (Fig 3E-F).

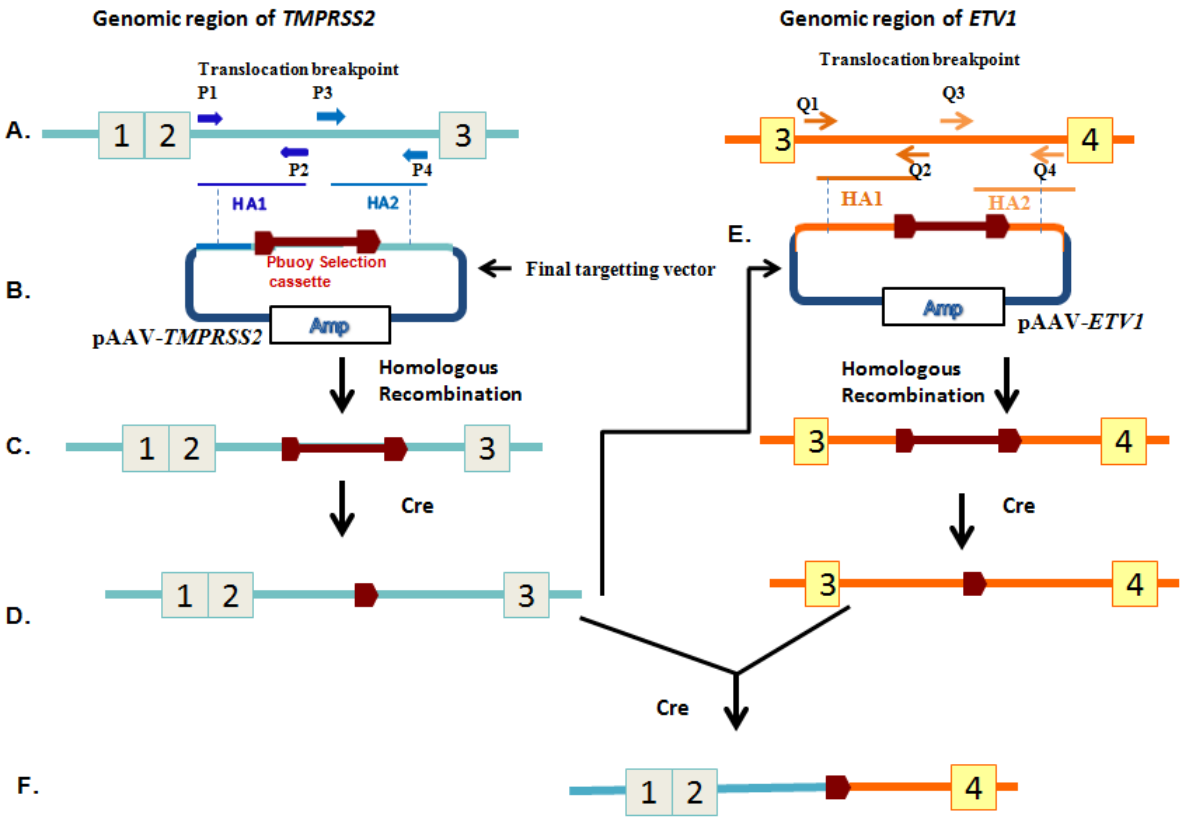

Figure 3: Overall schematic of the project. A) The blue part represents genomic region of *TMPRSS2* gene, the orange that of *ETV1*. The boxes symbolize the exons. The location of primers that were used to amplify the homology arms (HAs) are shown with small blue and orange arrows and are listed in table 2. The selection cassette is shown in dark brown flanked by two rectangles representing *loxP* sites. Sequences shown as "HA1" and "HA2" were amplified and stitched together as shown in Fig 4 to form the final targeting vectors (B and E) for two genes. The pAAV-*TMPRSS2* was delivered in the cell so that the HA sequences of the targeting vectors and the homologous sequences in the genome were recombined, resulting in integration of the selection cassette in the intended region of the targeted intron (C). Cre-mediated recombination will then excise the selection cassette retaining one *loxP* site in each gene (D). Finally translocation is expected to occur as a consequence of Cre mediated site directed recombination that would produce the fused gene (F).

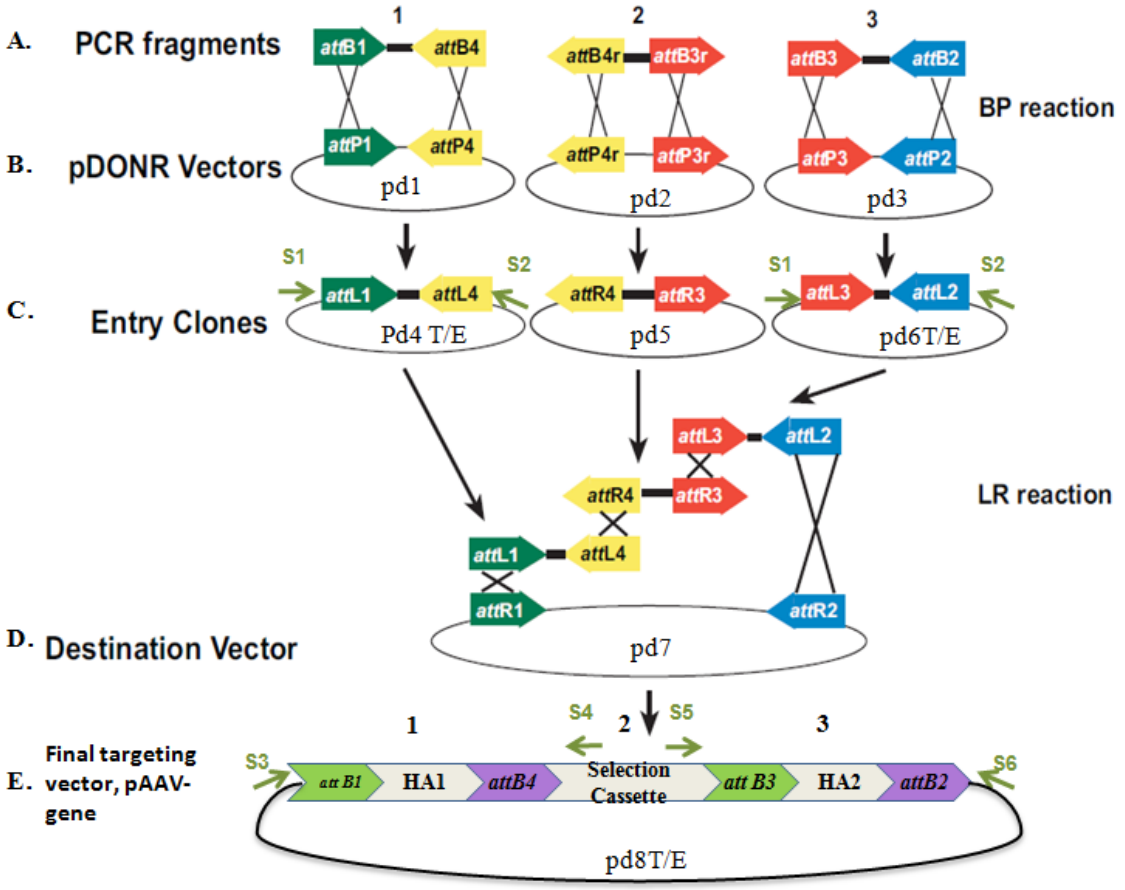

Figure 4: Development of the gene targeting vector by multisite gateway cloning.
(A) The PCR amplified HA1 and HA2, denoted 1 and 3 respectively, were different for the two genes *TMPRSS2 and ETV1*. The pbuoy selection cassette, the same for both genes, is denoted 2. All three elements were flanked by different *att*B sites. Three MultiSite Gateway® Pro Donor vectors (B) were used in separate *attB-attP* (BP) recombination reactions to generate three entry clones with the cloned segment flanked by different *attL* and *attR* sites (C). pAAV recipient vector called destination vector (D) was used to perform *attL-attR* recombination reaction to create the final targeting vector containing three DNA elements in proper order and orientation (E). All plasmids denoted here as pd are listed in table 1. S1- S6 (green arrows) denote positions of primers used for PCR screening of constructs and all these primers are listed in table 3.

Amplification and Purification of Homology Arms:

The sequences from the regions around the intended translocation region, called "homology arms" (HAs) were amplified from *TMPRSS2* (Fig 5A) and *ETV1* (Fig 5B) from genomic DNA of target prostate epithelial cells, RWPE1 using appropriate *att*B-tailed primers listed in table 2. Touchdown PCR method was employed to specifically amplify the region of interest. The PCR products were analyzed on 1% agarose gel.

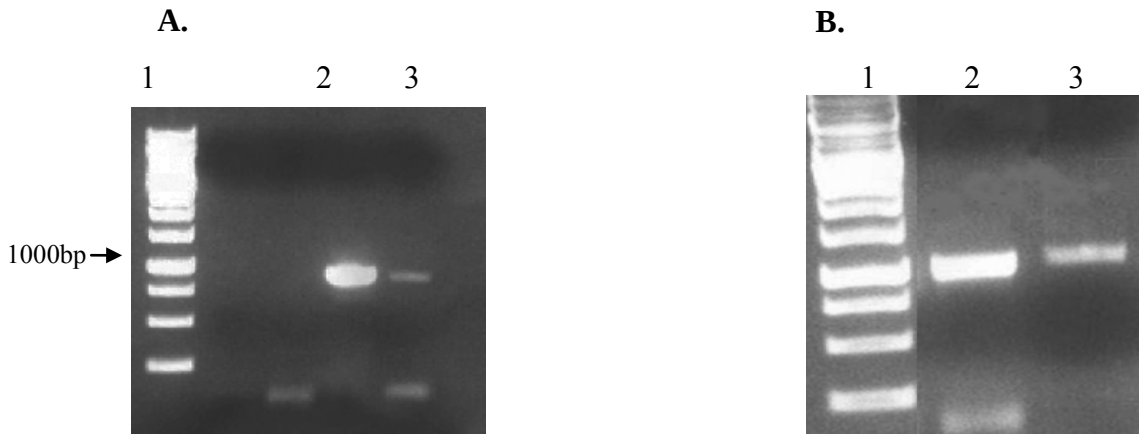

Figure 5: Homology arm amplification. A. lane 1, 1 kbp DNA ladder; lane 2 *TMPRSS2* HA1 amplified with P1, P2 primers (table 2); lane 3 *TMPRSS2* HA2 with P3, P4 primers. **B.** Lane 1, 1 kbp DNA ladder; lane 2 *ETV1* HA1 amplified with Q1, Q2 primers; lane 3 *ETV1* HA2 amplified with Q3, Q4.

Construction of entry clones:

The *att*B – *att*P (BP) recombination reaction (A → B in fig 4) was carried out to clone these *att*B flanked HAs in proper pDONR vectors containing *att*P sites to make entry clones (figure 4C). The amplified HA1 (1 in fig 4A) of *TMPRSS2* and *ETV1* was cloned in pd1 (pDONR 221 P1-P4) vector yielding entry clone pd4T (*TMPRSS2)* and pd4E (*ETV1*). Similarly the amplified HA2 (3 in fig 4A) of *TMPRSS2* and *ETV1* was cloned in pd2 (pDONR 221 P3-P2) vector and the resulting plasmids were called entry clone pd6T and pd6E. Entry clones pd4 (HA1) and pd6 (HA2) for *ETV1* (Fig 6) and *TMPRSS2* (Fig 7) were subject to PCR screening using S1, S2 primers (table 3, fig 4C) to find the positive clones with the correct insertion. The entry clone pd5 contained the pbuoy selection cassette (2 in fig 4A) which have two *loxP* sites flanking a neomycin phosphotransferase resistance gene sequence under control of internal ribosome entry site (IRES) shown in fig 10. It was required for downstream selection of positive clones with correct insertion of pbuoy selection cassette in the genome after homologous recombination.

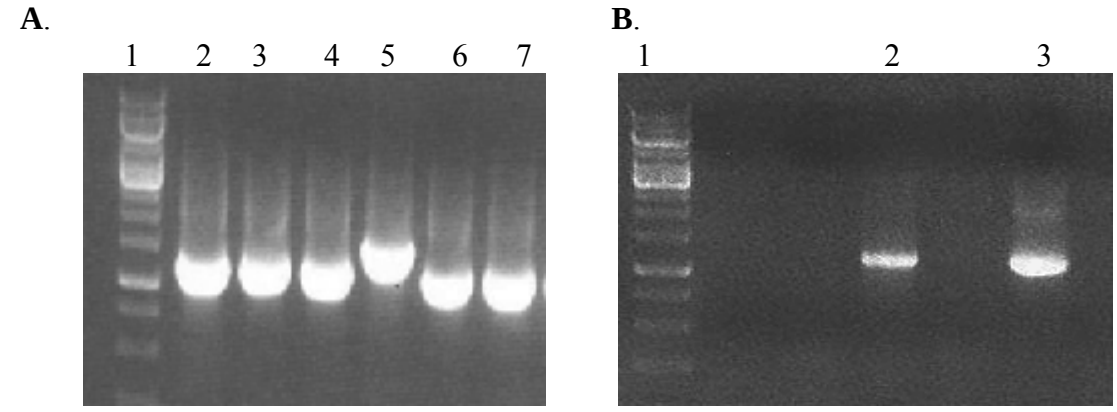

Figure 6: PCR screening of entry clones of *ETV1*. A. lane 1, 1 kbp DNA ladder; lane 2-7
PCR amplicons using S1, S2 primers from entry clone pd4E containing HA1 purified from six different bacterial colonies; B. lane 1, 1 kbp DNA ladder; lane 2-3 PCR amplicons using S1, S2 primers from entry clone pd6E containing HA2 purified from two different bacterial colonies.

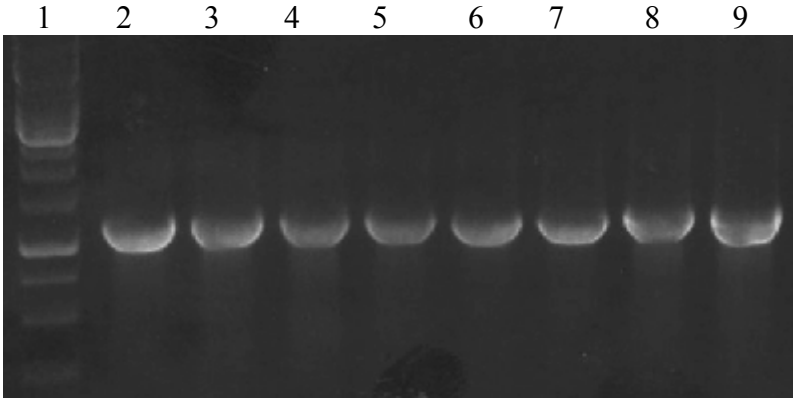

Figure 7: PCR amplification of *TMPRSS2* entry clones. Lane 1, 1 kbp DNA ladder; lane 2-5 PCR amplicons using S1, S2 primers from entry clone pd4T containing HA1 purified from four different bacterial colonies; lane 6-9 PCR amplicons using S1, S2 primers from entry clone pd6T having HA2 purified from four different bacterial colonies.

Construction of final targeting vector:

The three entry clones were stitched together by *att*L →*att*R (LR) recombination reactions into the destination vector pAAV-*ccdB*, separately for *TMPRSS2* and *ETV1* genes (step C →D in fig 4) to yield plasmids pAAV-*TMPRSS2* (pd8T containing *TMPRSS2* inserts) and pAAV-*ETV1* (pd8E containing *ETV1* inserts). The purified plasmids were screened by PCR using internal and external primer sets (Table 3) to confirm the presence and correct orientation of inserts. The PCR products for pd8E (pAAV-*ETV1)* (Fig 8) and pd8T (pAAV-*TMPRSS2)* (Fig 9) were analyzed on 1% agarose gel.

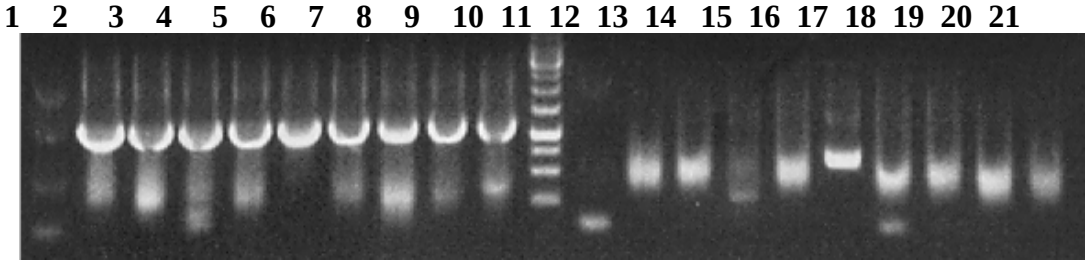

Figure 8: PCR screening of *ETV1* targeting vectors. Lane 1 and 11, 1 kbp DNA ladder; lane 2-10 PCR amplicons using S3, S4 primers from pd8E (pAAV-*ETV1*) to check HA1 insertion from ten different bacterial colonies; lane 12-21 PCR amplicons using S5, S6 primers from pd8E (pAAV-*ETV1)* to check HA2 insertion from ten different bacterial colonies;

The PCR positive *ETV1* targeting vectors were then sequenced to ensure the correct insert. The sequencing result showed that in pd8E (pAAV-*ETV1*), the HA1 of *ETV1* had not been correctly inserted in the pAAV recipient vector. HA2 was fine, but somehow HA1 had been replaced with HA1 of *TMPRSS2*. The whole construct development for *ETV1* was started over again.

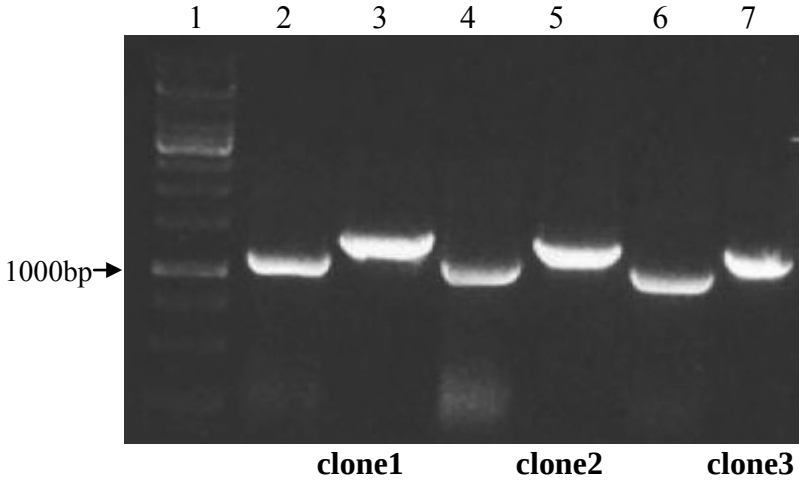

Figure 9: PCR screening of *TMPRSS2* targeting vectors. Lane 1, 1 kbp DNA ladder; lane 2, 4, 6 PCR amplicons using S3, S4 primers from pd8T (pAAV-*TMPRSS2*) to check HA1 insertion from three different bacterial colonies; lane 3, 5, 7 PCR amplicons using S5, S6 primers from pd8T (pAAV-*TMPRSS2*) to check HA2 insertion from three different bacterial colonies.

The PCR-positive pAAV-*TMPRSS2* (pd8) clones were also subjected to sequencing, showing that the HAs and selection cassette had been correctly integrated here in pAAV vector. Thus, the final targeting vector for *TMPRSS2* was ready to introduce into the RWPE1 cells.

Targeting RWPE1 cells with rAAV-*TMPRSS2*

rAAV virions containing pAAV-*TMPRSS2* were produced using a helper-free system that allows the production of infectious recombinant human adeno-associated virus-2 (AAV-2) virions without the use of a helper virus. Three plasmids were required for generation of infectious rAAV: the targeting vector pd8T (pAAV-*TMPRSS2*) and the plasmids that contain the trans-elements required for packaging (pAAV-RC and pHelper). The remaining adenoviral gene products were supplied by the AAV-293 host cells, which are HEK293-derived cells with improved adeno-associated virus production capabilities (Grimm *et al*, 1999). This rAAV-*TMPRSS2* was used to infect the prostate cells, RWPE1. However, the RWPE1 cells died two weeks after infection with rAAV-*TMPRSS2*. The targeting was repeated twice following the same procedures with less amount of geneticin (450 μg/ml) in the selection medium. All of these efforts were in vain. Geneticin inhibits protein synthesis in the eukaryotic cells. Resistance against this antibiotic is conferred by the neomycin phosphotransferase gene that was placed in the selection cassette. Only the expression of this *neo* gene would allow the cells to thrive in the growth media containing geneticin.

DISCUSSION:

The successful implementation of somatic cell gene targeting is a challenging endeavor. Targeting one allele takes three months of time, so it is a time consuming work. The whole work is a multistep process necessitating that each step is critically monitored before moving to the subsequent steps with proper controls. Methodologically, there are three basic steps to recreate the *TMPRSS2-ETV1* gene fusion: construction of targeting vectors, delivery of the vectors into the target cell and finally recombination to produce the gene fusion. All these three steps have been done for both of the genes as shown in fig 3. The final targeting vector for *TMPRSS2* was successfully generated and delivered into the RWPE1 cells. An error in constructing the *ETV1* targeting vector, premature death of targeted cells and lack of sufficient time prevented production of the final gene fusion.

Touchdown PCR reactions were utilized to precisely amplify the genomic region of interest. The early phase of thermal cycles favors accumulation of amplicons whose primer-template complementarity is the highest. During subsequent cycles the stepwise transition to a lower temperature ensure high yields by making use of the desired amplicons in the reaction that outcompetes any non-specific products (Korbie *et al*, 2008). The specific amplification of the HAs was crucially important for all the downstream steps because it would direct the homologous recombination to insert the selection cassette in the correct locus.

The observed targeting frequency in known cell lines using rAAV methods ranges from 0.2% to 70% per round of targeting. It is reasonable to anticipate a frequency of approximately 5–10% for a previously untested locus (Rago *et al*, 2007). In the current study previously untested loci and cell lines were targeted, which need optimization. The RWPE1 was selected as target cell line because the project required a karyotypically stable normal prostate cell in which *TMPRSS2-ETV1* translocation could be generated to follow the exact cellular effect rendered by that translocation. However there are no experimental reports suggesting how this cell line would behave upon rAAV-mediated transduction. In this study the cells essentially died after two weeks of targeting using a tolerable dose of geneticin (400-500 µg/ml) (Wilson *et al*, 2005). These prostate epithelial cells are non-transformed, so they do not grow on top of each other but need contact with the substrate (Tobias Sjöblom, personal communication). The premature death of targeted cells might be due to several reasons. Firstly, there might be no integration of the selection cassette in the targeted locus. Secondly, it might be possible that the integration occurred in the targeted locus but the endogenous promoter that was supposed to control the expression of selectable marker gene was not strong enough to transcribe the neomycin resistance gene to a sufficiently high level to combat geneticin in the growth medium. The *TMPRSS2* expression level can be significantly increased by induction with androgen hormone (Mani *et al*, 2009). The later reason seems to be highly probable. One way to circumvent this problem is by promoter induction. The cells will be treated with androgen after targeting to induce the endogenous promoter activity. Another way to overcome this hurdle is to use some alternative cell lines for the same experiment.

Future Perspective

If cells that are viable after targeting can be obtained, then positive clones where selection cassette has been successfully integrated in region of *TMPRSS2* where *in vivo* translocations occur will be selected by PCR screening. The screening primers have been designed (Table 2); one primer placed outside the selection cassette and the other one inside so that wild type clones and successfully targeted clones yield amplicons of different length.

Once the targeted cells containing the desired insert are found, Cre-mediated recombination will be performed. The *cre* gene will be expressed in the targeted clones from a recombinant adenovirus. The Cre enzyme works in two sequential steps, as shown in fig 3: First it would excise the selection cassette via *loxP* mediated recombination. This would leave behind a single *loxP* site in the genome. After checking this Cre excision of the selection cassette, the cells will be targeted to insert *loxP* site in the translocation breakpoint region of *ETV1* gene (fig 3, lower right part). The same steps would be repeated to place a single *loxP* site in the *ETV1* translocation breakpoint region. Then Cre mediated recombination would yield the fused gene of interest. The whole plan is schematically shown in figure 3.

Adequate time is considered necessary to target *TMPRSS2* and *ETV1* alleles iteratively. In the near future this translocation will be attempted in normal prostate cells, which will provide a valuable model system to dig deep into the molecular mechanism of this rearrangement.

MATERIALS AND METHODS

Biological materials

Target cells

The normal prostate epithelial cell RWPE1 was the target cell line. These cells have similar properties as normal epithelial cells and behave similarly in response to growth factors. These cells are responsive to androgen hormone. Most importantly they have a stable karyotype (Kleinman, 1997). RWPE-1 cells were maintained in serum free Keratinocyte-SFM (Serum free media) medium (Invitrogen Cat. No 10725) which contains 25 mg/ml of bovine pituitary extract (BPE) and 2.5 µg/ml recombinant epidermal growth factor (r-EGF), plus antibiotics (penicillin, 10000 U/ml, streptomycin 10000 µg/ml) (Invitrogen, catalog 15090). Cells were passaged upon confluence and seeded at 2×10^6 cells/ T-75 flask.

rAAV packaging cells

AAV-293 are HEK293-derived cells with improved adeno-associated virus production capabilities and were used for rAAV packaging (Stratagene, Catalog 240071). These cells were grown in a 75 cm^2 flask in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 5% penicillin–streptomycin (PS). (Invitrogen, Catalog 41966)

Bacterial cells

One Shot Mach1™ T1 phage-resistant *E. coli* cells (Invitrogen, Catalog C8620-03) were used to transformation and cloning. They were the fastest growing chemically competent strains.

Plasmids

Multisite gateway cloning plasmids were provided with the kit from Invitrogen (Catalog 12537-100). The plasmids needed to produce AAV were from Stratagene (Catalog 240071). All plasmids are shown in table 1.

Table 1: Plasmids

Plasmids	Features[a]	Source and Reference
pd1 (pDONR221 P1-P4)	Backbone vector for BP recombination to make entry clone 1; *attP1,P4* sites, *kanR*[b], *ccd*B gene	Multisite Gateway Pro (Invitrogen)
pd2 (pDONR221P4r-P3r)	Backbone vector for BP recombination to make entry clone 2; *attP4r,P3r* sites, *kanR*[b], *ccd*B gene	Multisite Gateway Pro (Invitrogen)
pd3 (pDONR221P3-P2)	Backbone vector for BP recombination to make entry clone 3; *attP2,P3* sites, *kanR*[b], *ccd*B gene	Multisite Gateway Pro (Invitrogen)

pd4T (pDONR221L1-HA1-L4)	Entry clone1; HA1 of *TMPRSS2*, *attL1,L4* sites, *kanR*[b]	This study
pd4E (pDONR221 L1-HA1- L4)	Entry clone1; HA1 of *ETV1*, *attL1,L4* sites, *kanR*[b]	
pd5 (pDONR221 R4-HA2-R3)	pBuoy selection cassette or entry clone 2; *attR4,R3* sites, NeoR[c], *loxP* sites, IRES[d], epitope[e], *kanR*[b]	Lab stock
pd6T (pDONR221 L3-Neo-L2)	Entry clone 3; HA2 of *TMPRSS2*, *attL3,L2* sites, *kanR*[b]	This study
pd6E (pDONR221 L3-Neo-L2)	Entry clone 3; HA2 of *ETV1*, *attL3,L2* sites, *kanR*[b]	This study
pd7 (pAAVR1-Cm^R-ccdB-R2)	Destination vector is the backbone vector for LR recombination to make the targeting vector; *attR1,R2* sites, *AmpR*[f], *ccd*B gene	Lab stock
pd8T (pAAV-*TMPRSS2)*	*TMPRSS2*-targeting vector; HA1 and HA2 of *TMPRSS2, attB1,B2,B3,B4* sites, NeoR[c], *loxP* sites, IRES[d], epitope[e], *AmpR*[f] ,	This study
pd8E (pAAV-*ETV1)*	*ETV1*-targeting vector; HA1 and HA2 of *ETV1, attB1,B2,B3,B4* sites, NeoR[c], *loxP* sites, IRES[d], epitope[e], *AmpR*[f]	This study
pd9 (pEXP7-tet)	Positive control for BP recombination; *TetR*[g]	Multisite Gateway Pro (Invitrogen)
Pd10 (pENTR L1-pLac-LacZα-L4)	Positive control for LR recombination; *att L1,L4* sites, *kanR*[b]	Multisite Gateway Pro (Invitrogen)
pd11 (pENTR R4-pLac-Spec-R3)	Positive control for LR recombination; *att R4,R3* sites, *kanR*[b]	Multisite Gateway Pro (Invitrogen)
pd12 (pENTR L3-pLac-Tet-L2)	Positive control for LR recombination; *att L2,L3* sites, *kanR*[b]	Multisite Gateway Pro (Invitrogen)
pd13 (pHelper plasmid)	adenovirus *E2A, E4,VA* gene	AAV helper-free System (Stratagene)
pd14 (pAAV-RC plasmid)	AAV-2 *rep, cap* genes	AAV helper-free System (Stratagene)

[a] Features relevant for my work
[b] Kanamycin resistance gene
[c] Neomycin resistance gene

[d] Internal ribosome entry site
[e] Hemagglutinin epitope
[f] Ampicillin resistance gene

Polymerase chain reaction amplification

All the primers were designed using Primer3 software and ordered as HPLC purified oligos (Sigma). Primer sequences are shown in tables 2 – 3 and fig 4 shows the regions they amplify. *TMPRSS2* and *ETV1* loci were amplified from genomic DNA obtained from RWPE1 cells using Platinum Taq DNA Polymerase High Fidelity (Invitrogen), as per manufacturer's recommendations. Touchdown PCR was performed with 6 ng of genomic DNA according to the following protocol: 96^0C for 2 min, 3 cycles of 96^0C for 10 sec, 64^0C for 10 sec, 72^0C for 90 sec, 3 cycles of 96^0C for 10 sec, 61^0C for 10 sec, 72^0C for 90 sec, 3 cycles of 96^0C for 10 sec, 58^0C for 10 sec, 72^0C for 90 sec, 3 cycles of 96^0C for 10 sec, 45 cycles of 57^0C for 10 sec, 72^0C for 90 sec, 1cycle of 72^0C for 10 min. The HA1 and HA2 reactions were pooled separately and purified using Silica Bead DNA Gel Extraction Kit (Fermentas, catalog K0513). The DNA concentration was measured using Nanodrop spectrophotometer.

Table 2 Primers A

Purpose	Primers	Sequence 5'-3'	Product size (bp)
Amplification of *TMPRSS2* HA1	P1 (*TMPRSS2* HA1 F[a])	***GGGGACAAGTTTGTACAAAAAAGCAGGCT*** GGGGACAAGTTTGTACAAAAAAGCA GGCTGTGAAAGCGGGTGTGAGG[d]	895
	P2 (*TMPRSS2* HA1 R[b])	***GGGGACAACTTTGTATAGAAAAGTTGGGTG*** GGGGACAACTTTGTATAGAAAAGTT GGGTGCGCACAAACTTTCTGGACAT[e]	
Amplification of *TMPRSS2*HA2	P3 (*TMPRSS2* HA2 F)	***GGGGACAACTTTGTATAATAAAGTTG*** GGGGACAACTTTGTATAATAAAGTT GACCCTGAGTGGTCAAGTGCTGAAT CCTGGTGGCTTGTTTG[f]	879
	P4 (*TMPRSS2* HA2 R)	***GGGGACCACTTTGTACAAGAAAGCTGGGTA*** GGGGACCACTTTGTACAAGAAAGCT GGGTACTGGTGGGAATAAGGGAAG[g]	
PCR screening of *TMPRSS2* loci after integration of selection cassette	st1 (*TMPRSS2* HA1 Scr[c] F)	GCCCGGAGGTGAAAGCGGGT	1141
	st2 (*TMPRSS2* HA1 Scr[c] R)	CCGTGAGGTCAGGC*ATT*G	
Amplification of *ETV1*HA1	Q1 (*ETV1* HA1 F)	***GGGGACAAGTTTGTACAAAAAAGCAGGCT*** GGGGACAAGTTTGTACAAAAAAGCA GGCT CCTCATGCAGAATAGGCTCA[d]	911
	Q2 (*ETV1* HA1 R)	***GGGGACAACTTTGTATAGAAAAGTTGGGTG*** GGGGACAACTTTGTATAGAAAAGTT GGGTGTCTTCTTGGGCTGAGATCAAA TCACCTGCCAGATGGAAATA[e]	

Amplification of *ETV1* HA2	Q3 (*ETV1* HA2 F)	[f]***GGGGACAACTTTGTATAATAAAGTTG*** GGGGACAACTTTGTATAATAAAGTT GGCCCTTCC*ATT*TCTCCTCCTAA	941
	Q4 (*ETV1* HA2 R)	***GGGGACCACTTTGTACAAGAAAGCTGGGTA*** GGGGACCACTTTGTACAAGAAAGCT GGGTAC*ATT*CTCTGACAGACCCAGG[g]	
PCR screening of *ETV1* loci after integration of selection cassette	se1 (*ETV1* HA1Scr[c] F)	AGCC*ATT*CAGTTACCAACCTG	1109
	se2 (*ETV1* HA1Scr[c] R)	TCTTCTTGGGCTGAGATCAAA	

[a] F, forward primer
[b] R, reverse primer
[c] Scr, screening primer
[d] ***GGGGACAAGTTTGTACAAAAAAGCAGGCT,*** *attB1* recombination *site*
[e] ***GGGGACAACTTTGTATAGAAAAGTTGGGTG***, *attB4* recombination site
[f] ***GGGGACAACTTTGTATAATAAAGTTG,*** *attB3* recombination site
[g] ***GGGGACCACTTTGTACAAGAAAGCTGGGTA,*** *attB2* recombination site

Table 3 Primers B

Use	**Primers**	**Sequence 5'-3'**	**Product size from pd4/6/8T (bp)**	**Product size pd4/6/8E (bp)**
PCR screening of pd4/6 entry clones to check the integration of HAs in vector	S1 (M13F[a])	GTAAAACGACGGCCAG	HA1: 1139 HA2:1124	HA1: 1155 HA2: 1185
	S2 (M13R[b])	CAGGAAACAGCTATGAC		
PCR screening of pd8 targeting vector to check the integration of HA1 in vector	S3 (AAV-Neo F)	GCCTTTTGCTCACATGTCCT	HA1: 1175	HA1: 1138
	S4 (AAV-Neo R)	AGGGAGTACTCACCCCAACA		
PCR screening of pd8 targeting vector to check the integration of HA2 in vector	S5 (Neo-AAV F)	TCGCCTTCTTGACGAGTTCT	HA2: 1425	HA2: 1489
	S6 (Neo-AAV R)	TGACGTATGCGGTGTGAAAT		

[a]F, forward primer
[b]R, reverse primer

Cloning:

Entry Clones

10 µl *att*B-*att*P (BP) recombination cloning reactions was set up in 1.5 ml microcentrifuge tubes using 100 ng of the *attB* flanked PCR amplified HAs and 150 ng of pDONR221 DNA; pd1 (pDONR221 P1-P4) for HA1 amplicons and pd3 (pDONR221 P3-P2) for HA2 amplicons. pd9 (50 ng/µl) and pd1 (150 ng/µl) plasmids were used as positive control. pd1 (150 ng/µl) was used as negative control. 2 µl of BP Clonase II enzyme mix (Invitrogen, Catalog 11789-020) was added to each reaction followed by incubation at 25 °C for 18 hr. The reaction was terminated by adding 1 µl proteinase K to each reaction and incubating at 37 °C for 10 min. The BP clonase enzyme which has the lambda integrase property, catalyzed the *att* site specific recombination reaction to replace *ccdB* gene in the pDONR vector by the specific HAs flanked by appropriate *attB* sites producing entry clones pd4T/E and pd6T/E (Fig 4A-C). The detailed map of entry clone pd6 is shown in fig 10.

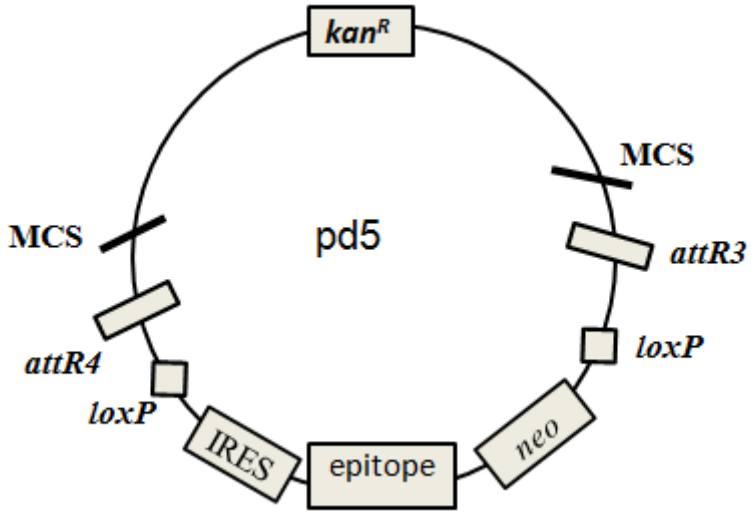

Figure 10: Map of pd5 (pDONR221 R4-Neo-R3). The vector contains multiple cloning site (MCS), kanamycin resistance gene *kan*^{R}, *attR* recombination sites and the selection cassette which is flanked by two *loxP* sites. The other components of the puoy selection cassette are internal ribosome entry site (IRES), hemagglutinin epitope tag and neomycin resistance gene (*neo)*. The IRES is a gene regulatory part that controls translation of the downstream genes. The hemagglutinin epitope is expressed in the cell membrane upon correct integration and expression of the selection cassette. The neomycin resistance gene is expressed to permit positive selection by geneticin.

Targeting clones:

The entry clones pd4T, pd6T carrying *TMPRSS2* HA1, HA2 inserts and the entry clones pd4E, pd6E with *ETV1* HA1, HA2 were separately mixed with pd7 (pAAV R1-Cm^R-ccdB-R2) recipient vector (fig 11), and LR Clonase enzyme. (Invitrogen, Catalog 12538-120). The 10 µl *att*L-*att*R (LR) recombination reactions was set up in 1.5 ml microcentrifuge tubes between 10 fmoles of each entry clone and 20 fmoles of pd7 destination vector. pd10 (10 fmole), pd11 (10 fmole), pd12 (10 fmole), pd7 (20 fmole) were the components used to set up a positive control LR reaction. pd4T/E (10 fmole), pd6T/E (10 fmole), pd7 (20 fmole) plasmids were used as negative controls in the LR reaction. The positive control was added to make sure that the LR recombination occurred correctly among the control vectors. In the

negative control LR reaction, no recombination would occur as it lacked one essential plasmid containing selection cassette. After transformation, lots of colonies supposed to grow in positive control plates whereas the negative control plates should not have any colonies due to retention of *ccdB* gene for lack of recombination. 2 µl of LR Clonase II enzyme mix was added to each reaction which was incubated at 25 °C for 18 hr. The reaction was terminated by adding 1 µl proteinase K to each reaction and incubating at 37 °C for 10 min. Recombination between *attL* sites in the entry clones and *attR* sites in the destination vector generated final targeting construct called pd8T (pAAV-*TMPRSS2*) and pd8E (pAAV-*ETV1*) (Fig 4E) by removal of *ccdB* gene from the pAAV recipient vector.

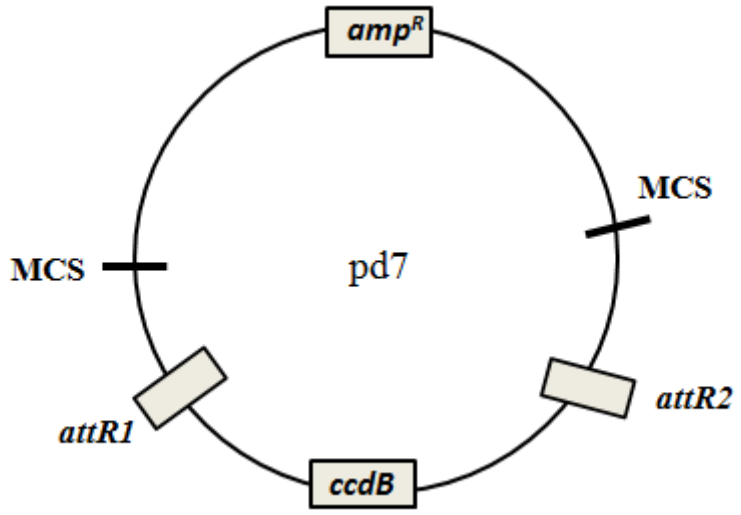

Figure 11: Map of pd7 (pAAV R1-Cm^R-ccdB-R2) destination vector. The pAAV recipient or the back bone vector contains the *ccdB* gene which is replaced by the gene in the selection cassette flanked by two HAs because of site specific recombination catalyzed by LR clonase. The vector also contains ampicillin resistance genes *amp^R*, the multiple cloning sites (MCS) and *attR* recombination sites.

Transformation and plasmid extraction:

Half of each BP and LR reaction was added to 50µl of one shot mach1 chemically competent *E coli* cells and heat shocked at 42 °C for 30 sec. 250 µl of LB medium (1 liter Luria broth contained 10 g tryptone, 5 g yeast extract, 10 g NaCl) was added and the samples incubated at 37 °C for 1 hour. The transformed cells from the respective reactions were spread on LB-agar (1 liter LB-agar prepared by mixing 10 g tryptone, 5 g yeast extract, 10 g NaCl and 20 g agar) plates containing 50 µg/ml kanamycin to select positive entry clones (BP reactions) and 50 µg/ml ampicillin for targeting clones (LR reactions). The positive control BP reaction was spread on LB-agar plate having 20 µg/ml tetracycline. The plates were incubated at 37 °C overnight. On the following day 3-5 clones were grown in 5 ml of LB containing 50 µg/ml ampicillin and kanamycin respectively by incubating at 37 °C for 8-12 hours. The cultures were then centrifuged at 6800 x g for 15 min at 4°C. The supernatant containing LB was removed and the DNA extracted from each clone using gene JET™ plasmid miniprep kit (Fermentas, catalog K 0502). The DNA concentration was measured using a Nanodrop, spectrophotometer. The clones were screened by PCR using primers S1-S6 (Table 3; location of the primers shown in fig 4). Touchdown PCR was performed with 20 ng of purified plasmid DNA according to following protocol: 96^{0}C for 2 min, 3 cycles of 96^{0}C for 10 sec, 64^{0}C for 10 sec, 72^{0}C for 90 sec, 3 cycles of 96^{0}C for 10 sec, 61^{0}C for 10 sec, 72^{0}C for 90

sec, 3 cycles of 96^{0}C for 10 sec, 58^{0}C for 10 sec, 72^{0}C for 90 sec, 3 cycles of 96^{0}C for 10 sec, 45 cycles of 57^{0}C for 10 sec, 72^{0}C for 90 sec, 1cycle of 72^{0}C for 10 min. In order to analyze the PCR products 1% agarose gel containing ethidium bromide (ETBr) was run in 1XTAE (40 mM tris-base, 20 mM acetic acid and 1 mM EDTA) buffer at 120 volt for 40 min and then exposed to UV. The PCR positive clones were then subject to sequencing from Uppsala genome centre (UGC).

Transfection and virus production

Stratagene's AAV Helper-Free System (Stratagene, Catalog 240071) was used to deliver the final construct in the target cell line. 2.5 µg of pd13 (pHelper), pd14 (pAAV-RC) and pd8T (pAAV-*TMPRSS2)* plasmids were mixed and Opti-MEM reduced-serum medium (Invitrogen) was added to a total volume of 750 µl. In a separate tube, 54 µl of 2 mg/ml lipofectamine (Invitrogen, Catalog 18324-012) was diluted in Opti-MEM to a total volume of 750 µl. The DNA solution was added dropwise to the lipofectamine solution and the samples were then incubated at room temperature for 15 min. In the meantime, 70-80% confluent AAV-293 cells washed with Hank's Buffered Salt Solution (HBSS) (Invitrogen, Catalog H6648) and 7 µl DMEM were added to the cells and incubated for 15 min. The DNA-lipofectamin solution was then added dropwise to the cells and mixed gently. The tissue culture plate was incubated for 3-4 hours, and then the transfection medium was replaced with DMEM growth medium. The cells were returned to the 37°C incubator for 72 hr. The signs of viral production, cell sloughing and color change in the medium from red to orange or yellow, were monitored.

AAV293 cells were pooled together with the medium into 15 ml conical tube. The cell suspension was subjected to four rounds of freeze-thawing (freezing: dry ice-ethanol bath, thawing: 37 °C water bath followed by brief vortex), 10 min each. Cellular debris was collected by centrifugation at 12,000 × g for 10 minutes at 4 °C. The supernatant containing the recombinant adeno-assiciated virions (rAAV-*TMPRSS2*) was recovered and stored at -80°C.

Infecting the RWPE1 cells with rAAV-*TMPRSS2*

When the RWPE1 cells reached a confluency of 60% - 80% in a 75 cm^2 flask, they were targeted with rAAV-*TMPRSS2*. The first step was to wash the cells with 5 ml HBSS. Then 350 µl of the cleared virus-containing lysate was directly added to the cell monolayer. 4 ml of growth medium was further added to the flask, which was then kept in a 37 °C incubator. After 2-3 hour 8 ml of growth medium was added and the cells were allowed to grow for 48 h. The targeted cells were washed with HBSS and trypsinized to harvest in 150 ml keratinocyte-SFM medium/PS containing 500 µg/ml geneticin (Invitrogen). 100 ml of this selection medium containing trypsinized cells was distributed to five 96-well plates. The remaining 50 ml of the cell containing selection medium was diluted three fold in the same way. The plates were wrapped with thin plastic and incubated at 37 °C for 2-4 weeks for homologous recombination to occur between the construct and target locus. The wells that had single colonies were identified and marked. The positive wells containing transgenic cell clones were consolidated in a new 96 well plate when the majority of colonies reached a size that occupied 30–60% of the well bottom (Rago, 2007).

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