
Rational Design and Construction of a Knock-Out Vector for Targeting the Decoy Type 2 Interleukin -1 Receptor

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Abstract: Given the limited data describing the phenotype for type 2 interleukin -1 receptor (Il1r2^{-/-}) in mouse strains and based on the decoy role of the gene product (IL-1R2), we hypothesized that IL-1R2 may have a unique but similar inhibitory role to IL-1R antagonist (IL-1Ra) in vivo. Despite the anti-inflammatory function of IL-1R2, its role in disease in vivo remains unclear. Therefore, we designed and implemented a knock-out construct template for a mouse embryonic stem (ES) cell line that can be used to easily make knockout mice. Bacterial artificial chromosome (BAC) clone of Il1r2 from mouse strain AB2.2 and ES cells from same strain were obtained and a long chain PCR was performed to isolate homologous arms containing homologous segments (8kb and 2kb). Short segments were cloned out for use as probe sequences and a construct with deletion in exon 3 was made so it can be removed in vivo. The *NeoR* in cloning vector was then flanked with loxP elements. The two homologous arms were successfully amplified with a truncation in exon 3 of the gene and the wild type cloning plasmid (p1049) was serially modified with loxP elements. One of the successfully transformed plasmid DNA was used as the starting material for the ligation of the subsequent loxP-PacI linker. This plasmid (p1049XL) was amplified in *E. coli* DH5 α cells and DNA extracted. The loxP-PacI linker was successfully ligated into the plasmid and transformed clones screened with MspI and compared to the virtual digest of the theoretical plasmid containing the insert and subsequently sequenced. This study has provided the basic ingredients for making an Il1r2-deficient mouse in order to adequately characterize the phenotype. By assembling the complete knock out construct from templates already provided in this study for the knock out in embryonic stem cells, Il1r2-deficient mice could be made.

Keywords: IL-1R2, Embryonic Stem Cell, BAC Clone, Long Chain PCR, Loxp Elements, Phenotype, Homologous Segments

1. Introduction

The pathogenesis of a variety of diseases in humans is mediated via the activities of the pro-inflammatory cytokine IL-1. Unlike other cytokines, 2 endogenous inhibitors tightly regulate the biological or pro-inflammatory activities of IL-1 [1, 2]. These include IL-1 receptor antagonist (IL-1Ra), which competitively blocks the interaction between IL-1 and its signaling receptor IL-1R type 1 (IL-1R1); and the less documented IL-1 receptor type 2 (IL-1R2), which acts as a decoy receptor for IL-1 [2]. Though structurally similar to the

IL-1R1, IL-1R2 is incapable of conducting IL-1 transmembrane signaling because it has a short 29 amino acid cytoplasmic domain and lacks the Toll/IL-1R region [2, 3]. Hence, it has been termed the prototypical decoy receptor [3]. Interestingly, IL-1R2 has a higher affinity for IL-1 than the IL-1R1 and this further promotes the inhibitory activity of IL-1Ra [4].

By competing with IL-1R1 for IL-1 and forming an IL-1R2/IL-1R accessory protein (IL-1R2/IL-1RAP) complex, IL-1R2 negatively regulates IL-1-dependent intracellular signaling. The IL-1R2/IL-1RAP complex sequesters both the ligand (IL-1) and the accessory protein (IL-1RAP) that

initiate the IL-1-dependent intracellular signal transduction [5, 6].

The expression of IL-1R2 is more confined, with neutrophils being the main source in mouse, unlike the ubiquitous IL-1R1 [7]. IL-1R2 is expressed in both membrane-bound (decoy receptor) and soluble form (sIL-1R2, binding protein). During acute inflammatory stimulation, anti-inflammatory molecules including IL-4, IL-13 and glucocorticoids increase the surface expression of IL-1R2. In contrast, pro-inflammatory mediators including TNF- α , leukotriene B4, lipopolysaccharide (LPS) etc. initiates the proteolytic cleavage of membrane-bound IL-1R2 [7, 8-10] resulting in the release of sIL-1R2. Circulatory sIL-1R2 binds to (buffers) leaked IL-1 from inflammation sites [11]. These observations are indicative of an anti-inflammatory role of IL-1R2 [1] and its association with some diseases has been established.

Early stage endometriosis is associated with decreased concentrations of serum soluble IL-1R2 [12, 13], and an imbalance in IL-1/IL-1R2 expression in the endometrial tissues of women with endometriosis compared with healthy women. On transfecting these endometrial tissues with cDNA of IL-1R2, a reduction in the inflammatory activity of IL-1 via a decrease in its induction of monocyte chemoattractant protein-1 (MCP-1) and vascular endothelial cell growth factor (VEGF) was observed [14]. Furthermore, in situ hybridization showed significant decrease in the mRNA expression of IL-1R2 and a concomitant increase in IL-1 mRNA in these women [15].

The increased shedding of IL-1R2 seen in patients with Alzheimer's disease may also be a protective mechanism against the effects of IL-1 [16]. Increased cerebrospinal fluid levels of soluble IL-1R2 have been observed in individuals with Alzheimer's disease [17], and this could be a marker of disease progression [2]. The tissue specific anti-inflammatory activity of IL-1R2 and its therapeutic potentials in non-systemic chronic inflammatory conditions such as psoriasis and periodontitis have also been demonstrated [18, 19].

The above evidence indicates that IL-1R2 may have therapeutic and other possible potentials. However, these experiments may not give the true functional role of the gene in vivo as would the time-tested targeted gene deletion. Interestingly, despite the overwhelming evidence supporting the mitigating role of IL-1R2 on IL-1 induced inflammation, there is yet limited data on well-documented phenotype of IL-1R2^{-/-} mouse model. Additionally, due to the potential existence of significant regional variations in IL-1R2 expression within the entire body and within individual organs, tissue-specific IL-1R2^{-/-} mice are especially important to enhance our understanding of IL-1R2 bioaction [2].

The phenotype for the IL-1R2 gene is yet to be fully characterized because of paucity of IL-1R2^{-/-} mouse. This may be due to a combination of factors including low understanding and enthusiasm on the therapeutic potential of IL-1R2 and previously unsuccessful attempts at knocking out the gene. However, recent evidence has shown that IL-1R2 is

a very important regulator of IL-1 function and as such may play a key role in the pathophysiology of dysregulation of IL-1 [2]. Recently, an IL-1R2-deficient mice were created in which the role of endogenous IL-1R2 in a mouse model of K/BxN serum transfer-induced arthritis was investigated. It was reported that IL-1R2 plays a vital inhibitory role in local IL-1- and neutrophil-induced tissue inflammation and appears to be less important for systemic responses to acute LPS and IL-1 administration in contrast to IL-1Ra [1].

Therefore, we designed and implemented a knock-out construct templates for a mouse embryonic stem (ES) cell line that can be used to easily make knockout mice. To achieve this, Bacterial artificial chromosome (BAC) clone of *Il1r2* from mouse strain AB2.2 and ES cells from same strain were obtained and a long chain PCR was done to isolate homologous arms containing homologous segments (8kb and 2kb) (Figure 1a). Short segments were cloned out for use as probe sequences and a construct with deletion in exon 3 was made (Figure 1b), so it could be removed in vivo, the *NeoR* in cloning vector was then flanked with loxP elements (Figure 1c).

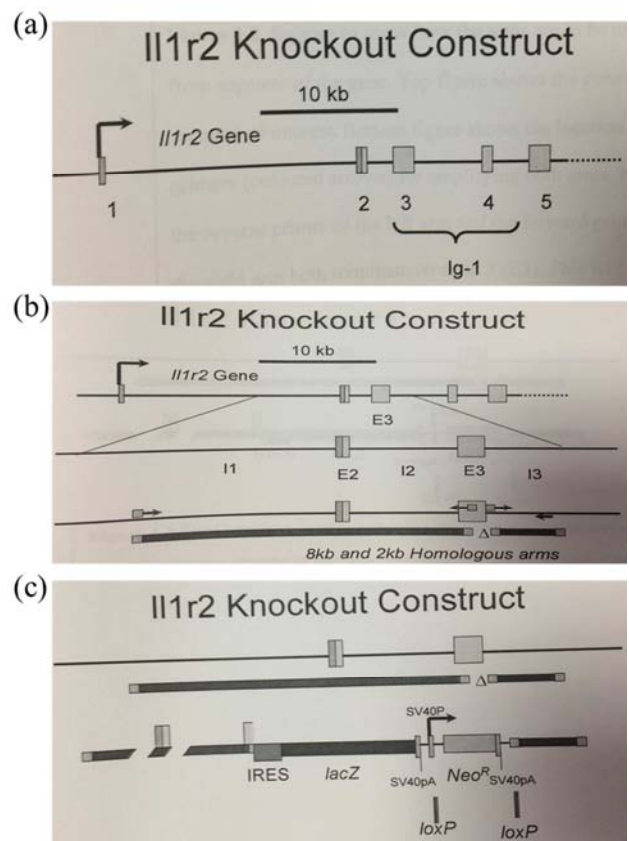


Figure 1. (a) Homologous arms and the introns and exons within the segment of interest. (b) Amplification of the arms from each segment of the gene. Genetic segments of interest (top) and location of the primers for amplifying both arms (bottom arrows). Note that both the reverse primer of the left arm and forward primer of the right arm terminate on exon 3 (E3). This produced 2 arms with a deletion in exon 3. (c) Final knockout construct showing the 2 homologous arms with the selection cassette in between. The *NeoR* is flanked on both sides by the loxP elements so that it can be removed by Cre-recombinase enzyme in vivo.

2. Materials and Methods

Unless otherwise stated, all solutions were prepared using sterile deionized water.

2.1. Bacterial Artificial Chromosome Extraction

Bacterial cultures to grow the BAC clone in *E. coli* DH10B for DNA extraction were prepared from 7.5% glycerol stock. Cultures were grown in 50 ml lysogenic LB broth containing 125 µg/ml chloramphenicol at 225 rpm, 37°C for 24 hours. The bacteria grow slowly in these conditions, but plasmid yields were improved compared to normal growth conditions.

DNA was extracted by centrifuging 10 ml of culture at 1500 g for 10 min at 4°C and the supernatant discarded. The pellet was then resuspended in GTE (50 mM Glucose/15 mM TrisHCl/10 mM Tris/10 mM EDTA) solution, lysed by adding freshly prepared solution of 0.2 M NaOH/1% NaDodSO₄ and mixed gently by inversion followed by addition of 3 M Potassium acetate/2 M acetic acid. This was then incubated on ice for 10 mins and centrifuged at maximum speed (13000 g) for 15 mins. DNA was precipitated from the supernatant in a fresh tube by adding isopropanol and centrifuging at maximum speed for 10 mins and supernatant discarded. The nucleic acid-containing pellet was then washed with 70% ethanol, dried and resuspended in 0.1 mM EDTA/6 mM TrisHCl/4 mM Tris (TE 0.1, filter sterile) and RNase A was added to a final concentration of 50 µg/ml of DNA.

Mini preparations for the 8 kb plasmid vector (wild type and modified) were done using essentially the same protocol.

2.2. Ligation Reactions

The 8 kb plasmid was sequentially modified by ligation reactions of digested plasmid with appropriate linkers. This was in order to achieve the desired modified plasmid with two loxP linkers in the same orientation to flank the g418 resistance cassette and its promoter and the left and right homologous arms. The loxP linkers were each designed to fit within a cleaved restriction site.

2.2.1. Ligation of Xba-loxP Linker

The p1049 has a single XbaI site adjacent to the NeoR gene. This was exploited to add the first loxP element. The plasmid was digested with XbaI to linearise the plasmid and then dephosphorylated. The linkers XbaLPR and XbaLPL (Table 1) synthesized by Eurogentec were designed to fit into a cleaved XbaI site. The linkers were annealed by heating the mixture in Styrofoam rack in a 100 ml beaker to boiling point and cooled on ice for 15 mins to allow for annealing. Ligation of Xba-loxP linker into plasmid was then performed using graded concentration of linkers (2000 fmol (5 µl), 600 fmol (1.5 µl), 200 fmol (0.5 µl) of Xba-loxP linker). 10 µl ligation mix contained 1 µg in 2 µl of linearised and dephosphorylated p1049, 1 µl of T4 DNA ligase buffer (GBCOBR), 1 µl of T4 DNA ligase from the same supplier, linkers and sufficient water to make a final volume. Two control reactions; without

Xba-loxP linker only and without both linker and ligase were also prepared to assess the efficiency of ligation. Mixtures were briefly mixed and centrifuged together and incubated at 14°C for a minimum of 16 hours.

2.2.2. Ligation of Pac-loxP Linker

The Pac-loxP linker was ligated into the plasmid that has been modified by the addition of an Xba-loxP linker. Essentially the same procedure was followed except that the modified plasmid (p1049XL) was linearised with PacI. The Pac-loxP linkers had in this case added PacI restriction site. These were, PacLPR and PacLPL synthesised by Eurogentec (Table 1).

2.3. Bacterial Transformation

Bacterial DNA transformation was done using competent *Escherichia coli* (DH5α) cells. Cells were thawed on ice and 100 µl of the competent cells were dispensed into 1.5 ml tubes on ice and 5 µl ligation mixture was then added and left on ice for 30 mins and then heat shocked in a water bath at 42°C for 2 mins. 200 µl of 2x TY (16 g bactotryptone/10 g yeast extract/5g NaCl) was gently added to each tube, gently mixed, and allowed to recover at 37°C for 40 mins on a shaker at 225 rpm. The transformation mix was plated on agar plates containing the appropriate antibiotic and incubated overnight at 37°C. The transformation efficiency was calculated using pUC19 colony count in which 40 ng of pUC19 was included in place of ligated DNA.

2.4. Restriction Endonuclease Enzyme Digest of p1049 Plasmid Vector and BACC DNA

Several restriction enzyme digests were carried out on the BACC (bMQ456A19) and the unmodified/modified plasmid vector. In each case, a virtual digest was also done using the online NEBcutter V2.0 software for comparison. These enzyme digests were used as screening tools in many instances.

2.4.1. XhoI and BamHI Digest of BACC DNA

To ensure that the Il1r2 gene was present without deletion, the BAC clone was digested with XhoI and BamHI and the fingerprint of the DNA compared with a simulated digest using NEBcutter V2.0 operating on the predicted sequence of bMQ456A19. A 40 µl reaction comprising 24 µl water, 4 µl of 10x buffer D and E for XhoI and BamHI respectively, 2 µl of XhoI and BamHI (Promega) and 10 µl of miniprep DNA was made, mixed and incubated at 37°C for 1 hour and restriction enzyme inactivated by heating. The sample was ran at 80V on a 0.6% agarose gel (Invitrogen) in 1x TAE (1 M Tris/0.4 M acetic acid/ 2 M EDTA) containing 0.2 µg/µl ethidium bromide. HindIII digested lamda DNA marker was included for size determination.

2.4.2. PacI and XbaI Digest of Plasmid Vector

The plasmid vector p1049 was digested with XbaI and the modified plasmid p1049XL was digested with PacI in order to ligate the Xba-loxP and Pac-loxP linkers respectively. The

digestion cuts the plasmid at the single PacI and XbaI site respectively leading to linearization of the circular plasmid for subsequent ligation of linkers. 36 µl of water, 10 µl XbaI buffer D (Promega), 4 µl of XbaI enzyme (Promega), 50 µl of p1049, mixed and incubated at 37°C for 1 hour and enzyme inactivated by heating to 65°C for 20 mins. Essentially the same procedure was used for the PacI digest.

2.4.3. XmnI Screening

In order to check for successful ligation of Xba-loxP into p1049, XmnI digest was done on p1049 and the DNA from transformed clones post ligation and compared to the virtual digest on NEBcutter V2.0. The addition of the 42 bp Xba-loxP linker on successful ligation is expected to increase the size of the plasmid by 42 bp and an added XmnI site within linker which is not in the wild-type plasmid.

About 23 clones were screened first by digesting with Asp700 which is an isoschizmer of XmnI but partial digestion was noticed so clones had to be screened using XmnI. The positive clones were selected for sequencing.

XmnI digest of DNA from clones was done by preparing a master mix of 12.75 µl of water, 2 µl of XmnI buffer and 0.25 µl of XmnI enzyme (Promega) per sample was made for the number of clones. 15 µl of the master mix was added to 5 µl of DNA sample from each clone and incubated for 1 hour at 37°C and then ran on a 0.8% agarose gel.

2.4.4. MspI Screening

MspI restriction endonuclease enzyme was used to screen plasmid extracted from clones after ligation of Pac-loxP linker into p1049XL. The same was done on sample p1049XL, which served as control for the screened colonies. Post transformation digest of miniprep DNA using MspI was done per reaction by preparing 5 µl of DNA prep, 2 µl of MspI buffer (Promega), 0.5 µl of MspI (Promega), 12.5 µl of water and incubating for 1 hr at 37°C. 12 µl of 6x loading dye (Promega) was added and 12 µl of each reaction loaded in a 2% agarose gel at 150V in ethidium bromide using 100 bp ladder.

2.5. Dephosphorylation Reaction

Prior to ligation of each linker into plasmid, dephosphorylation reaction of linearized plasmid was performed to remove the phosphate groups at the ends of the plasmid. This reduces the plasmid self-ligation which would

otherwise overwhelm the products. In each case the plasmid was first digested with the appropriate enzyme and subsequently subjected to dephosphorylation.

The 100 µl of digestion reaction of p1049 using XbaI enzyme was inactivated by heating to 65°C for 20 mins to which 5 µl of 20 mM Zinc acetate and 2 µl of calf intestinal phosphatase (Promega) was added. This was incubated at 37°C for 1 hour and the calf intestinal phosphatase was inactivated by adding 3 µl of 0.5 M EDTA and then extracted with 50/50 phenol/chloroform. DNA was precipitated with 100% ethanol, washed with 70% ethanol, dried and dissolved in 20 µl of TE (6mM/TrisHCl/4mM Tris/ 1mM EDTA).

A similar procedure as above was followed for p1049XL except that the plasmid was linearised with PacI enzyme and bacterial alkaline phosphatase (BAP, GIBCOBRL) was employed.

2.6. Polymerase Chain Reactions

2.6.1. Flanking Sequences

To test for the presence of II1r2 and to prepare probes for future use in southern blots, PCR was employed to amplify left and right flanking sequences outside the region of II1r2 that was to be used for homologous recombination. These primers (Table 1) were chosen to create probes with no repetitive elements that are identified in build 36 of the mouse genome with the University of California, Santa Cruz (UCSC) genome browser. The right (RFR2PR/RFR2PL) and left (LFR2PR/LFR2PL) flank primers synthesized by Eurogentec in 100 µM solution was diluted in TE0.1 to make 10 µM stock of each primer. 40 µl reactions were set up containing 2 µl of DNA, 4 µl of each of the four primer mixes, and 34 µl of the master mix. The PCR master mix was made of 240 µl of 5x Go-Taq Green reaction buffer (Promega), 750 µl of fresh sterile water, 240 µl of 10 mM dNTPs, 6 µl of Go-Taq polymerase (Promega). Three control reactions were also made containing LFR2PL/RFR2PR and RFR2PL/LFR2PR and TE0.1 only. For the reactions, serial dilutions of the approximately 40 ng/µl BAC DNA stock was made with TE0.1 to give 4 ng/µl, 1.2 ng/µl, 0.4 ng/µl, 0.12 ng/µl, and 0.04 ng/µl concentrations respectively. The amplification reaction was run for 25 cycles as follows: T1 (95°C, 2 mins), T2 (95°C, 40 secs), T3 (53°C, 40 secs), T4 (72°C, 40 secs), T5 (72°C, 30 secs), and then held at 15°C with the lid kept at 105°C to prevent evaporation.

Table 1. Primer and Linker Sequences.

Primer	Sequence 5' → 3'
XbaLPR	CTAGAATAACTTCGTATAGCATACATTATACGAAGTTATT
XbaLPL	CTAGAATAACTTCGAATAATGTATGCTATACGAAGTTATT
PacLPR	TAAATAACTTCGTATAGCATACATTATAGCAAGTTATTTAAT
PacLPL	TAAATAACTTCGTATAATGTATCGTATAGCAAGTTATTTAAT
LFR2PL	GTGGGTCATCTGAGCCTTT
LFR2PR	CAAGCAAACCCACAGGCACT
RFR2PR	AGGCAAGTGCTCTCTCCTGT
RFR2PL	GACCATGACTCTCCCCACAC
RAR2PR	CAGCGGCCGCCACAAGGTGCCACATCACAC
RAR2PL	TAGCGGCCGACATTCAGGTAAGGCTCTTTGGGTCT
SHORT LAR2PR	GAAACTGACAGTTGTCTCCATGGACTGT
SHORT LAR2PL	CACACCAACTAGAGAACCAGTTAGTC

2.6.2. PCR for 2 kb Right Arm

To amplify the 2kb right arm of the knock out construct, primers RAR2PR and RAR2PL (Eurogentec) were employed. A reaction master mix for 6 reactions containing 293 μ l of water, 30 μ l of 10x PfuUltra II Fusion HS DNA polymerase buffer (Stratagene), 7.5 μ l of 10 mM dNTPs mix was made. 2-, 4- and 8-fold dilution of BAC DNA (40 ng/ μ l) to give 20 ng/ μ l, 10 ng/ μ l and 5 ng/ μ l of miniprep DNA for each reaction. A 50 μ l reaction was set up in 6 thin walled PCR tubes comprised of 46 μ l reaction mix, 1 μ l of each primer pair, 1 μ l of miniprep BAC DNA, 1 μ l of PfuUltra II Fusion HS DNA polymerase (Stratagene). Three control reactions were also made two of which contained just one primer each and one without DNA. Each tube therefore contained 40.5 μ l of water, 5 μ l of 10x buffer, 0.5 μ l dNTPs (25 mM of each), 1 μ l of miniprep DNA, 1 μ l of polymerase and 1 μ l of each primer (except the controls). The amplification reaction was performed with the following criteria: T1 (95°C, 2 mins), T2 (95°C, 40 secs), T3 (58°C, 30 secs), T4 (72°C, 60 secs), T5 (72°C, 5 mins), run for 15 cycles and held at 15°C with lid at 105°C.

2.6.3. PCR for 8 kb Left Arm

Reaction master mixture for 3 reactions was made with 119.5 μ l of water, 15 μ l 10x PfuUltra II Fusion HS DNA polymerase buffer, 3.75 μ l of dNTPs. Three reactions including a control were set up using 46 μ l of reaction mix and 80 ng and 20 ng miniprep DNA, instead of the usual 5 ng, 10 ng and 20 ng. This was because previous reactions with these BAC DNA concentrations yielded no products on gel electrophoresis. Each reaction contained 40.5 μ l of dH₂O, 5 μ l 10x PfuUltra II Fusion HS DNA polymerase buffer, 0.5 μ l of dNTPs mix (25 mM of each), 1 μ l SHORT LAR2PR (10 μ M), 1 μ l SHORT LAR2PL (10 μ M), 2 μ l of miniprep BAC DNA, 1 μ l of PfuUltra II Fusion HS DNA polymerase. The control reaction had just one primer. The amplification parameters were; T1 (95°C, 2 mins), T2 (95°C, 20 secs), T3 (68°C, 20 secs), T4 (72°C, 3 mins), T5 (72°C, 3 mins), run for 30 cycles and held at 15°C, with lid at 105°C.

3. Results

3.1. PCR Amplification of BAC DNA Segments

Specific segments of BAC DNA were amplified by PCR for use as homologous arms in plasmid vector or as probes as well as for screening purposes.

3.1.1. Flanking Sequences

Flanking sequences outside the homologous arms were amplified for use as southern probes and to determine accuracy/completeness of the required gene segments. Specific primers that do not anneal to the repetitive sequences of the genome were designed. The primers for the left flank probe extended between bases 40039034 to 40039404 on the mouse chromosome 1 genome and gave a

371 bp product, while the right flank probe extended from 40053319 to 40053633 with an expected 315 bp product (Figure 2). There was no DNA amplification in the control samples.

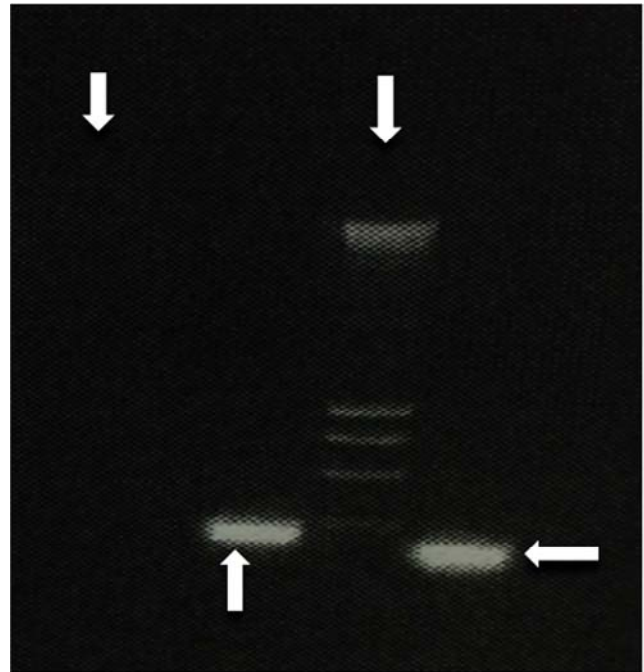


Figure 2. 1.4% gel electrophoresis of amplified left and right flanking sequences. The up pointing and left pointing arrows show the 371 bp left flanking and the 315 bp right flanking sequences respectively. The down pointing left arrow shows the control reaction with no amplicon and the 1 kb plus DNA ladder is indicated by the down pointing right arrow.

3.1.2. Probe Sequences

Apart from the flanking sequences, other primers were designed for use to amplify certain segments of the gene. Primers amplifying short segments of the DNA were designed for testing the 8 kb primers. The 8 kb primers showed great promise as the test primer used in conjunction with the 8 kb forward primer amplified the desired product length (Figure 3).

3.1.3. 2 kb Right Arm

Using primers RAR2PR and RAR2PL, the 2 kb right arm was amplified from the BAC DNA. Three different concentrations of DNA (5 ng/ μ l, 10 ng/ μ l and 20 ng/ μ l) were used. Two reactions using just one of the primer pair and water, and another without DNA were used as controls. The gel electrophoresis showed 3 clear bands of about 2 kb with decreasing intensity from 20 ng/ μ l to the 5 ng/ μ l DNA concentrations used. The bands were at the appropriate positions compared to the lambda DNA marker (Figure 4). The primer sequences were chosen so that the primer (RAR2PL) is located within the third exon of the il-1r2 gene, which codes for part of the extracellular immunoglobulin domain of the receptor.

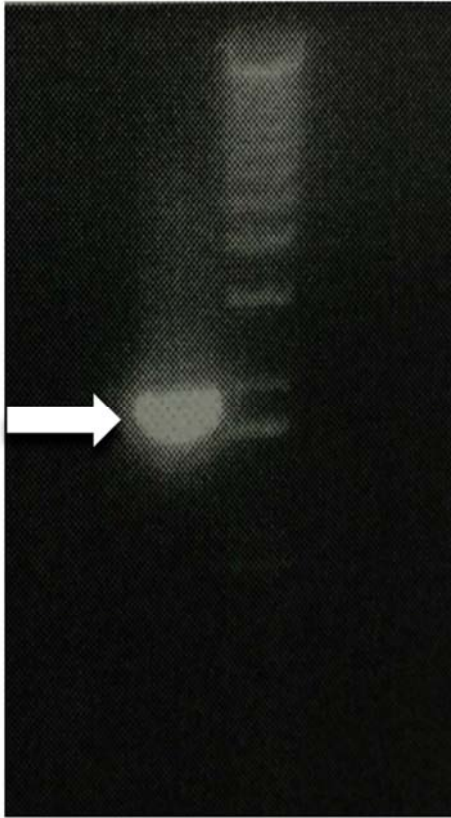


Figure 3. 0.8% gel electrophoresis showing amplicon corresponding to 1.8 kb. The PCR was done using a test primer with the 8 kb forward primer to prove its efficiency/accuracy.

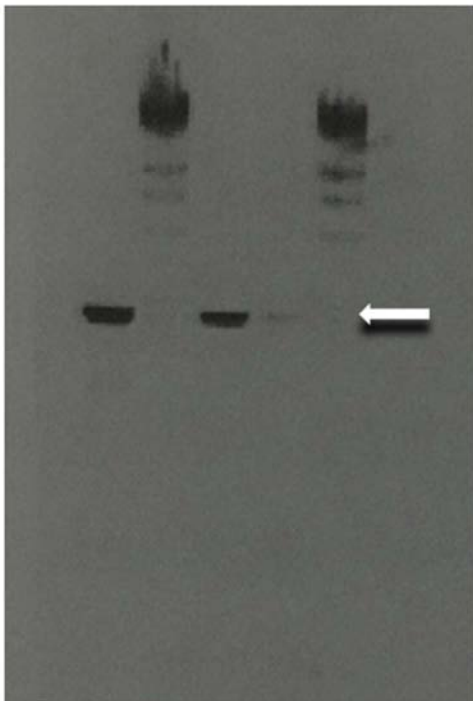


Figure 4. PCR products run on a 0.8% gel. The lanes show the 2 kb right homologous arm located at the right point using the Hind III digest of lambda marker. The DNA on gel show decreasing intensity (arrow) from left to right corresponding to the concentrations of BAC DNA in the starting reaction. No products were amplified in the control reactions indicating that primers were specific.

3.1.4. 8 kb Left Arm

The 8kb left homologous arm of the construct was amplified using ‘SHORT’ primers. Only the reaction with 8 ng of the BAC DNA yielded a positive result. The 20 ng and 40 ng reactions had no significant products on gel electrophoresis (Figure 5). The reverse primer in this case was located a few bases into the third exon just like the forward primer of the short arm in order to achieve the truncation of exon 3. This ensured that 167 bp of the 259bp exon 3 was deleted.

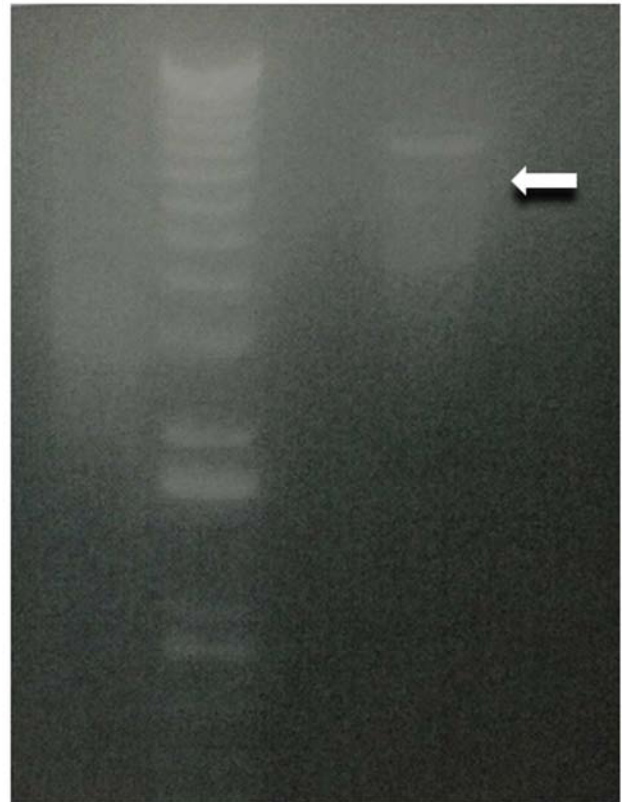


Figure 5. A 0.6% agarose gel showing the 8 kb (white arrow) left homologous arm, which correspond to the same size of band on the 1 kb plus DNA ladder. The lane with visible amplicon had 80 ng of DNA in the starting reaction while the lane to the left of the marker with a smear had 40 ng of DNA. The control lanes showed no trace of products.

3.2. LoxP Inserts

3.2.1. LoxP-XbaI Insert

To ensure the *NeoR* could be removed after the homologous recombination, two loxP flanking linkers were sequentially ligated in to the plasmid vector p1049. Since the vector had a single PacI and XbaI site flanking it respectively, ligation was done with these restriction sites attached to the linkers in order to preserve them post ligation. XmnI was used to screen for successful ligation of the loxP/XbaI insert because the enzyme contains a restriction site within the loxP linker, and a successful ligation would produce plasmid with an added XmnI site and consequently a change in the restriction map between wild type plasmid and plasmid with loxP/XbaI insert. The simulated digest of both

wild type plasmid and the theoretical successful insert showed two major bands of 5.9 kb and 2.2 kb for the wild type compared to 3 major bands of 4.9 kb, 2.2 kb and a 1 kb respectively (Figure 6). The XmnI screen of all clones yielded several candidates with the differentiating 1 kb band compared to the wild type plasmid (Figure 7).

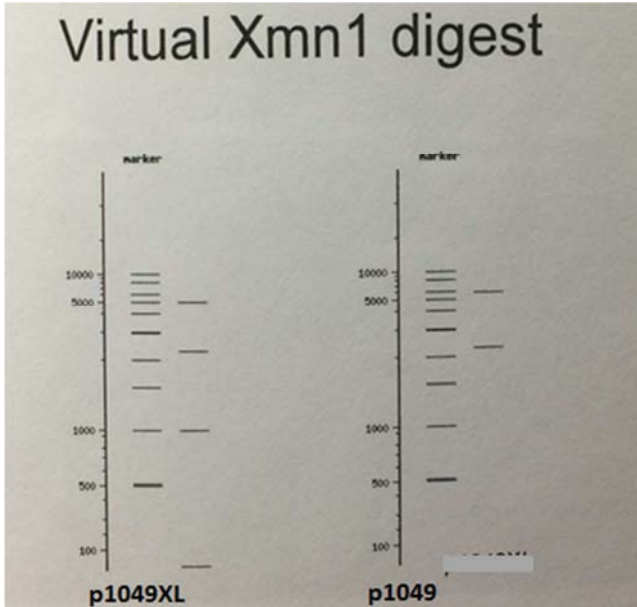


Figure 6. Simulated XmnI digest of both wild type plasmid (right) and the expected result on successful ligation with loxP-XbaI linker (left). Notice that the transformed plasmid has band shift from the 5.9 kb to 4.9 kb and an additional band at 1 kb not present in the wild type.

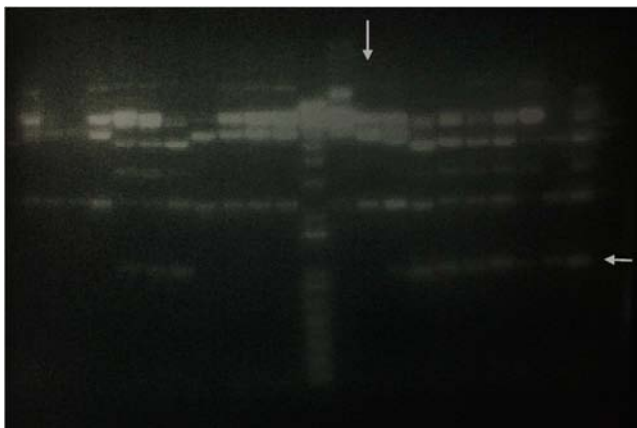


Figure 7. 0.8% gel of XmnI digestion of transformed clones after ligating loxP-XbaI linker into plasmid (p1049) using a 1 kb plus marker. The wild type plasmid was digested as control (top arrow) and showing the expected bands at 5.9 kb and 2.2 kb. The lanes with the 1 kb additional band (right arrow) and a slight shift in the 5.9 kb of the wild type to 4.9 kb position were the positive clones as a result of successful ligation.

3.2.2. LoxP-PacI Insert

One of the successfully transformed plasmid DNA was used as the starting material for the ligation of the loxP-PacI linker. This plasmid (p1049XL) was amplified in *E. coli* DH5α cells and DNA extracted as outlined previously. The loxP-PacI linker unlike the loxP-XbaI had no suitable commercially available restriction enzyme within for the

purposes of screening. However, MspI virtual digest of p1049XL and the theoretical plasmid containing successful insert (p1049XLPL) showed a discernable band shift (Figure 8). MspI was used to screen clones digest and run on a 1.4% gel as this showed the best band resolutions. The best band shifts of successful plasmids were selected for sequencing (Figure 9).

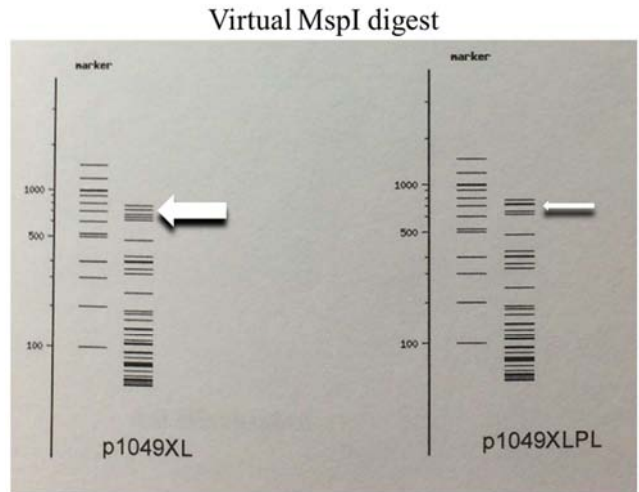


Figure 8. The expected band pattern using a simulation digest of both plasmids with MspI. The left pattern shows the p1049XL while the right is the expected result on successful ligation with loxP-PacI with a disappearance of one of the 5 bands between 600 and 800 kb bands.

MspI digest



Figure 9. MspI digest of transformed p1049XL plasmid showing some putative positive clones (white arrows) and some unsuccessful transformants (black filled arrows).

3.3. Gene Sequencing

To determine whether the inserts were at the right position and have the right orientation, the positive candidates were subsequently sequenced using NeoP2 primer. Of the 15 samples sequenced, only 2 had the loxP-XbaI linker successfully inserted without nucleotide deletions but the orientations were in the reverse and forward directions respectively. Two clones had the linker inserted in the forward direction but had 2 deletions in each one, while a few had multiple copies of linker inserted.

4. Discussion

The SV40 promoter used in our experiments is a strong mammalian promoter commonly used in mammalian expression cells [20] and has been employed previously to drive the selection markers. In addition to the SV40 promoter, lox P element were used to flank both the SV40 promoter and NeoR selection marker. This enabled both to be deleted by Cre-recombinase enzyme when desired. By deliberately avoiding the homologous arms or probe sequences ending in the unusually dense regions of the genes, amplifying the 8 kb left arm was a lot easier and a total length of 10 kb was provided for both arms. This length increases the homology between our cloning vehicle and the native gene with increased chance of homologous recombination.

Furthermore, the screening methods applied, and the modifications proposed from hindsight are important strategies towards achieving the set goals. XmnI was an efficient screening tool for loxP-XbaI linker insertion. This was because the loxP-XbaI linker had an XmnI restriction site within it in addition to the two sites contained in the wild type plasmid. This gave a different restriction map compared to the wild type. However, screening for the loxP-PacI insert was challenging as the only restriction enzyme found within the linker was scarce. MspI was used instead but the differences in map pattern were not as distinct as demonstrated with XmnI. This could be improved by adding overhang with rare cutting enzyme to the loxP/PacI linker to allow for easier screening.

5. Conclusions

This study has provided the basic ingredients for making an Il1r2-deficient mouse in order to adequately characterize the phenotype. By assembling the complete knock out construct from templates already provided in this study for the knock out in embryonic stem cells, Il1r2-deficient mice could be made.

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