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ENHANCEMENT OF EXTRA CHROMOSOMAL RECOMBINATION IN SOMATIC CELLS BY AFFECTING THE RATIO OF HOMOLOGOUS RECOMBINATION (HR) TO NON-HOMOLOGOUS END JOINING (NHEJ)

Gretchen M. Zaunbrecher¹, Bashir Mir², Patrick W. Dunne¹, Matthew Breen², and Jorge A. Piedrahita²

¹Department of Veterinary Anatomy and Public Health, College of Veterinary Medicine, Texas A&M University, College Station, TX. 77843

²Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC. 27606.

Abstract

Advancements in somatic cell gene targeting have been slow due to the finite lifespan of somatic cells, and the overall inefficiency of homologous recombination. The rate of homologous recombination is determined by mechanisms of DNA repair, and by the balance between homologous recombination (HR) and non-homologous end joining (NHEJ). A plasmid-to-plasmid, extra chromosomal recombination system was used to study the effects of the manipulation of molecules involved in NHEJ (Mre11, Ku70/80, and p53) on HR/NHEJ ratios. In addition, the effect of telomerase expression, cell synchrony, and DNA nuclear delivery was examined. While a mutant Mre11 and an anti-Ku aptamer did not significantly affect the rate of NHEJ or HR, transient expression of a p53 mutant increased overall HR/NHEJ by 2.5 fold. However, expression of the mutant p53 resulted in increased aneuploidy of the cultured cells. Additionally, we found no relationship between telomerase expression and changes in HR/NHEJ. In contrast, cell synchrony by thymidine incorporation did not induce chromosomal abnormalities, and increased the ratio of HR/NHEJ 5-fold by reducing the overall rate of NHEJ. Overall our results show that attempts at reducing NHEJ by use of Mre11 or anti-Ku aptamers were unsuccessful. Cell synchrony via thymidine incorporation, however, does increase the ratio of HR/NHEJ and this indicates that this approach may be of use to facilitate targeting in somatic cells by reducing the numbers of colonies that need to be analyzed before a HR is identified.

Introduction

The low efficiency of HR combined with the finite lifespan of somatic cells makes the process of gene inactivation lengthy and difficult. This, in turn, severely restricts the ability to fully exploit the utility of this technology in species such as cattle and swine. Currently, most targeting protocols use traditional types of enhancements such as promoter/polyA trapping and positive/negative selection to increase targeting efficiencies. While there are previous reports of enhancement using these enrichment schemes, the most realistic values of enhancement of HR are in the range of 2–5 fold with positive/negative selection and 100–500 fold with promoter

traps [1–3]. Yet, even with the availability of these enrichment schemes, targeting in somatic cells remains difficult.

Cells, in response to a DNA double strand break (DSB), activate two competing pathways of DNA repair, the NHEJ which typically occurs at the beginning of the S phase of the cell cycle and HR towards the end of the S phase. In yeast and ES cells, the HR pathway appears to be dominant, while in somatic cells the DSB is preferentially repaired by NHEJ [4]. Many proteins are involved in NHEJ, including Ku70, Ku80, DNA dependent protein kinase catalytic subunit (DNA-PKcs), and the Rad50/Mre11/XRS2 protein complex [5]. While multiple studies have explored the enhancement of HR by the over-expression of several proteins involved in HR, including Rad 51 [6], RecA [7], Rad 52 [8], and RuvC [9], there are no reports on the effects of suppression of NHEJ on targeting frequency. In order to determine whether inhibition of NHEJ would enhance HR we tested several NHEJ inhibitors including, a truncated Mre11 protein, an anti-Ku RNA aptamer and a mutant P53 protein. The truncated Mre11 and the RNA aptamer contain the binding site for Ku70 protein and are capable of sequestering Ku70, thus inhibiting its binding to Ku80 to form a functional heterodimer [10,11]. The mutant p53 is capable of disrupting the binding of p53 to Rad51, thus increasing availability of Rad51, which in turn results in increased HR [12,13].

In addition, telomere ends are known to sequester several of the proteins involved in DNA repair including Ku70/80, Mre11, and the Rad50/Mre11/XRS2 complex [14–17]. Thus we examined the effects of overexpression of telomerase on the rate of extra chromosomal recombination in somatic cells. Finally, we have recently reported that addition of a nuclear localization signal to the targeting construct combined with cell synchrony by thymidine incorporation increases the rate of HR at the hypoxanthine guanine phosphoribosyl transferase (HPRT) locus in fetal fibroblasts [18]. Culture of cells in the presence of thymidine causes accumulation of cells in the S-phase of the cell cycle and is considered a non-toxic method of cell synchronization with no reports of increasing the incidence of double stranded breaks or inducing aneuploidy [19]. We wanted to extend these observations to determine whether the same effect was seen in a plasmid-to-plasmid, extra-chromosomal recombination system.

Plasmid-to plasmid extra chromosomal recombination systems have been used in the past to study HR [20]. In the plasmid to plasmid recombination system used here, two truncated but overlapping plasmids containing the puromycin-N-acetyl-transferase (*pac*) gene, (referred heretofore as Puro) conferring resistance to puromycin, are co-introduced into the cell (Puro Δ 5' and Puro Δ 3'; Figure 1). Colonies resistant to puromycin, representing homologous recombination events across the overlap region, are then selected and quantified. Changes in the frequency of HR per million cells electroporated (overall HR) upon the addition of tests factors can then be measured against the no treatment control. In addition, to determine the effect of treatments on random insertion resulting from NHEJ the number of colonies after transfection with a linearized, completely functional puromycin resistance plasmid are quantified. Comparing the rates of HR and NHEJ assist in determining both the effectiveness of the treatment and whether it is acting at the HR or NHEJ level.

MATERIALS AND METHODS

Development of truncated puromycin vectors

Two truncated puromycin-N-acetyltransferase vectors, Puro Δ 5' and Puro Δ 3' were constructed by PCR amplifying selected regions of pGKpuro plasmid (Figure 1). Primer sequences were: for forward primer FP1 (5'-GCTTGCGGGTCATGCAC-3'), reverse primer RP1 (5'-GTAAAACGACGGCCAG-3'), forward primer FP2 (5'-CAGGAAACAGCTATGAC-3') and reverse primer RP2 (5'-CCGAGTACAAGCCCCACGG-3'). All oligonucleotides, unless specifically mentioned, were purchased from Genosys. FP1-RP1 primers generated Puro Δ 3',

a fragment containing the PGK promoter, a 5' end truncated puromycin-N-acetyltransferase, and lacks the poly A signal. FP2-RP2 generates PuroΔ5', a fragment containing a 3' truncated puromycin-N-acetyltransferase and the poly A signal, but lacking the PGK promoter. The two fragments share approximately 580 base pairs (bp) homology in the puromycin-N-acetyltransferase gene, but neither fragment codes for a functional gene. However, recombination between these two fragments result in a fully functional puromycin-N-acetyl-transferase gene (Figure 1). A successful recombination event can then be scored as a puromycin resistant colony.

PCR conditions were initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 15 sec, 30 sec annealing at 62°C (for FP1 and RP1) or 58°C (for RP2 and FP2), and elongation at 69°C for 2 min followed by a final elongation at 69°C for 7 min. The resulting products were cloned into a Topo pCR2.1 vector (Invitrogen). NLS-PuroΔ5' and NLS-PuroΔ3' plasmids were constructed by cloning an 180bp NLS fragment containing FseI restriction sites at the both ends into PuroΔ5' and PuroΔ3' plasmids via a synthetic linker [18].

Development of Mre11, mutant p53, and Anti-Ku aptamer

Preparation of Mre11 construct—The truncated Mre11 plasmid was developed using porcine fetal fibroblast (PFF) cDNA as the template for the following primers: Mre11F1: GGATTTATGGAGAAAGATGCAG and Mre11R1: CCACAGACATTGAACGTCCAAAG. (just changed font size to match with the other primer sequences) These primers were designed from conserved sequences in the mouse (Accession# NM_018736) and human (Accession# BT_006730) and amplified a 408 bp product that encompasses amino acids 24–160 (based on mouse open reading frame) using the following PCR conditions: initial denaturation at 94°C for 2 minutes, 30 cycles of denaturation at 94°C for 10 seconds, annealing at 55°C for 30 seconds, and elongation at 69°C for 2 minutes followed by a final elongation at 69°C for 7 minutes. The 408bp PCR product was then cloned into a Topo pCR2.1 vector, sequenced to confirm identity, and re-amplified with the forward primer, Mre11F2: CGCGCGCCATGGACTCCGGATTTATGGAG, containing homology to the Mre11F1 plus additional NcoI and BspEI sites, and the reverse primer, Mre11R2: CGTATAGCGGCCGCCACAGACATTGAACG, containing homology to Mre11R1 plus an additional NotI site. The resulting 438 bp product was partially digested with NotI and NcoI (NcoI site 235 bp within amplicon) and subcloned into a pEF/myc/nuc expression vector (Invitrogen). This mutant Mre11 construct is the porcine homologue of the one used previously by Goedecke et al. (1999). Expression of Mre11 from this plasmid was confirmed by RT-PCR 24–48 hr after electroporation (data not shown).

Preparation of p53 construct—A mutant p53 was generated by amplification of p53 using hemi-nested primers: Forward primer: ATCCCAGGACGGTGACACC; reverse primer1: ACCTGCACCAAGCAGAGGTC; reverse primer2: AGGCTGAGCAGATGAAGACTCC. The PCR conditions were as follows: initial denaturation at 94°C for 2 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and elongation at 72°C for 2 minutes followed by a final elongation at 72°C for 7 minutes. Five units of Taq polymerase (Promega), 20mM of each dNTP (Roche), and 2mM of magnesium chloride was used. The resulting fragment was then cloned into a pCR2.1 vector (Invitrogen). A Valine¹³⁵ to Alanine¹³⁵ point mutation was introduced using the oligo AAGACCTGCCAGCGCAGCTGTGGGTC. The mutant p53 cDNA was then subcloned into a pDNA3 under the control of the CMV promoter and sequenced to confirm that the modification was accurate.

Preparation of anti-KU aptamer—An anti-Ku RNA Aptamer was purchased from Dharmacon Research, Inc. containing the sequence: A*G*G*UCGGCAUACAGAGUCCGAAUGCGCGUAACUUCG*A*C*U. The oligonucleotide was made in the stable 2'-O-ACE protected form to protect the aptamer from endonucleases. The (*) nucleotides possess a thioate linkage instead of phosphodiester bond for exonuclease protection. This aptamer is analogous to the one used previously by Yoo and Dynan (1998).

Isolation of porcine fetal fibroblasts

Purebred Duroc gilts on days 35, 36, or 57 of pregnancy were hysterectomized and the fetuses removed. For day 35 and 36 fetuses, the head and viscera were removed and the remaining tissue was minced with a sterile razor blade while with the day 57 fetuses, the skin was removed from the hind leg and the muscle was minced. The tissue was added to 10 ml of 0.05% trypsin (Gibco) supplemented with 0.9mM potassium chloride, 0.9mM dextrose, 0.7mM sodium bicarbonate, 0.1mM EDTA (all from Sigma), and 20mM sodium chloride (EM Science). The tissue/trypsin solution was shaken at 37°C for 15 minutes a total of three times. After incubation, the supernatant was collected, pooled, and pelleted. The cell pellet was resuspended in DMEM/F12 media (Gibco) supplemented with 10% fetal bovine serum (FBS) and 5% calf serum (CS) (both from Hyclone), 30mM sodium bicarbonate, 0.5mM pyruvic acid, and 2mM N-acetyl-L-cysteine (all from Sigma). In addition, 100 units (U) penicillin, 100 ug streptomycin, and 250 ng amphotericin (Gibco) were added to inhibit microbial growth. The cells were placed in the appropriate number of 10cm tissue culture plates (Corning), incubated in a 5% CO₂ incubator at 39°C, expanded once and frozen in 50% FBS, 40% media, and 10% DMSO (Sigma) for long time storage and future use.

Generation of Telomerase expressing cells

Day 57 porcine fetal fibroblasts were virally transfected with green fluorescent protein (GFP) alone, and GFP plus human telomerase reverse transcriptase (hTERT-GFP), using procedures described elsewhere [21]. After viral transfection GFP-positive cells were sorted, plated, and expanded for future studies. Functional telomerase activity in the fibroblasts was determined using the TRAPeze Telomerase Detection Kit (Intergen). A population doubling experiment was conducted comparing the GFP- and hTERT-GFP-expressing cells. Population doublings were calculated using the formula: $[\log_{10}(\# \text{ cells harvested}) - \log_{10}(\# \text{ cells seeded})] \times 3.3219$.

Electroporation and cell synchronization conditions

Cell cultures were re-established from frozen cells and grown in antibiotic free media as described above. The cells were expanded until appropriate numbers were obtained, trypsinized, counted, and aliquoted to $3-10 \times 10^6$ cells per electroporation. Cells were pelleted and resuspended in 0.8 ml of cold F10 Nutrient Mixture (HAM) (Gibco) and placed in a 4 mm gap electroporation cuvette. DNA was delivered into the cells via electroporation (450 V, 1 ms, 4 pulses).

Experiments were replicated 3 (n=3) to 6 (n=6) times as indicated in the text, and in each case, 5 nM each of linearized PuroΔ3' and circular PuroΔ5' plasmids were used. For each replication, cells to be electroporated were combined into a single pool, and divided into the appropriate treatment groups to ensure no cell line, cell density, or cell cycle differences existed between treatments. To determine the frequency of NHEJ in each replication, a complete and functional linearized Puro plasmid (5nM) was utilized. For the p53, AntiKu, Mre11, and NLS experiments, 5nM of each oligo or circular plasmid were added during electroporation. For the thymidine treatment, 2mM of thymidine (Sigma) was added to the cells at 50–70% confluency for 24 hours prior to electroporation. After each electroporation, cells were plated at $1-2 \times 10^6$ cells per 10cm tissue culture plate and allowed to recover and attach overnight.

Puromycin at a concentration of 2 µg/ml was added to all plates approximately 24 hours after electroporation and cells grown under selection until puromycin resistant colonies were visualized (7–14 days). Plates were washed with phosphate buffered saline, and incubated in a 1:1 ratio of methanol:acetone at -20°C for 20 minutes, air dried and stained with trypan blue.

Cell cycle determination and karyotypic analysis

To determine percentage of cells arrested in the S phase of the cell cycle, propidium iodide staining following manufacture guidelines (DNAcon3-Consul^{T.S.}) was performed 0, 12, 24, and 36 hours after a 24 hour incubation in 2mM thymidine. Cells were analyzed on a FACSCalibur (Becton-Dickinson) flow cytometer, equipped with a 15mW air-cooled argon laser, using CellQuest (Becton Dickinson) acquisition software. Propidium iodide fluorescence was collected through a 585/42-nm bandpass filter and list mode data were acquired on a minimum of 20,000 single cells defined by a dot plot of PI-width versus PI area. Data analysis was performed in ModFit LT (Verify Software House) using PI-width versus PI area to exclude cell aggregates.

In addition, karyotype analysis was performed on hTERT-GFP, GFP-expressing cells, and thymidine synchronized cells. Metaphase spreads were prepared using conventional methods of colcemid arrest, followed by hypotonic exposure and methanol/acetic acid fixation. Chromosomes were counterstained in 80 ng/ml 4',6-diaminidino-2-phenylindole (DAPI) and mounted in antifade solution (Vectashield, Vector Laboratories). Digital images were acquired using a fluorescence microscope (Axioplan 2ie, Zeiss) equipped with a cooled CCD camera (CoolSnapHQ, Photometrics, Tuscon, AZ), both driven by dedicated software (SmartCapture 2.3.1 Digital Scientific, Cambridge, U.K.). Between 10–25 well spread metaphase preparations from replicates of the hTERT-GFP, GFP-expressing cells, and thymidine synchronized cells were used to determine diploid copy number.

Statistical analysis

Data was tested for homogeneity of variance by plotting the residuals and log-transformed if needed. Averages of at least three replications were subjected to ANOVA to determine differences between the means of the different treatments. If a difference was found, Fisher's Least Significant Difference analysis, using SPSS software, was performed to determine which treatments were significantly different at the $p < 0.05$. To determine the interaction between addition of NLS and cell synchrony with thymidine the data was analyzed by two-way ANOVA.

RESULTS

Effects of Mre11, anti-Ku, and mutant p53 on the ratio of HR/NHEJ

The number of puromycin resistance colonies arising from recombination between Puro $\Delta 5'$ and Puro $\Delta 3'$ in the presence of one of the repair molecules, truncated Mre11, anti-Ku aptamer, or mutated p53, were compared to the number of colonies resulting from the no treatment added control. Fig 2 shows the results from four independent replicate experiments. As can be seen the truncated Mre11 and anti-Ku aptamer were unsuccessful in increasing HR/NHEJ ratios (CTL 1.86 ± 0.7 ; Mre11 1.18 ± 0.35 ; Anti-ku 1.32 ± 0.34). The only treatment to have an effect on HR/NHEJ was the mutated p53 treatment with an increase in plasmid to plasmid HR from 1.86 ± 0.7 the control to 5.59 ± 1.81 ($P < 0.05$)

Effects of telomerase on life span and the ratio of HR/NHEJ

An hTERT-GFP expression plasmid [21] was virally transfected into porcine fetal fibroblasts and was shown to stably express an active telomerase protein via the TRAPeze Telomerase

Detection Kit. In addition, a cell line expressing GFP alone was similarly transfected to control for all genetic and cell culture manipulations. Only cell extracts from hTERT-GFP-transfected cells exhibited the expected laddering pattern after PCR while all other samples, GFP alone transfectants and heat-inactivated samples, showed no hTERT activity (Figure 3). A population doubling experiment was also undertaken to determine if this active human telomerase could extend the natural life span of the fibroblasts. The human telomerase expressing cells became senescent after 21 doublings while the GFP expressing cells continued dividing to 39 population doublings at which time the experiment was terminated.

In order to determine if hTERT expression affected HR, the plasmid-to-plasmid recombination rates between hTERT-GFP-expressing, GFP-expressing, and normal non-transfected cells were compared. Average results of three independent experiments (n=3) on the same cell lines showed that overall HR was higher ($p<0.05$) in the unmanipulated cells (2.5 ± 1.2 HR), than in GFP-expressing cells (0.3 ± 0.1) or the hTERT-GFP-expressing (0.4 ± 0.1) (Figure 4). When the data was expressed as HR/NHEJ, however, there were no differences between any of the treatment groups (Control cells 4.3 ± 2.3 ; GFP CTL cells 4.4 ± 3.8 ; hTERT cells 6.4 ± 2.8). This was due to the fact that NHEJ, as indicated by the number of surviving colonies per million cells electroporated with the linearized, complete puro plasmid, were much lower in the +hTERT-GFP and +GFP cells than in the untransformed cells (9.2 ± 2.7 , 9.7 ± 4.4 , and 67.3 ± 8.3 , respectively; Mean $\pm$ SE).

Metaphase spreads of the hTERT-GFP-expressing cells showed 25% of the cells had normal diploid karyotypes ($2n=38$), 25% of cells with variable number of chromosomes (not tri- or tetraploid), and 50% tetraploid cells. While all the GFP-expressing cells contained small extra fragments that did not resemble normal chromosomes, 15% were determined to be diploid, 50% were triploid with extra small fragments, and 35% aneuploids of variable number with all being over 57 chromosomes.

Effects of cell synchrony and nuclear DNA delivery on ratio of HR/NHEJ and chromosomal stability

To study the effect of cell cycle on the rates of HR, cells were synchronized in late S phase by overnight incubation in 2mM thymidine. Propidium iodide staining and FACS analysis was used to determine the cell cycle stage of the cells prior to, during, and after thymidine treatment. As shown in Table 1, there was a 5-fold increase in cells in the S-phase after 24hr incubation with thymidine. Following removal of thymidine the cells quickly recuperated. Karyotypic analysis of thymidine treated cells did not detect any increases in aneuploidy compared to untreated cells (data not shown).

To determine the effect of faster delivery of the transgene to the nucleus on HR, an NLS was added to the plasmid-to-plasmid recombination system. Additionally, the interaction between cell synchrony and the use of NLS was tested. The mean of six experiments (n=6), indicated that there were no differences in the rates of overall HR among any of the groups (Figure 5). In contrast, when the rates of HR/NHEJ were calculated, thymidine synchronization has a drastic and significant effect (noNLS-no Thy 1.8 ± 0.5 ; no NLS- yes Thymidine 11.5 ± 4.2 ; yes NLS-no Thymidine 2.3 ± 0.1 ; yes NLS-yes Thymidine 14.8 ± 4.7 $P<0.05$). (also in picture of figure five you need to change the right hand legend from Ration to Ratio) This was due to a reduction in the colony number per million cells electroporated with the linearized, complete Puro plasmid from 61.8 ± 3.0 in the no-thymidine group to 15.1 ± 4.9 in the thymidine-treated group indicating the rate of NHEJ was lowered in the cells synchronized in late S phase. To determine whether there was any interaction between NLS and thymidine synchronization on both overall HR and HR/NHEJ ratio, a two-way-ANOVA was performed. This analysis confirmed that the effects of thymidine were mostly through NHEJ. It also indicated that NLS

had a positive, but non-significant ($P < 0.29$), effect on the overall rate of HR ($1.50 \pm .21$ with NLS vs. $1.15 \pm .25$ without NLS).

Karyotype analysis of at least ten metaphase spreads from duplicate harvests of control and thymidine synchronized cells, indicated that there was no difference in diploid chromosome number in the two cell populations (data not shown).

DISCUSSION

Since the original reports of targeting in mammalian cells [22,23], gene targeting in mouse ES cells has become widely applied. However, attempts to develop a viable, pluripotent ES cell line in livestock species have had little success [24–26]. With the birth of Dolly, a viable lamb resulting from the transfer of a nucleus from a somatic cell to an enucleated oocyte [27], the use of somatic cells as carrier cell lines to produce a transgenic animal could be realized. This possibility was further advanced with the production of the first targeted transgenic livestock species [28]. The birth of COL1A1 targeted sheep, demonstrated that it was indeed possible to make the considerable in-vitro cell manipulations needed for targeting, and still have the capability of producing viable offspring. Most recently, other groups [29–31] have reported successful targeting of the $\alpha 1,3$ galactosyltransferase (GT) in pigs, and multiple loci in cattle [32]. Yet, HR in somatic cells remains a formidable task.

Several studies have looked at the over-expression of several proteins involved in HR and their effects on HR rates [6–9,13]. However, there has been no report of studies on suppression of NHEJ pathway molecules and their effects on the rates of HR. From a practical perspective NHEJ plays a critical role independent of the overall rate of HR. For instance two treatments that have the same rate of HR but one has a 100 fold lower rate of NHEJ, translates into most of the surviving colonies being targeted events. This means the investigator has to examine only a few transgenic colonies to identify the targeted event. Thus, reducing the rate of NHEJ has a significant effect on the efficiency of targeting.

Therefore, the effect of several molecules affecting the NHEJ pathway on the rate of HR was assayed via a plasmid-to-plasmid recombination system. Results of this study indicate a significant role of p53 in enhancing plasmid to plasmid HR. A similar effect on HR rate, of constitutively expressed mutant p53 has been reported previously [13]. This enhancement is likely due to the mutant p53's ability to interfere with both normal p53 tetramer formation and with p53tetramer/Rad51 interactions [12]. However, tight control must be exerted on the transient nature of the mutated p53 due to its potential ability to cause aneuploidy in the transfected cells thus making the targeted cells unfit for cloning.

Among the many proteins involved in NHEJ, Ku 70/80, p53, DNA-PK, and the Rad50/Mre11/XRS2 complex have been studied extensively. Results indicate that these proteins as well as others are involved in many different telomeric functions [14–16,33]. Among cell types shown to possess higher rates of HR there is a common aspect in that they all express telomerase. ES cells [34], telomerase-positive somatic cells [34], regenerating liver cells [8,35], and Fanconi's anemia cells [36,37] all have both a high rate of HR and express telomerase. Similarly, spermatocytes undergoing meiosis, a form of HR, also express telomerase [38,39]. Yet, to date, there have been no studies on the relationship between the expression of telomerase and the enhancement of HR. To examine this relationship, porcine fetal fibroblasts (PFF) virally co-transfected with the human telomerase reverse transcriptase (hTERT) and the green fluorescent protein (GFP), and shown to express a functional hTERT, were compared to the same PFF either non-transformed or transfected with GFP alone. As shown in Figure 4 the overall HR rates were significantly lower in the hTERT-GFP and GFP-expressing cells ($P < 0.05$). However, when the HR/NHEJ rates were calculated there were no differences between the

three groups. This is because the number of random insertions were drastically reduced in both transformed cells lines.

Also, while hTERT was shown to be active via TRAP assay (Figure 3), no cellular life extension was seen. This same cDNA has been shown to increase both life span and telomere lengths of human fibroblasts [40]. However, Zhu et. al. [41] reported life extension but no telomere elongation in previously transformed human fibroblasts. In addition, others found that transformations with hTERT alone was ineffective in immortalizing normal human mammary fibroblasts but required the addition of Simian virus 40 (SV40) large T antigen [42]. While there have been no reports of hTERT expression in porcine cell lines, ectopic expression of hTERT has been reported in both sheep and bovine cell lines [43,44]. However, only in sheep has hTERT resulted in elongation of life span [43,45].

Our results indicate that thymidine synchronization of somatic cells in the late S phase is effective in reducing the tendency of NHEJ DNA repair thus allowing for a higher ratio of HR/NHEJ to occur (Figure 5). In this plasmid-to-plasmid recombination model it accomplishes this by a reduction in the number of random insertions rather than by an increase in the number of homologous recombinants as has been reported previously by us and others [17,18]. Additionally, although we saw a small increase in the overall HR in the presence of NLS, it was non-significant ($P < 0.29$). This differs from our recent observations on the effect of NLS on targeting in a chromosomal locus [18], which showed a drastic and significant effect of NLS on overall HR. Our interpretation of these results is that DNA entry into the nucleus is not a rate-limiting step in a plasmid-to-plasmid recombination system where multiple target sequences are available. In contrast, chromosomal loci with only two alleles available for targeting have a more stringent requirement for rapid entry of the targeting DNA into the nucleus. Thus, while extra-chromosomal homologous recombination models can be useful in elucidating some of the key rate limiting steps in homologous recombination, it is important that those results be confirmed in a chromosomal targeting model. Similar discrepancies between chromosomal and plasmid-to-plasmid recombination has been reported previously by others [46]. Additionally, we did not detect any gross chromosomal rearrangements in thymidine-treated cells. This supports previous observations indicating that thymidine block does not cause an increase in DSBs, as do other S-arrest compounds such as hydroxyurea [17].

Taken together, our results show a reduction of random (NHEJ) insertions by thymidine synchronization has a beneficial effect in the efficiency of targeting as fewer colonies need to be analyzed to identify correctly targeted colonies. The results presented here, combined with our previous results in a genomic loci [18], demonstrate that the thymidine block is an effective mean of increasing the efficiency of homologous recombination in cultured somatic cells. As such it should facilitate the development of genetically modified animals such as pigs and cattle that can be used in medical and agricultural biotechnology.

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Figure 1. Schematic diagram of the plasmid-to-plasmid recombination system
a) Puro Δ 3'; b) Puro Δ 5'; c) loci after HR between Puro Δ 3' and Puro Δ 5'. Neither the Puro Δ 5' nor the Puro Δ 3' truncated plasmids alone are able to confer puromycin resistance. Both vectors share 580bp of homologous sequence, and recombination between these two plasmids results in a fully functional puromycin resistant gene. FP1, RP1, FP2, and RP2; Location of primers for generation of Puro Δ 5' and Puro Δ 3'. Parallel bars, region of homology

Figure 2. Effect of NHEJ inhibitors, Mre11, anti-Ku aptamer, and mutant p53 (p53*) on plasmid to plasmid recombination in somatic cells

The rate of recombination, as determined by reconstitution of the Puro gene, was determined in four separate experiments and the data analyzed by ANOVA. Overall HR (Red bars), referring to the number of puromycin resistant colonies per million cells electroporated with $\Delta 5' + \Delta 3'$, did not differ between the control, MRE11, and anti-KU treatments. However, an approximate 2-fold increase was detected when the mutant p53 was utilized. A similar pattern was observed for HR/NHEJ (%; Blue bars). HR/NHEJ refers to the ratio of targeted colonies relative to random insertions. NHEJ was determined from the number of colonies obtained

with a linearized complete puromycin-resistance plasmid. Columns of the same colour with different superscripts are significantly different at $P < 0.05$. Error bars = standard error

Figure 3. Expression of human telomerase reverse transcriptase (hTERT) by hTERT-GFP transformed fetal fibroblasts

Cell extracts for cells transformed with hTERT-GFP or GFP alone were collected and hTERT activity measured using the trap assay as indicated in material and methods. The presence of a DNA laddering pattern is indicative of hTERT activity. Replicates of hTERT-GFP, GFP, and their heat inactivated (Inact.) cell extracts are shown. A positive control supplied by the kit manufactures is also included. Arrow at left indicates location of first band of ladder. Additional bands of increasing molecular weight are seen in the hTERT-GFP and the positive control samples. None of the heat-inactivated samples show any hTERT activity as expected. A residual band of activity is seen in the GFP samples but is significantly lower than on the hTERT-GFP samples or the controls.

Figure 4. Effect of expression of hTERT in porcine fetal fibroblasts on plasmid to plasmid recombination in somatic cells

Fetal fibroblasts were transformed with a hTERT-GFP, or GFP as described in the text, and the rate of homologous recombination determined using the plasmid-to-plasmid recombination assay. The rate of recombination, was determined in three separate experiments and the data analyzed by ANOVA. Overall HR (Red bars) was significantly higher in the control untreated group, than in the cells expressing hTERT-GFP or GFP alone. When the rates of HR were corrected for NHEJ rates (HR/NHEJ; Blue bars) there were no differences among the treatment groups. Columns of the same colour with different superscripts are significantly different at $P < 0.05$. Error bars = standard error

Figure 5. Effect of thymidine S-phase synchronization (Thy) and a nuclear localization signal (NLS) on plasmid to plasmid recombination in somatic cells

The rate of plasmid-to-plasmid recombination was determined in six separate experiments and the results analyzed by ANOVA. Neither thymidine synchronization nor addition of NLS had a significant effect on overall HR (red columns). In contrast, cells treated with thymidine, independent of addition of NLS, had a 5-fold greater rate of HR/NHEJ (blue bars) than unsynchronized cells. Columns of the same colour with different superscripts are significantly different at $P < 0.05$. Error bars = standard error.

Table 1

Effect of thymidine synchronization on percentage of cells in different stages of the S-cycle

	Early S	Mid S	Late S	S-Phase Total
No Thymidine	6.5	2.6	2.8	11.9
+12 hr Thymidine	40.0	10.6	2.3	53.0
+24 hr Thymidine	22.5	33.4	10.4	66.0
-12 hr Thymidine	7.3	2.7	25.4	35.0
-24 hr Thymidine	6.4	1.5	23.3	31.1
-36 hr Thymidine	4.7	2.6	14.7	22.0

+ Indicates number of hours after addition of thymidine to the culture media.

- Indicates hours after removal of thymidine from the +24 hr cultured cells.