Published in final edited form as:

J Invest Dermatol. 2013 March ; 133(3): 685–691. doi:10.1038/jid.2012.351.

Local delivery of gene-modifying triplex-forming molecules to epidermis

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Abstract

Epidermal keratinocytes are particularly suitable candidates for *in situ* gene correction. Intraperitoneal administration of a triplex-forming oligonucleotide (TFO) was shown previously to introduce DNA base changes in a reporter gene in skin, without identifying which cells had been targeted. We extend those previous experiments using two triplex-forming molecules (TFMs), a peptide nucleic acid (PNA-Antp) and a TFO (AG30), and two lines of transgenic mice that have the chromosomally integrated λ*supFG1* shuttle-reporter transgene. Successful *in vivo* genomic modification occurs in epidermis and dermis in CD1 transgenic mice following either intraperitoneal or intradermal delivery of the PNA-Antennapedia conjugate. FITC-PNA-Antp accumulates in nuclei of keratinocytes and, after intradermal delivery of the PNA-Antp, chromosomally modified, keratin 5 positive basal keratinocytes persist for at least 10 days. In hairless (SKH1) mice with the λ*supFG1* transgene, intradermal delivery of the TFO, AG30, introduces gene modifications in both tail and back skin and those chromosomal modifications persist in basal keratinocytes for 10 days. Hairless mice should facilitate comparison of various targeting agents and methods of delivery. Gene targeting by repeated local administration of oligonucleotides may prove clinically useful for judiciously selected disease-causing genes in the epidermis.

INTRODUCTION

Disease-causing mutant epidermal keratinocytes are particularly suitable candidates for localized gene targeting strategies. First, keratinocytes are accessible, making targeting *in situ* possible and repeated targeting practical. Accessibility also makes targeting *ex vivo* followed by selection of correctly targeted cells and transplantation feasible (Petek *et al*., 2010). Second, even localized or rare corrections can be amplified by improved biological fitness. For example, a single corrected cell can expand to a greater than 1 cm patch of normal skin in spontaneous, epidermal revertent mosaicism, a naturally occurring

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CONFLICTS Authors have no conflicts to declare.

phenomenon now recognized in ichthyotic (Choate *et al*., 2010) and blistering genodermatoses (Jonkman and Pasmooij, 2009; Jonkman *et al*., 1997; Smith *et al*., 2004). Third, a number of genodermatoses have regional variability in expression or phenotypic severity, implying that successful targeting of keratinocytes in a localized area could be clinically beneficial even to a patient with generalized cutaneous disease.

Heritable, sequence-specific targeting of DNA by triplex-forming oligonucleotides (TFO) was first demonstrated 20 years ago using a psoralen-linked TFO that bound to and mutated λ phage DNA (Havre *et al*., 1993). Since then, TFOs have been used to target extrachromosomal DNA in mammalian cells (Sandor and Bredberg, 1994; Wang *et al*., 1995), chromosomes of mammalian cells (Gunther *et al*., 1995; Vasquez *et al*., 1999) and, following intraperitoneal injections, DNA of many organs, including skin (Vasquez *et al*., 2000). TFOs by themselves induce mutations or recombinations in DNA (Faruqi *et al*., 1996; Sandor and Bredberg, 1995) and require the participation of DNA repair proteins (Faruqi *et al*., 2000; Vasquez *et al*., 2002; Wang *et al*., 1996). TFOs administered along with short, homologous donor oligonucleotides can introduce predictable, sequence-specific base changes in genes in close proximity to the triplex (Chan *et al*., 1999). The combination of a triplex-forming molecule (TFM) and an adjacent homologous donor oligonucleotide has been used to restore function to mutant HPRT (hypoxanthine phosphoribosyl transferase) (Majumdar *et al*., 2008) and to predictably change the sequence in β-globin (Chin *et al*., 2008; Lonkar *et al*., 2009) and CCR5 (C-C chemokine receptor 5) (Schleifman *et al*., 2011).

Clinical success of any gene targeting strategy will be dependent on delivery of the oligomer to target cells or tissue, and each target cell may present unique obstacles. Some progress has been made in improving TFO delivery by physical and chemical means. TFO-induced recombination occurs in 1% of cultured cells following intranuclear injection compared to only 0.003% following transfection with cationic lipids (Luo *et al*., 2000). Peptide nucleic acids (PNA) are more versatile triplex-forming molecules than phosphodiester oligonucleotides, but are difficult to deliver to cells. Coupling a PNA to a cell penetrating peptide, Antennapedia, (PNA-Antp), increases targeting of the *supFG1* gene 10–20-fold in cultured cells (Rogers *et al*., 2004) and, after intraperitoneal injection, in bone marrow-derived cells and whole tissues (Rogers *et al*., 2012). Those previous studies demonstrate that chromosomally integrated *supFG1* genes are well-suited for evaluating sequence-specific targeting of the reporter gene because the *supFG1* assay is both quantitative and reproducible.

The goal of this report is to demonstrate that: a) site-specific targeting occurs in keratinocyte DNA; b) that chemically distinctive triplex-forming molecules can be used to target keratinocyte DNA; c) and that triplex-induced targeting can be achieved by local delivery that is not dependent on strain of mouse or skin location. This will set the stage for quantitative comparisons of different triplex-forming molecules and/or local delivery methods.

RESULTS

For these experiments, we utilized transgenic mouse models that contain multiple copies of the λ*supFG1* shuttle vector integrated into mouse chromosomal DNA (Figure 1). The vector DNA can be isolated from mouse genomic DNA and packaged into phage particles for subsequent analysis for induced mutations. *SupFG1* encodes an amber suppressor tRNA whose function can be scored as a blue-white screen in indicator bacteria. We employed previously described oligomers that form triplex structures with a purine-rich target sequence in *supFG1* DNA. The PNA oligomer (PNA-Antp) forms a PNA:DNA:PNA triplex invasion complex (Faruqi *et al*., 1998) while the polypurine DNA oligomer, AG30, binds in the major groove oriented parallel to the purine rich strand of the *supFG1* duplex (Vasquez *et al*., 2000) (Figure 1). These molecules are known to induce site-specific targeting of the *supFG1* gene in various tissues of mice, but targeting of specific epithelial cells has not been demonstrated.

Gene targeting in epidermis and dermis following intraperitoneal injection of triplex-forming PNAs

Intraperitoneal injections of PBS, PNA, or PNA-Antp were administered on days one and three to AV *supFG1* transgenic mice at a concentration of 20 mg/kg (115–150μg, depending on size of mouse) for each dose. Ten days post-treatment, tail skin was harvested and analyzed for targeted mutations in the *supFG1* gene. Mutation frequencies in whole skin induced by PNA-Antp (34×10^{-6}) were determined to be 6-fold over background, and significantly more than that induced by the PNA molecule alone (15×10^{-6}) (Figure 2). In order to examine the extent of targeting in the individual skin layers, the epidermis was enzymatically separated from the dermis prior to analysis. In those experiments, systemic administration of the PNA-Antp conjugate induced more genomic modification in both epidermis and dermis than the PNA alone or the PBS controls (Figure 2). We analyzed mutations that were induced following PNA-Antp treatment by sequencing the shuttle reporter obtained from both the epidermis and dermis. The majority of the mutations were located precisely within the PNA binding site of *supFG1* and consisted of mainly single base substitutions, insertions or deletions, as previously observed in other cells and tissues (Rogers *et al*., 2012) and consistent with the occurrence of sequence-directed genome modification (Figure 2, inset). By contrast, mutations in the PBS controls are spread over the entire *supFG1* sequence, as previously reported (Gunther *et al*., 1995; Vasquez *et al*., 2001).

Gene targeting in epidermis following intradermal injection of triplex-forming PNAs

To minimize the quantity of triplex-forming molecules needed to target cells in skin and reduce potential for targeting irrelevant cells, we treated AV mice with two 10μg doses of PNA or PNA-Antp by intradermal injection into the tail on days 1 and 3. Ten days after treatment, tail skin samples were harvested and analyzed for targeted mutations in the *supFG1* gene in both the epidermis and dermis. Mutation frequencies in tail epidermis treated by intradermal injection with PNA-Antp averaged 59×10^{-6} (Figure 3a), a substantial 12-fold increase compared to the control PBS treated mice and double the frequency observed following intraperitoneal administration of more than ten times the amount of PNA-Antp. Mutations in clones isolated following intradermal injections of PNA or PNA-

Antp cluster in and around the triplex-binding site, while those following injection or PBS are scattered throughout the *supFG1* sequence. Mutation frequencies in ear epidermis following injection of PNA-Antp in tail were the same as the PBS controls, and those mutations were scattered along the *supFG1* sequence, not concentrated at the triplex-binding site. Intradermal injection of PNA alone had no effect on mutation frequency in epidermis compared to PBS-treated mice (Figure 3a). In the dermis, intradermal delivery of both the PNA alone and the PNA-Antp conjugate resulted in a 5-fold increase in targeting frequencies compared to the PBS control (Figure 3a), and a greater increase relative to PBS than that achieved by intraperitoneal administration.

Gene targeting of basal keratinocytes with PNA-Antp

Since keratinocytes will be the likely target of many gene modification strategies in the skin, we wanted to be certain that mutations could be made in basal keratinocytes. FITC-PNA-Antp was injected intradermally into mouse tails and imaged 5 hours later. In broad stretches of the epidermis above the injected area, but not at distant sites, nearly all epidermal nuclei were fluorescent using laser confocal fluorescence microscopy (Figure 3b). Basal keratinocyte nuclei were accentuated when viewed by fluorescence microscopy on cross sections of skin (Figure 3c). Intradermal injections of PNA-Antp were made into tails and epidermis isolated ten days later, as described above. Single cell suspensions were prepared from epidermis of several animals, pooled, stained with antibody to keratin 5, and sorted by FACS. Mutation frequency was 2-fold greater in the PNA-Antp-treated keratinocytes than in the PBS-treated keratinocytes (Figure 3d).

Gene targeting in back and tail epidermis of hairless mice with triplex-forming oligonucleotide, AG30

In anticipation of investigating different methods for delivery or different gene-modifying molecules, we bred our reporter gene into a hairless background and tested gene targeting using the TFO, AG30, in back and tail skin. FITC-AG30 injected intradermally into SKHAV *supFG1* transgenic mouse tails and imaged 1 hour later revealed nuclear accumulation in broad stretches of epidermis, as seen using laser confocal fluorescence microscopy (Figure 4a). Gene targeting by AG30 in keratinocytes was measured 10 days after intradermal injection into tails. Single cell suspensions were prepared from epidermis of several animals, pooled, stained with antibody to keratin 5, and sorted by FACS. Mutation frequency was 4-fold greater in keratin 5 positive keratinocytes from tails treated with AG30 than in those from tails treated with PBS (Figure 4b). The larger surface that back skin provides compared to tail would be advantageous for testing. To demonstrate that a gene targeting oligomer has biological activity in skin other than tail skin, AG30 was injected intradermally into the backs of SKHAV mice and whole epidermis harvested 10 days after the second injection. AG30 induced a 2.7-fold increase in frequency of mutation (19.5×10^{-5}) compared to PBS (7.1×10^{-5}) in back epidermis (Figure 4c), which is the same fold increase seen in tail epidermis in this experiment (Figure 4d). Mutation frequencies in ear epidermis following injection of AG30 in tail were the same as the PBS controls, and those mutations were scattered along the *supFG1* sequence, not concentrated at the triplex-binding site.

DISCUSSION

These studies extend previous reports that systemically administered, sequence-specific TFOs target skin (Vasquez *et al*., 2000) and confirm that the transduction peptide, Antennapedia, enhances the *in vivo* efficacy of sequence-specific, gene-modifying PNAs (Rogers *et al*., 2012). Gene targeting by triplex-forming molecules is now well-established in cultured cells and in mice *in vivo*. Experiments in this report add two important and novel observations to this growing literature. First, local delivery of these gene-modifying compounds can be at least as effective as systemic delivery. Local delivery will be important in future efforts aimed at increasing efficiency of targeting, reducing cost, and minimizing targeting or toxicity to off-target cells or tissues. Second, to our knowledge there has been no previous demonstration that triplex-forming molecules can induce sequence-specific gene modification in a keratinocyte or, for that matter, in any epithelial cell. It is now clear that systemic administration of AG30 or PNA-Antp targets whole tissues (Rogers *et al*., 2012; Vasquez *et al*., 2000) and bone marrow-derived cells, including hematopoietic stem cells and myeloid and lymphoid cells (Rogers *et al*., 2012). Analysis of whole tissues leaves open the question of whether some non-epithelial cell type common to the tissues targeted, such as endothelial cells, fibroblasts or migratory bone marrow-derived cells, were the modified cells. Our studies now demonstrate that TFMs target genes in both the epidermis and dermis, and that at least some of the targeted cells in the epidermis are basal keratinocytes. This result implies that *in vivo* local delivery of TFMs has the potential to permanently modify genes that cause disease in the epidermis.

Proliferating keratinocytes might be expected to be especially susceptible to TFM-mediated gene modification because it is well-established that triplex-mediated targeting strongly favors cells passing through the S or M phases of the cell cycle (Majumdar *et al*., 2003). That those targeted cells would persist for more than a week after TFM delivery, the time it takes for mouse epidermis to completely turn over, could not be anticipated; persistence that long implies that the TFM-induced gene modifications that we measured originated in proliferating, not post-mitotic cells or in cells committed to differentiate. The lower targeting frequency in $K5^+$ keratinocytes compared to intact epidermis is likely explained by epidermal kinetics; over time, an increasing fraction of targeted, transient amplifying basal cells commit to differentiation and are desquamated. For gene modifications to become permanent within the epidermis, keratinocyte stem cells will need to be targeted, and that may require an additional therapeutic step of inducing those stem cells to divide just prior to administering the TFM. It is possible that the PNA-Antp or the AG30 also targeted *supFG1* in post-mitotic keratinocytes or other cells in epidermis, such as melanocytes or dendritic cells, and contributed to the higher targeting seen in whole epidermis. This seems unlikely since those cells are generally non-mitotic, and there is no evidence that active transcription of the transgene, another way of making a gene accessible for targeting (Macris and Glazer, 2003), occurs in epidermis of these animals.

Two different triplex-forming molecules that target the same *supFG1* sequence were used in our experiments. TFMs bind to the major groove in polypurine:polypyrimidine stretches of duplex DNA in a sequence-specific manner according to a well-established binding “code” (Knauert and Glazer, 2001; Letai *et al*., 1988). Polypurine TFOs, such as AG30, bind in a 5′

to 3′ orientation antiparallel to the purine strand of the duplex and polypyrimidine TFOs bind parallel to the purine strand of the duplex. PNAs form triplex DNA according to the same base binding code as TFOs but utilize an amide backbone rather than a phosphodiester backbone, giving them several advantages, including increased binding affinity and resistance to nucleases (Nielsen, 2010). Compared to TFOs, delivery of PNAs to cells in culture has required more extreme measures, such as cell permeabilization with streptolysin-O (Faruqi *et al*., 1998) or electroporation. We had previously shown that covalent coupling of the PNA to the cell penetrating peptide from antennapedia increased uptake and targeting efficiency *in vitro* (Rogers *et al*., 2004) and *in vivo* (Rogers *et al*., 2012). We now show that this peptide greatly increases targeting of epidermal keratinocytes *in vivo*. By contrast, the Antennapedia peptide did not improve targeting of dermal cells. These results emphasize that delivery of gene targeting oligomers may be very different *in vivo* than *in vitro*, and that delivery properties are cell-type specific. Uptake and biological activity in dermis of the PNA lacking the Antp is consistent with at least one other example of *in vivo* biological activity of an unmodified PNAs used to induce exon-skipping in the dystrophin gene in muscle cells (Yin *et al*., 2008)

Taken together, experiments reported here establish the *supFG1* transgenic mouse as a useful and robust model in which to test and compare different methods of local delivery and biological efficacy of different gene-modifying small molecules to keratinocytes. Local delivery of a therapeutic agent could be advantageous in several ways, including the potential for increased local concentration and decreased side effects in non-target tissues. It has been suggested that high hydrodynamic pressure is important for delivering siRNA by intradermal injection into mouse paw (Gonzalez-Gonzalez *et al*., 2010). Our finding that gene modification occurred to the same extent in back as in tail skin following intradermal injection, where delivery of 50μl should result in quite different post-injection pressures, implies that pressure may not be necessary for delivering gene-modifying molecules to epidermis.

To date, triplex-mediated targeting of DNA occurs at low frequency. Some rare events, however, can have a clinically significant impact. In several instances of epidermolysis bullosa, spontaneous heritable genetic events in keratinocytes lead to expanding, clonal regions of skin that do not blister (Jonkman and Pasmooij, 2009; Jonkman *et al*., 1997; Smith *et al*., 2004). In ichthyosis with confetti caused by a rare class of mutations in KRT10, spontaneous loss of heterozygosity eliminates the mutant keratin gene through a recombination event that results in expanding clones of normal-appearing skin (Choate *et al*., 2010). In diseases such as epidermolysis bullosa and ichthyosis with confetti, low frequency gene correction strategies like TFM-mediated gene targeting could have a substantial therapeutic impact.

MATERIALS AND METHODS

Triplex-forming oligomers

PNA oligomers were obtained from BioSynthesis, Inc (Lewisville, TX). The PNA oligomer, used for targeting the *supFG1* gene, was synthesized with the sequence JJJ JJT TJJ T-O-O-O-TCC TTC CCC C $(Lys)_3$ [O=8-amino-2,6,dioxaoctanoic acid], and designed to bind as a

clamp to the homopurine strand of positions 167–176 of *supFG1*, as previously described (Faruqi *et al*., 1998; Rogers *et al*., 2012). Pseudoisocytosine (J), which mimics N-3 protonated cytosine, was used to ensure pH-independent binding to G in the Hoogsteen mode of triplex formation in the PNA:DNA:PNA triplex. The cell penetrating peptide, antennapedia, was covalently linked to the PNA at the C-terminal lysine via continuous synthesis with the following sequence, JJJ JJT TJJ T-O-O-O-TCC TTC CCC C-O-O-KKKKKWKMRRNQFWIKIQR (PNA-Antp). For the labeled PNA-Antp conjugates, FITC was conjugated to the PNA at the N-terminus via two O linkers. The DNA oligomer, AG30, was prepared by Midland Certified Reagent Co (Midland, TX) with a 3′-proplyamine and is designed to bind to the homopurine strand of positions 167–196 of *supFG1* as previously described (Vasquez *et al*., 2000). FITC was covalently linked to the 5′ end of AG30.

Mice

The AV *supFG1* mice were derived in a CD1 background (Charles River Laboratories Inc, Wilmington, MA), as described (Rogers *et al*., 2012). DNA dot blot analysis demonstrated ~ 50 copies of the λ shuttle vector DNA in their genomes (data not shown). The presence or absence of the *supFG1* reporter gene in the resulting pups was determined by polymerase chain reaction as described previously (Narayanan *et al*., 1997). SKHAV *supFG1* mice were derived by breeding AV mice to SKH1 mice (Charles River Labs, Troy, NY) and back crossing transgenic offspring to SKH1 until the transgene, identified by PCR as described above, was in a hairless background. All procedures were conducted according to animal protocols approved by the Yale University Institutional Animals Care and Use Committer, using the guidelines set by the Institutional Animal Care and Use Committees of the National Institutes of Health and Yale University.

Imaging

Transgenic AV or SKHAV mice were injected intradermally in the tail with FITC- PNA-Antp or FITC-AG30, 10μg in 50μl PBS. Mice were anesthetized with ketamine-xylazine and *en face* images of living tissue *in vivo* were obtained 1 and 5 hours after treatment using a VivaScope 2500 (Lucid Inc., Henrietta, New York, USA) confocal imager equipped with a 630nm reflectance laser and a 488nm fluorescence laser(Gonzalez-Gonzalez, *et al* 2011). Aquasonic H100 (Parker laboratories, INC. Fairfield,NJ) ultrasound transmission gel was used as immersion medium between the objective lens and the tissue cassette. The mouse tail was placed over the glass window of the tissue cassette after adding Crodamol STS (Croda Inc., Edison, NJ) as index matching fluid (index of Refraction 1.47). The Z-depth position was adjusted using the 630 nm reflectance laser to identify the stratum corneum. The 488 laser was then used obtain Z stacks of 750×750μm slices of 5μm thickness. Images shown are from slices where most of the cells are basal or spinous keratinocytes. Mice were also sacrificed and tissue samples harvested, embedded in O.C.T. embedding medium (Sakura, Tokyo, Japan) and frozen. 6μm tissue sections were prepared, mounted onto glass slides and examined by fluorescence microscopy using a Zeiss Axiovert 200 microscope and AxioVision 45 software.

TFO delivery to mice

Intraperitoneal injections of PNA (20 mg/kg), PNA Antp (20mg/kg) or PBS were administered to AV mice at 2–3 weeks of age in 200 μl solution of PBS. Injections were given on days one and three, at the same time each day, and tissue was harvested 10 days after the last treatment, according to a recently reported protocol known to successfully target other organs (Rogers *et al*., 2012). Intradermal injections of PNA Antp (10 μg), AG30 (50 μg), or PBS were given to AV or SKHAV mice at 3 weeks of age in volume of 50 μl PBS. Injections were given on days one and three, at the same time each day and tissue was harvested 10 days after the last treatment.

Tissue preparation

Tail or back skin in 1×2cm pieces was removed to fascia and, where indicated, epidermis was separated from the dermis by overnight exposure at 4°C to 2.5 % Dispase II (Roche, Indianapolis, IN) in PBS (Adams *et al*., 2005). To isolate keratin 5 positive keratinocytes, epidermal sheets were incubated in 0.25% trypsin for 10 minutes at 37°C, filtered sequentially through 100μm and 40μm strainers (BD Biosciences, San Jose, CA) fixed with Cytofix/Cytoperm (BD Biosciences, San Jose, CA) for 30 minutes at room temperature, washed with Perm/Wash (BD Biosciences, San Jose, CA), incubated with rabbit anti mouse keratin 5 (Neomarkers Thermo Fisher, Kalamazoo, MI) for 30 minutes at 4 °C, washed, incubated with FITC conjugated anti rabbit IgG (BD Biosciences, San Jose, CA) for 20 minutes at 4 °C and washed again. Fluorescence activated cell sorting (FACS) analysis was performed using a FACSVantage SE (BD Biosciences, San Jose, CA). High molecular genomic DNA pooled from 6 8 mice was isolated from these $K5^+$ cells and analyzed for induction of mutation.

supFG1 mutagenesis assay

Genomic DNA was isolated from tissues or cells and analyzed for induction of mutations as previously described (Rogers *et al*., 2012; Vasquez *et al*., 2000). Briefly, genomic DNA was incubated with λ *in vitro* packaging extracts for shuttle vector rescue and reporter analysis by a blue white bacterial screen and sequencing of representative mutant plaques to characterize the types of mutations induced by TFO treatment. Each treatment group consisted of three mice and standard errors were calculated for the mutation frequency values as indicated by the error bars, except for the pooled $K5^+$ cells in the PNA Antp experiment where the frequencies are the mean of two experiments ± standard deviations.

ACKNOWLEDGEMENTS

Authors are grateful for the fertile intellectual environments created by Peter Glazer and the GO Delivery! Project organized by Roger Kaspar, and for assistance from Denise Hegan. This work was supported by the NIH (RC2ARO58955 (Roger Kaspar, Christopher Contag and LMM), K22 CA120049 (FAR) and Yale Skin Disease Research Center P30 AR41942 (FAR).

Abbreviations

TFM triplex forming molecule

TFO	triplex forming oligonucleotide
PNA	peptide nucleic acid
Antp	antennapedia
HPRT	hypoxanthine phosphoribosyl transferase
CCR5	C-C chemokine receptor 5

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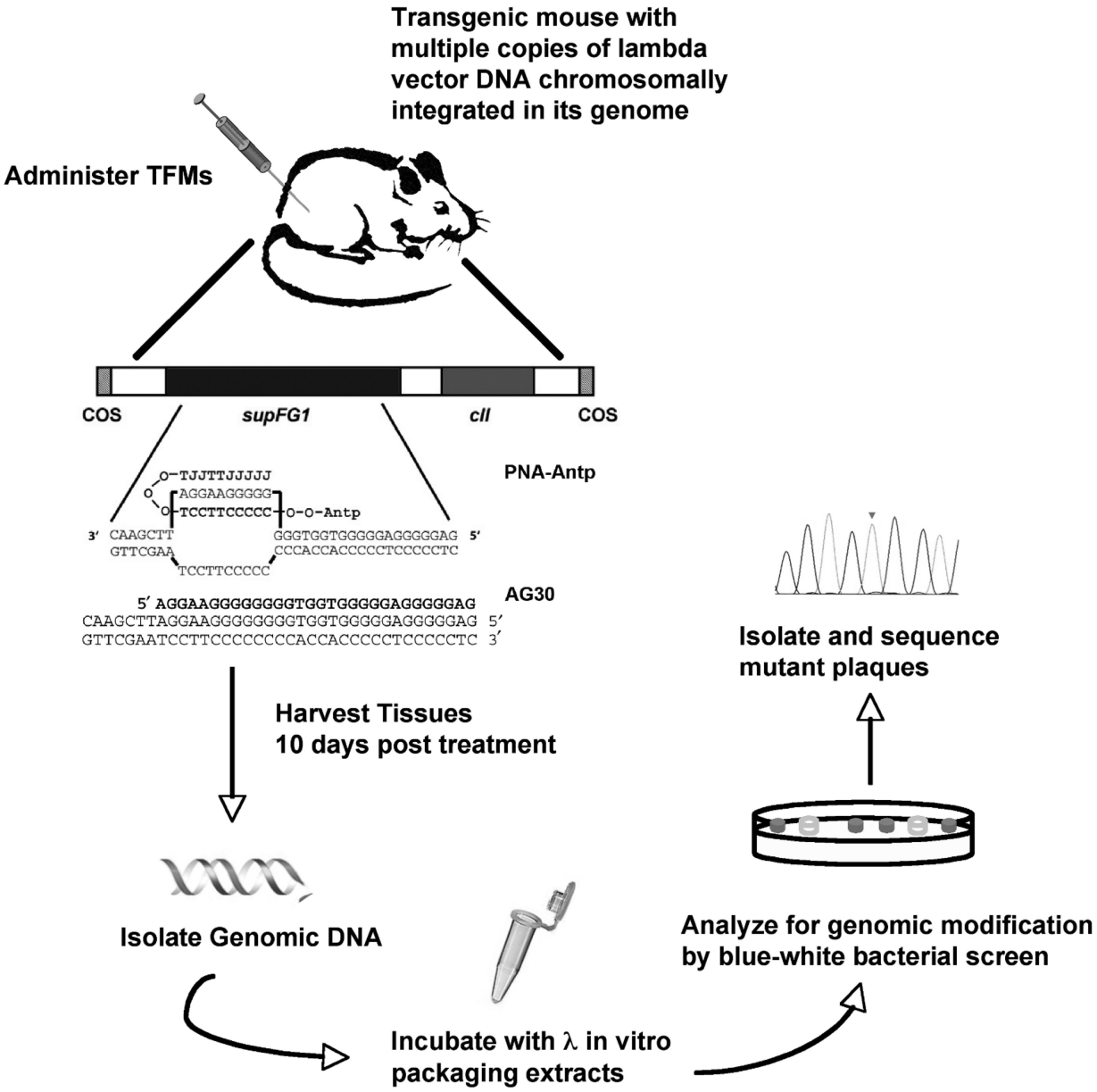

Figure 1. Graphic summarizing experimental design and methods

AV and SKHAV mice have the chromosomally integrated *supFG1* reporter gene. The PNAs (used in the AV mice) form a strand invasion complex by binding to the homopurine strand at position 167–176 of the *supFG1* gene and generating a PNA:DNA:PNA triplex. The AG30, used in the SKHAV mice, form a triplex structure at positions 167–196 of *supFG1*.

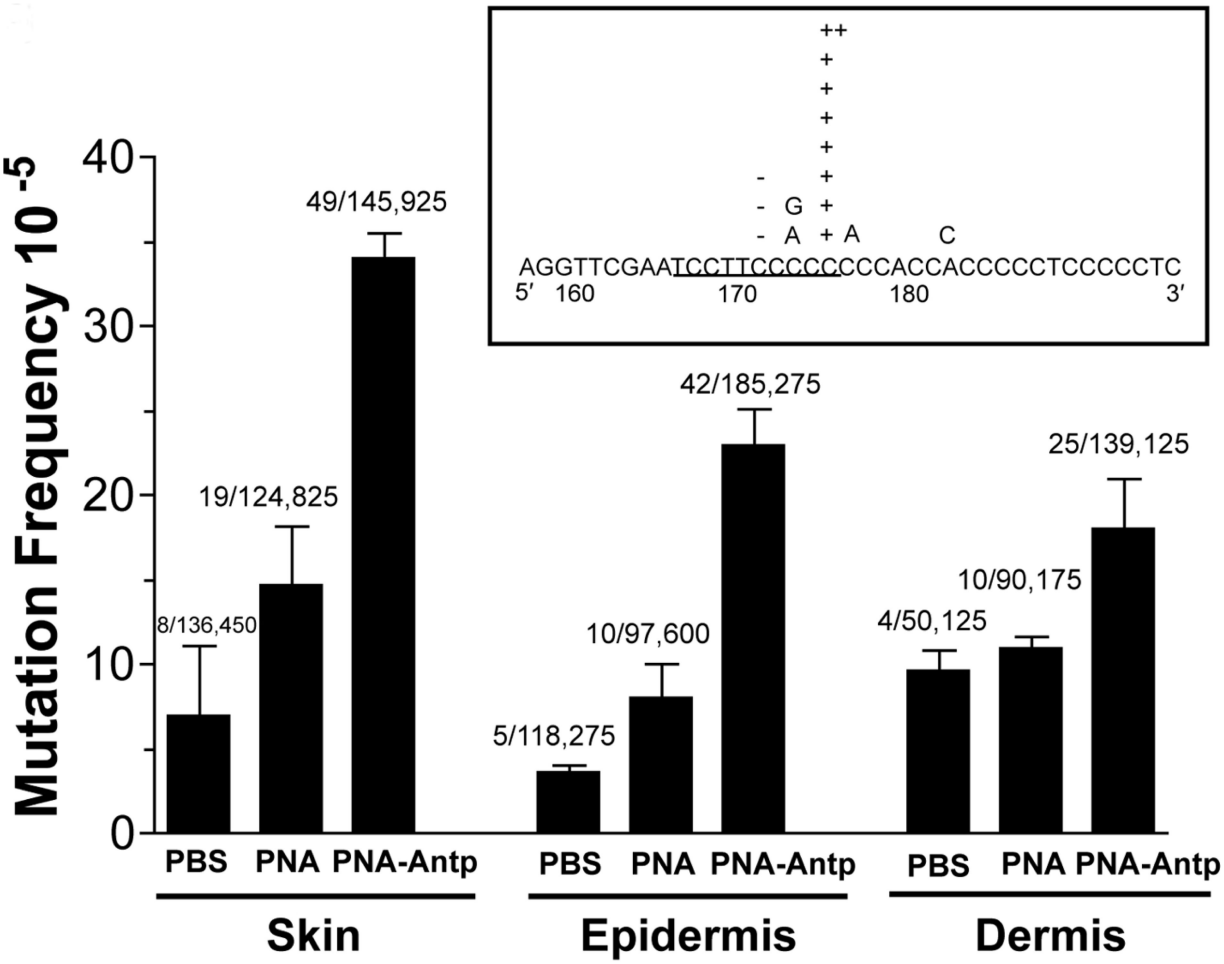

Figure 2. Gene targeting in epidermis and dermis of mice after intraperitoneal delivery of triplex-forming PNAs

AV mice received PNA, PNA-Antp or PBS by intraperitoneal injection. Ten days later mutations were measured in the *supFG1* transgene recovered from genomic DNA of intact tail skin or dispase-separated epidermis and dermis. Numbers over bars are mutants plaques over total plaques counted. Frequencies are averages +/− SEM from individual mice. Inset shows sequence analysis of mutations induced by treatment with PNA-Antp. The PNA target site is underlined and (+) and (−) represent single base pair insertions and deletions.

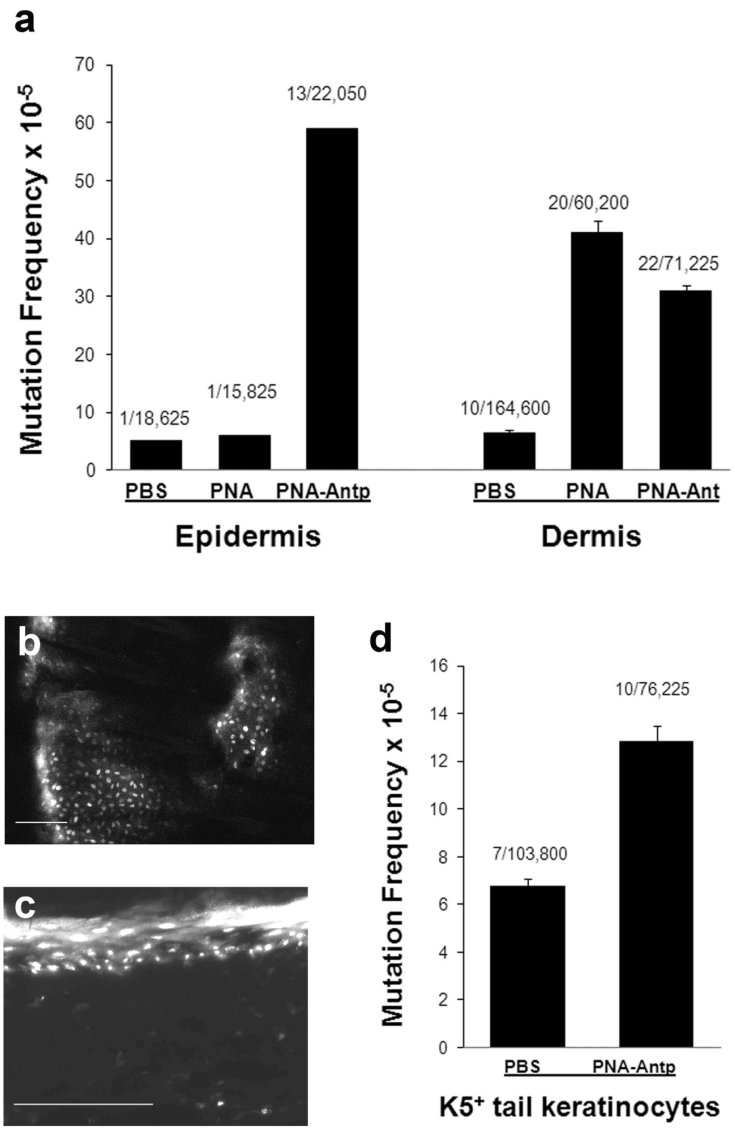

Figure 3. Gene targeting in epidermis, dermis and tail keratinocytes of mice after intradermal delivery of triplex-forming PNAs

(a) AV mice received PNA, PNA-Antp or PBS by intradermal injection. Ten day later mutations were measured in the *supFG1* transgene recovered from genomic DNA of dispase-separated epidermis and dermis. Numbers over bars are mutants plaques over total plaques counted and frequencies are averages +/− SEM from individual mice. **(b and c)** Imaging performed 5 hours after intradermal injection of FITC-PNA-Antp into tail. Image oriented with caudal end of tail to right. Bars = 50μm. **(b)** *En face* confocal image of a tail scale showing nuclear fluorescence in basal keratinocytes at edges of the image and little fluorescence in the papillary dermis in the center of the image. **(c)** Epifluorescence image shows nuclear fluorescence throughout skin, stronger in epidermis than dermis. **(d)** AV mice received PNA-Antp or PBS by intradermal injection. Ten days later mutations were measured in the *supFG1* transgene recovered from genomic DNA of keratin 5 positive keratinocytes pooled from tails of 4–6 mice. Numbers over bars are mutant plaques over

total plaques counted and frequencies are averages +/− SD from two separate experiments using pooled keratinocytes.

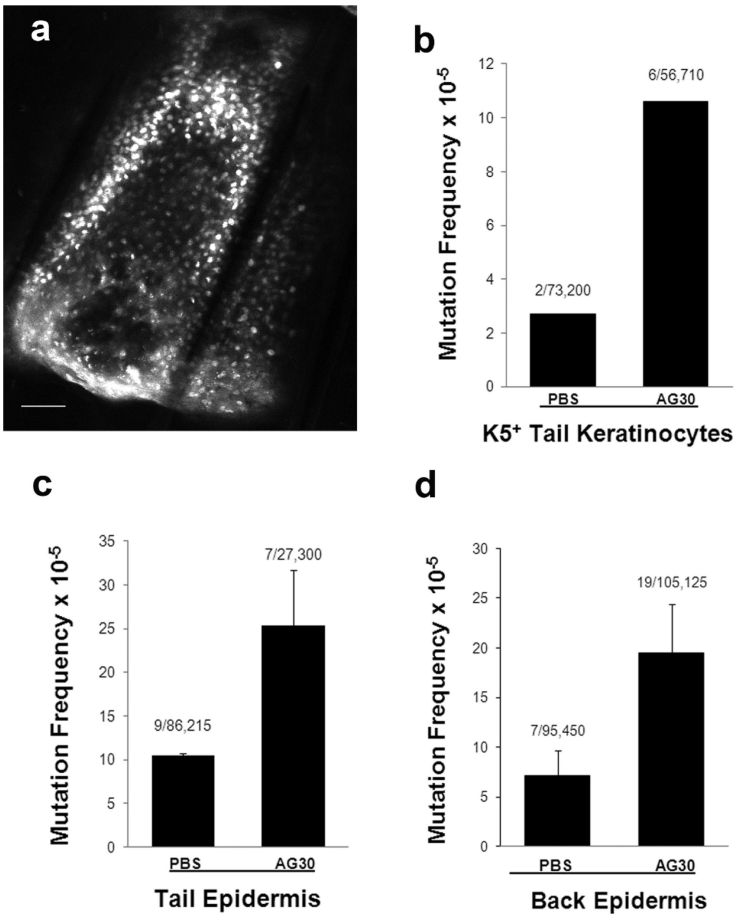

Figure 4. Gene targeting in tail and dorsal epidermis of hairless mice after intradermal delivery of triplex-forming oligonucleotide, AG30

(a) Confocal fluorescence microscopy of epidermis performed 1 hour after intradermal injection of FITC-AG30. The caudal end of the tail is in upper right corner and nuclear fluorescence is seen in the *en face* confocal slice that goes through basal keratinocytes at the edges of a tail scale. Bar = 50μm. (**b**) SKHAV mice received AG30 or PBS by intradermal injection. Ten days later mutations were measured in the *supFG1* transgene recovered from genomic DNA of keratin 5 positive keratinocytes pooled from tails of 4–6 mice. (**c and d)** SKHAV mice received AG30 or PBS by intradermal injection. Ten days later mutations were measured in the *supFG1* transgene recovered from genomic DNA of tail (**c**) or back (**d**) epidermis. Numbers over bars are mutants plaques over total plaques counted and frequencies are averages +/− SEM (in c and d) from individual mice.