Bigenic Cre/loxP, puΔtk conditional genetic ablation

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ABSTRACT

Genetic ablation experiments are used to resolve problems regarding cell lineages and the in vivo function of certain groups of cells. We describe a two-component conditional ablation technology using a mouse carrying an X-linked puΔtk transgene, which is only activated in cells expressing Cre. Ablation of the Cre-expressing cells can be temporally regulated by the time of ganciclovir (GCV) administration. This strategy was demonstrated using a Col2Cre transgenic line. Differentiating chondrocytes in bigenic animals could be ablated at different developmental stages resulting in disorganized growth plates and dwarfism. Macrocephaly, macroglossia and umbilical hernia were also observed in ablated 18.5 dpc embryos. Crosses between the puΔtk selector line and existing cre lines will facilitate numerous temporally regulated tissue-specific ablation experiments.

INTRODUCTION

Cell ablation experiments are used to resolve questions regarding cell lineages, physiological function, differentiation potential and interactions between groups of cells (1–3). Different strategies have been used to achieve ablation in vivo. Surgical ablation can be used to remove a specific organ or an embryonic precursor during development (4,5). Laser surgery can be used to ablate single cells in organisms with well-defined cell lineages and with a limited number of cells such as Caenorhabditis elegans (6). Ablation can also be achieved genetically, based on the temporal and spatial expression pattern of a transgene in a group of cells in a transgenic animal (1,7,8). Genetic ablation targeted by the expression of molecular markers can be more precise than surgical techniques, because molecular differences between adjacent cells are often not obvious anatomically (2,9).

Genetic ablation in transgenic mice utilizes tissue-specific promoters to drive suicide genes, such as diphtheria toxin A chain (DTA) or Herpes Simplex Virus type I thymidine kinase (HSV1 tk) (1–3,9). When DTA is used, the cells in the target tissue are killed when DTA is expressed. Embryonic expression of DTA ablates cells and tissues during development which prevents analysis of target tissues in later development or adult life (3,10). When HSV1 tk is used, temporal control of the ablation is possible, because killing depends on the administration of a prodrug, such as ganciclovir (GCV) or 1-(2-deoxy-2-fluoro-1-beta-D-arabinofuranosyl)-5-iodouracil (FIAU). Thus, ablation that causes embryonic or neonatal lethality can be avoided, enabling the physiological function of target cells to be examined in adults (1,8). Despite the advantages of HSV1 tk, its use is constrained by the infertility of male transgenic mice carrying HSV1 tk (11–13).

Here, we described a mouse strain, puΔtk, which responds to Cre expression by activating a conditional suicide gene, puΔtk. PuΔtk is a fusion protein between puromycin N-acetyltransferase and a truncated version of HSV1 tk, which does not cause male infertility in transgenic mice (14). The puΔtk selector allele can be used with the existing tissue-specific cre lines so that conditional cell ablation experiments can be performed in a variety of tissues. We demonstrate ablation at different developmental stages in both embryos and adults using a Col2Cre transgene.

MATERIALS AND METHODS

puΔtk selector construction

Two adjacent loxP sites were inserted into pBSII-KS- by a three-way ligation between a PstI and XhoI double digested pBSII-KS- plasmid and two small DNA fragments composed of two sets of oligonucleotides containing loxP sites as well as an AscI site or an SfiI site. 3295 5'-GGG GGG CCG ATC AGG CCA TAA CTT CGT ATG ATC TAA ATA CGA AGT TAT C-3', 3296 5'-TCC AGA TAA CTT CGT ATG TAT C'GTA AGT GAT CCT GAC C'TG ATC GGC C-3', 3297 5'-GAT AAC TTC GTA TAG CAT ACA TTA-3'. A neomycin (neo) coding region and the bovine growth hormone poly(A) signal (bpA) was then introduced between the AscI and SfiI sites, so that it was flanked by the two loxP sites. The loxP-neo-bpA-loxP cassette was then excised from this vector and inserted into a pYTC37 (14) derived plasmid in a position downstream of the phosphoglycerate kinase-1 promoter (PGK) and upstream of the puΔtk coding region to create the selector cassette.

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PGK-\text{loxP-neo-bpA-}\text{loxP-}\pu\Delta\text{tk-bpA}. An \text{Hprt} replacement targeting vector was constructed by inserting this cassette into \text{Hprt} exon 3 of an \text{Hprt} targeting vector (15) in an orientation opposite to that of endogenous transcription. The function of the \text{loxP} sites in this vector were tested by transforming constructs into the BNN132 \textit{Escherichia coli} strain (BD Biosciences Clontech, CA).

Generation of \pu\Delta\text{tk} selector mice

The targeting construct was linearized with \text{PvuI} and electroporated into the AB1 ES cell line maintained on irradiated SNLP-7/4 feeders (16,17). \text{G418} selection was initiated 24 h after electroporation and maintained for 4 days. Subsequently, the cells were selected in both \text{G418} and 6-thioguanine for 6 days. Targeted clones were picked and confirmed by Southern blot analysis of HindIII-digested genomic DNA, probed with an external genomic fragment (15). The functionality of the \text{loxP} sites in the \pu\Delta\text{tk} selector ES cell line was tested by the acquisition of both puromycin resistance and FIAU sensitivity following electroporation of a CMV-\text{cre} expression cassette. The \text{Hprt} \pu\Delta\text{tk} selector allele was established in mice following blastocyst injection and germ line transmission of the BC85-D4 clone.

Mice

Heterozygous \pu\Delta\text{tk} selector females were mated with \text{Col2Cre} heterozygous males (18) to generate \text{Col2Cre}, \pu\Delta\text{tk} progeny. \text{GCV} (Roche, CA) was administered daily at a dose of 100 mg/kg by intraperitoneal injection. For embryonic chondrocyte ablation, \text{GCV} was administered to pregnant female \pu\Delta\text{tk} selector mice beginning either at 4.5 dpc, 12.5 dpc or 14.5 dpc and repeated daily until 18.5 dpc. For adult chondrocyte ablation, \text{GCV} was administered daily to 2-week-old pups for either 3 or 6 weeks.

RT-PCR

Dissected tissues from a 2-week-old \text{Col2Cre}, \pu\Delta\text{tk} compound heterozygous male were frozen in liquid nitrogen. Total RNA was extracted using Trizol (Invitrogen, CA) and reverse transcribed by MMLV reverse transcriptase (Superscript II; Invitrogen). Primers used for reverse transcription are either poly(T) for \beta-actin or 5893 5'-GCT CTA GAG CTC AGT TAG CCT CCC CCA TCT CCC GGG C-3' for \pu\Delta\text{tk}. Primers for PCR reactions for \beta-actin and \pu\Delta\text{tk} were 5641 5'-GAG AAG AGC TAT GAG CTG CCT-3', 5642 5'-ATC CTG TCA GCA ATG-3', 5891 5'-ATG ACA AGC GCC CAG ATA AC -3' and 5892 5'-ATG CTG CCC ATA AGG TAT CG -3'.

BrdU labelling

Five-week-old mice were injected intraperitoneally with 100 \muM of 100 \muM Bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO) in phosphate-buffered saline 1 h before the tibia was isolated. The tibia was fixed for 6 h in 4% paraformaldehyde (PFA) (Sigma) and processed for paraffin sections (19). Seven micrometer sections were prepared and BrdU incorporation was visualized using a staining kit (Zymed, CA). Equivalent sections were selected and BrdU positive cells were counted in the same regions from all genotypes. This data was expressed as a percentage of the total number of chondrocytes in the proliferating zone or the extended 'hypertrophic chondrocyte-like' region in the ablated bigenic mice.

X-ray analysis

X-ray analysis was performed using a Faxitron Specimen Radiography System Model MX-20 (Faxitron, IL). Before analysis the skin was removed, the mice were eviscerated and fixed in PFA.

Morphometry and histology

Femur length measurements were performed on cleaned bones with a dial caliper (Scienceware, NJ). For histological analysis, tibias were fixed in 4% PFA overnight, dehydrated in graded ethanols and embedded in methyl methacrylate at low temperature (20). Sagittal 7 \mu m sections of the calcified tibia were cut with a tungsten carbide blade and collected on precoated slides (Superfrost Plus, NH). Sections were stained according to the Von Kossa method with a Van Gieson counterstain to analyse mineralized and osteoid bone. They were also stained with Weigert's haematoxilin/fast green/safranin O for bone and cartilage analysis (21,22).

\textit{In situ} hybridization

Sample preparations for paraffin sections and the \text{Col2\alpha1}, \text{Col10\alpha1} \textit{in situ} hybridization were carried out as described previously (23,24). Brie fly, dissected tibias were fixed in 4% PFA overnight, dehydrated in graded ethanols and embedded in methyl methacrylate at low temperature (20). Sagittal 7 \mu m sections of the calcified tibia were cut with a tungsten carbide blade and collected on precoated slides (Superfrost Plus, NH). Sections were stained according to the Von Kossa method with a Van Gieson counterstain to analyse mineralized and osteoid bone. They were also stained with Weigert's haematoxilin/fast green/safranin O for bone and cartilage analysis (21,22).

RESULTS AND DISCUSSION

Construction of the \pu\Delta\text{tk} selector mouse

To generate an allele which can be combined with existing \text{cre} lines to initiate ablation in different tissues, we constructed a cassette which responds to \text{Cre} by activating the conditional suicide gene, \pu\Delta\text{tk} (Figure 1A). \pu\Delta\text{tk} avoids the complication of male infertility frequently seen in transgenic mice carrying full-length HSV1 \text{tk} (14). The \pu\Delta\text{tk} selector construct is driven by the PGK promoter which has a broad expression pattern at different developmental stages (26). It was shown that the PGK promoter can drive ubiquitous expression of a reporter gene in many embryonic organs, including the liver, kidney, intestine, heart, limb and rib cartilage in the transgenic mice (27). In an un-induced state, the PGK promoter drives a \text{loxP} flanked \text{neo} cassette. When \text{Cre} is
expressed, recombination between the loxP sites deletes the neo-bpA cassette, resulting in juxtaposition of the puDTk cassette and the PGK promoter resulting in expression of puDTk. The suicide gene puDTk is only activated in cells which express Cre and their descendants. These cells can be killed by prodrugs, such as GCV or FIAU. These same cell lineages can also be selected in vitro, because the puDTk fusion gene enables positive selection in puromycin (28).

The puDTk cassette was targeted into the endogenous Hprt gene on the X-chromosome (Figure 1A and B). This locus is an excellent site for inserting transgenes, because a single copy transgene can be easily targeted to this locus in ES cells and Hprt null mice have a very mild phenotype (29–31). The location of the puDTk construct on the X-chromosome enables this allele to be tracked in mice, reducing the need for molecular genotyping. X-inactivation in females allows partial ablation experiments to be conducted in situations where the ablated cell type is essential for survival, while complete ablation can be achieved in males.

The targeted puDTk selector ES cell line BC85-D4 was tested for its response to Cre following transient expression. The clone BC85-D4 was used to establish germ line chimaeras. The puDTk selector allele was only found in the female descendents from transmitting chimaeras consistent with its X-linkage. Hemizygous puDTk selector males were obtained in the next generation, and these did not show any

Figure 1. Construction of puDTk selector and the experimental design. (A) The use of puDTk selector in conditional genetic ablation experiments. puDTk is activated in the Cre-expressing tissue of a bigenic mouse. Tissue-specific ablation can be performed in a temporally regulated manner by controlling the timing of GCV administration. (B) Targeting strategy. (C) Southern blot confirming targeting. Genomic DNA isolated from wild-type and puDTk selector embryonic stem cells, digested with HindIII and probed with a 5' external probe, showing the wild-type (7.1 kb) and the puDTk selector (6.8 kb) alleles.
overt phenotype and their fertility was normal. This is consistent with the previous observations of Hprt null mutant mice and the fertility of male mice carrying the puΔtk cassette (14,31).

GCV killing of proliferating chondrocytes

To test the effectiveness of the puΔtk cassette in mediating Cre dependent ablation in vivo, compound heterozygous mice carrying the puΔtk selector allele and chondrocyte-specific Cre cassette (Col2Cre) were generated (Figure 1C).

Heterozygous puΔtk selector females were crossed to heterozygous Col2Cre (18) males to generate progeny with all four possible genotypes. Owing to random X-inactivation, mosaic expression of the selector cassette is expected in females, and therefore only male progeny were used. Cartilage-specific puΔtk mRNA expression was confirmed by RT–PCR performed on RNA samples isolated from different tissues from a 2-week-old Col2Cre, puΔtk male (Figure 2A).

Figure 2. Cartilage-specific puΔtk activation and GCV-mediated killing of proliferating chondrocytes in a Col2Cre, puΔtk compound heterozygous male. (A) RT–PCR shows that puΔtk is only activated in cartilage. (B) Schematic representation of the normal growth plate. R, resting zone; P, proliferating zone; H, hypertrophic zone; and O, ossifying zone. (C) BrdU immunohistochemistry reveals complete killing of proliferating chondrocytes in a Col2Cre, puΔtk compound heterozygous male. In contrast to the tibia growth plate in the wild-type mouse, the tibia growth plate of the bigenic animal is totally disorganized. No obvious zones of chondrocytes representing the different stages of differentiation exist, instead an extended region comprised of enlarged, hypertrophic chondrocyte-like cells was observed. Arrows indicate BrdU+ cells.

Bigenic males were treated daily with GCV starting at 2 weeks of age. GCV-mediated killing of the proliferating chondrocytes was evaluated by BrdU incorporation at 5 weeks. In wild-type, Col2Cre and puΔtk selector mice, 12–13% of the chondrocytes in the proliferating zone of the tibia growth plate were labelled by BrdU (Figure 2C). Thus, GCV treatment did not appear to affect proliferating chondrocytes. In contrast, no BrdU-labelled chondrocytes could be detected in tibia growth plate sections from puΔtk, Col2Cre compound heterozygous animals (Figure 2C). These sections also revealed that these mice had disorganized growth plates with no proliferation zone. Thus, GCV treatment mediated complete killing of the proliferating chondrocytes in compound heterozygous mice.

Proliferating chondrocyte ablation blocks long bone growth

The impact of proliferating chondrocyte ablation on long bone growth was analysed. All four different genotypes of mice were treated with GCV from 2 weeks of age and as early as 3 weeks after the initiation of GCV treatment the growth in length of the long bones in compound heterozygotes had ceased. Mice of the other three genotypes were unaffected (data not shown). Whole body X-ray photographs were taken when the mice reached 8 weeks of age (Figure 3A). These revealed dwarfism, reduced bone density, dome-shaped rib cages and bowing of the extremities in GCV-treated compound heterozygous mice. Where the growth plates ought to have been increased opacity was noted. Thus, GCV ablation of proliferating chondrocytes appeared to block long bone growth.

Detailed analysis of Von Kossa stained sections from GCV-treated Col2Cre, puΔtk mice revealed that both primary and secondary spongiosa were absent (data not shown) and that the growth plates were disorganized, with no obvious proliferating chondrocyte zone and an extended hypertrophic chondrocyte-like region (Figure 3C and D). Chondrocytes within the hypertrophic chondrocyte-like region were enlarged but did not appear to have undergone apoptosis. Blood vessel invasion was not observed, indicating that the long bone growth process was blocked. However, analysis of Von Kossa stained tibia sections indicated that the mineralization process was not affected (Figure 3C and D).

Abnormal hypertrophic chondrocyte differentiation

The extended hypertrophic chondrocyte-like region in the proliferating chondrocyte-ablated bigenic mice was examined in more detail. Tibia sections stained with safranin O, which stains cartilage matrix glycoprotein, showed that the cartilage matrix was relatively normal after the proliferating chondrocytes had been ablated (Figure 4).

Markers of proliferating chondrocytes (Col2α1) and hypertrophic chondrocytes (Col10α1) (32) were tested by in situ hybridization on tibia growth plate sections. Col2α1 transcripts were absent in the GCV-treated bigenic animals, indicating complete ablation of proliferating chondrocytes. Col10α1 transcripts were absent in the tibia growth plates of the GCV-treated bigenic animals, suggesting that the enlarged chondrocytes observed in the extended hypertrophic
chondrocyte-like region are not terminally differentiated hypertrophic chondrocytes. One possible explanation for this phenotype is that proliferating chondrocytes are essential for the proper function or differentiation of hypertrophic chondrocytes (32,33), implicating signalling between proliferating and hypertrophic chondrocytes. Interestingly, normal mineralization was observed in the growth plate of the GCV-treated bigenic animals (Figure 3D). Thus, Col10a1 expression is not necessary for the mineralization process, consistent with the observation of normal mineralization in Col10a1 null mice (34,35).

The reversibility of GCV-mediated long bone growth block was examined. Drug treatment was stopped after 3 weeks of administration and tibias were isolated and examined 3 weeks later. The extended hypertrophic chondrocyte-like region in the growth plate was absent and replaced by a thinner growth plate with normal organization (Figure 4). The normal proliferating chondrocyte zone and the hypertrophic chondrocyte zone were restored, and in situ hybridization analysis revealed that both Col2a1 and Col10a1 expression patterns had returned to normal. Primary and secondary spongiosa was also observed in the tibia section after the recovery period. Thus, the GCV treatment did not kill or prevent resting chondrocytes from restoring a functional growth plate, and chondrocytes lying in the extended hypertrophic chondrocyte-like region were able to terminally differentiate into functional hypertrophic chondrocytes that could participate the long bone growth.

**Embryonic GCV-mediated chondrocyte ablation**

To test whether this conditional ablation system can be used during development, GCV was administered to puDtk selector females after mating with Col2Cre males. Daily GCV treatment was initiated 1 day after implantation (4.5 dpc) and the embryos were examined at E18.5. The sex of embryos was assessed based on anatomic features and was confirmed using a Y-chromosome-specific probe (36).

The GCV-treated bigenic 18.5 dpc embryos were smaller than their littermates (Figure 5A). On average, they were 70% of the normal length and 50% of the normal weight. They exhibited multiple cranial–facial defects and an abnormal skin texture. The GCV-treated bigenic animals also exhibit an umbilical hernia (Figure 5D and E), cleft palate, (Figure 5F) and under ossified vertebrae (Figure 5G, H and I). Dome-shaped heads and other head abnormalities were observed (Figure 5A, F and I). These features are reminiscent of those seen in mouse mutants with chondrocyte developmental defects (37–39).
We explored the consequences of initiating ablation at different embryonic stages. GCV treatment was initiated at E4.5, E12.5 and E14.5, which gave different degrees of chondrocyte ablation. GCV treatment initiated at 4.5 dpc results in the most severe phenotype. There is almost no ossification seen in the ribs and vertebrae of these embryos. These embryos also have a dome-shaped head resulting from the imbalance between the development of a membranous roof and the endochondral base of the cranium (38). The roof of the cranium is least affected since it is comprised of membranous bones that develop without a cartilage intermediate. Ossification defects were much less severe when ablation is initiated at 12.5 dpc or 14.5 dpc. Our results indicate that GCV can cross the placenta, and embryonic cell ablation can be performed at a variety of different embryonic stages.

The use of a bigenic system allows the regulatory element (tissue-specific cre) and the effector element (the puΔk selector cassette) to be separated in the parents (10). Such separation prevents prodrug-mediated ablation of maternal tissues in pregnant females. In these experiments, the bigenic embryos exhibited severe ablation of proliferating chondrocytes, but there was no discernable effect on the pregnant females. To our knowledge, this is the first reported use of the HSV1 tk/GCV to ablate cells during embryonic development in vivo.

One limitation of HSV1 tk/GCV system is that GCV-mediated killing is limited to proliferating cells (40–42). Recently, several other conditional suicide gene/prodrug systems have been used in cell ablation experiments in vivo. DTB-r/DT and Ntr/CB1954 are two such systems in which killing is independent of cell proliferation, in principle these gene/prodrug systems can be used in place of HSVtk/GCV (43,44). Alternatively, DTA, M2 and barnase suicide genes can be used with inducible expression systems (such as the Tet-on/Tet-off system) to mediate conditional killing (3,45,46). It is possible to build effector strains similar to the puΔk selector described here, using different conditional suicide genes that can be activated by site-specific recombination systems, such as the Cre/loxP or Flp/frt (47,48).

Figure 4. Abnormal hypertrophic chondrocyte differentiation in proliferating chondrocyte-ablated mice. Safranin O/fast green staining and Col2α1 and Col10α1 in situ hybridization analyses (from the left to the right) of the tibia growth plates from wild-type (Wt) and bigenic mice before GCV treatment (2wk) after a 3 week GCV treatment (5wk) and after a 3 week recovery following a 3 week GCV treatment (8 week). Glycoproteins stained by safranin O are detected in both the wild-type and bigenic cartilage matrix after the 3 week GCV treatment. Col2α1 is normally expressed in proliferating chondrocytes and Col10α1 is expressed in hypertrophic chondrocytes. The enlarged hypertrophic ‘chondrocyte-like cells’ observed in the growth plate of the ablated bigenic mouse do not express Col2α1 or Col10α1. This analysis suggests that the hypertrophic ‘chondrocyte-like cells’ are neither proliferating chondrocytes nor terminally differentiated, fully functional hypertrophic chondrocytes. SO, safranin O; FG, fast green.
The increase in the repertoire of cre lines will expand the utility of the effector lines described here to different cell lineages.

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