The *Terminator* mouse is a diphtheria toxin receptor knockin mouse strain for rapid and efficient enrichment of desired cell lineages

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Abstract

Biomedical research often requires primary cultures of specific cell types, which are challenging to obtain at high purity in a reproducible fashion. Here we engineered the murine Rosa26 locus by introducing the diphtheria toxin receptor flanked by loxP sites. The resultant strain was nicknamed the *Terminator* mouse. This approach results in diphtheria toxin receptor expression in all non-Cre expressing cell types, making these cells susceptible to diphtheria toxin exposure. In primary cultures of kidney cells derived from the *Terminator* mouse, over 99.99% of cells were dead within 72 hours of diphtheria toxin treatment. After crossing the *Terminator* with the Podocin-Cre (podocyte specific) mouse or the Ggt-Cre (proximal tubule specific) mouse, diphtheria toxin treatment killed non-Cre expressing cells but spared podocytes and proximal tubule cells, respectively, enriching the primary cultures to over 99% purity based on both Western blotting and immunostaining of marker proteins. Thus, the *Terminator* mouse can be a useful tool to selectively and reproducibly obtain even low abundant cell types at high quantity and purity.

Integrated Introduction, Results, and Discussion

An enormous amount of research is currently performed using immortalized cell lines that have been isolated from the organs of various mammalian species and maintained in culture for long periods. These cell lines provide a ready platform for the *in vitro* dissection of signaling pathways, metabolic cycle analysis, cell proliferative and survival responses, channel function, etc. However, this approach is continuously confounded by two problems: 1) immortalized cells exhibit altered proliferative and apoptotic responses as a result of either abnormal gene expression (SV40 e.g.) or selection (MDCK cells, e.g.); and 2) continuous cell culture typically leads to significant epigenetic and phenotypic changes and

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the dysregulated expression of many genes when compared to the *in vivo* expression profile of the parent cell type[1, 2, 3].

These problems can be partially overcome using primary cultures of freshly isolated cells. However, these cells are hard to be efficiently isolated in large numbers with high purity, and attempts to expand primary cultures to obtain large numbers of cells are frequently unsuccessful or lead to either replicative senescence or the selective expansion of abnormal clonal populations. Low abundant cell types are particularly difficult to be purified and analyzed in primary culture.

Generation of the Terminator mouse

To overcome these problems and provide rapid and efficient isolation of large numbers of primary cells of any type, we have developed a mouse in which the diphtheria toxin receptor (DTR) gene is knocked into the Rosa26 locus, thus ubiquitously expressed, but can be turned off by expression of the Cre recombinase[4] (Figure 1A). By mating this mouse, nicknamed the *Terminator* mouse (*Rosa-DTR^{flox}*), to an appropriate Cre strain, DTR expression is permanently removed from cells that express Cre at any time prior to diphtheria toxin selection. Upon Cre mediated recombination, GFP expression is designed to be turned on, driven by the Rosa26 promoter (Figure 1A)[4]. After gene targeting in embryonic stem cells, a total of 8 clones were identified that carried the correctly modified Rosa26 locus and two were subjected to blastocyst microinjection to establish chimeras. Four male chimeras were generated and 3 had germ line transmission. The *Terminator* mouse can be genotyped by a pair of primers that amplify the Rosa26 promoter and the inserted DTR, with the sense primer complementing a genomic area outside of the targeting vector (Figure 1B). The *Terminator* mice are viable, fertile, and healthy (>14 months) without any macroscopic or microscopic abnormalities observed (data not shown).

DTR is ubiquitously expressed

Multiple organs including the brain, heart, intestine, kidney, liver, lung, pancreas, skeletal muscle, gonads, and spleen were isolated and subjected to Western blotting. DTR is detected in all tested organs from the *Terminator* mouse, while wild type mice do not have detectable levels of DTR (Figure 2A, and data not shown).

To test the sensitivity of cells isolated from the *Terminator* mouse to diphtheria toxin (DT), kidneys were minced, digested in collagenase and the resultant cells seeded onto multiple 10 cm dishes. DT (100ng/ml) was added to the culture medium starting on day 2 and cultures washed every 48 hours to remove dead cells. While wild type kidney cells remained healthy after DT treatment, the vast majority of kidney cells from the *Terminator* (*Rosa-DTR^{flox}*) mouse had detached within 24 hours after DT treatment. By 72-96 hrs, the treated dishes had fewer than 10 viable cells/dish remaining, while the untreated dishes were nearly confluent (2B, C). This suggests that the DTR is expressed by nearly all renal cells in the *Terminator* mouse, but that rare cells do survive DT selection even in the absence of Cre expression.

Primary culture of podocytes using the *Podocin-Cre;Rosa-DTR^flox* mouse

To test our hypothesis that the *Terminator* mouse can assist in the enrichment of primary cultures of a particular type of cells, primary cultures were examined from whole kidneys of *Podocin-Cre;Rosa-DTR^flox* double transgenic mice[5]. In the absence of DT treatment, whole kidney cultures exhibited a typical mixed phenotypic picture (Figure 3A). DT treatment resulted in death of many of the adherent cells by 96 hours, and the surviving cells exhibited typical podocyte features (Figure 3A). Western blot analysis of surviving cells revealed enriched expression of the podocyte marker Wt1 and down regulation of the proximal tubule marker megalin in the DT treated samples (Figure 3B). Immunofluorescence revealed that greater than 99% of the cells that survived DT treatment exhibited Wt1+ expression, whereas untreated cultures have approximately 22% WT1+ cells (Figure 3C, D). Podocyte enrichment was further confirmed by quantitative RT-PCR analysis of Wt1 and podocin expression (Figure 3E, F).

Primary culture of proximal tubular cells using the *Ggt-Cre; Rosa-DTR^flox* mouse

To test the efficacy of cell type specific enrichment of another common cell type, *Terminator* mice were mated to *Ggt-Cre* mice to delete DTR expression in proximal tubular cells[6]. Total kidney digests from *Ggt-Cre;Rosa-DTR^flox* mice were seeded onto multiple culture dishes. On day 2, half of the plates were treated with DT (100ng/ml). 96 hrs later, cells from DT treated dishes had increased expression of megalin and markedly reduced expression of Wt1 as compared to control dishes (Figure 4A). Immunofluorescence staining of whole kidney cultures from *Ggt-Cre;Rosa-DTR^flox* mice following DT treatment revealed loss of cells with detectable desmin expression (mesangial/interstitial cells), Wt1 expression (podocytes), and Tamm-Horsfall protein expression (thick ascending limb cells), whereas the number of E-cadherin expressing epithelial cells was enriched (Figure 4B). In contrast, control cultures continued to show the expected mix of all cell types. Of note, GFP expression was not detected in any cells, suggesting that the IRES-GFP cassette does not express at sufficiently high levels to be easily detected.

In summary, the *Terminator* mouse provides a powerful genetic tool to rapidly and efficiently enrich mixed cell cultures for individual types as long as an appropriate promoter is available for selective Cre expression. To our knowledge, this is the first mouse tool for such purposes. This approach will allow investigators to rapidly derive purified cell lines from wild-type or transgenic mice for in vitro studies, and obviate the need to immortalize these cells or use them at high passage. Kabgani et al reported primary culture of podocytes with proven origin by cell sorting[7]. Our approach has a few advantages over that method, including a higher degree of initial cell purity and avoidance of the stress of cell sorting. This technology can also be applied to isolation of very rare cell types as long as a tissue specific Cre mouse strain is available, as well as isolation of common cell types in an organ specific manner (cardiac endothelial cells using the Tie2-Cre, e.g.). Moreover, embryonic stem cells (ESC) carrying the *Rosa-DTR^flox* locus and an appropriate Cre promoter (*Pax2-Cre;Rosa-DTR^flox* for embryonic kidney, e.g.) would assist in high-throughput screening of small compounds to induce efficient ESC differentiation toward a desired cell lineage. However, it should be noted that the DT selection process does result in transient exposure

to large numbers of detached and dying cells that could release factors that at least temporarily influence the phenotype of the surviving DTR negative cells.

Supplemental Concise Methods

Animals

Mouse protocols were approved by the Yale University Institutional Animal Care and Use Committee (IACUC). The Ggt-Cre mouse was kindly provided by Dr. Eric Neilson (Feinberg School of Medicine, Chicago, IL), and the Podocin-Cre mouse by Dr. Lawrence Holzman (University of Pennsylvania, Philadelphia, PA).

ES cell targeting and blastocyst injection

To allow for potential transplantation studies using C57Bl/6 recipients or any other experiments calling for a defined genetic background, C57Bl/6 ES cell line (obtained from ATCC) was used for gene targeting following standard protocols. Identified ESC clones were verified for chromosome integrity by karyotyping before being injected.

Primary culture of podocytes and proximal tubular cells—Donor mice (6~8 weeks old) were anesthetized with ketamine and perfused with 1×PBS via left ventricle before kidneys were isolated. Kidney capsule was removed, minced into ~2 mm × 2 mm × 2 mm cubes and incubated with collagenase B (Roche, 0.2 mg/ml) or Liberase TM (Roche, 0.2mg/ml) for 45 min with occasional agitation at 37°C. The digested mixture was then filtered through a 40 μM cell strainer and seeded onto multiple plates. For podocyte culture, plates were pre-coated with rat tail collagen. Podocytes were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin/streptomycin, HEPES (10 mM), sodium pyruvate (1 mM) and sodium bicarbonate (1mM), while proximal tubular cells were cultured in DMEM high glucose medium, supplemented with 10% FBS and penicillin/streptomycin.

48 hrs after seeding, DT containing medium (Sigma, 100ng/ml) was applied to the culture. After starting DT treatment the medium was changed every 48hrs ×2, followed by every 72 hours until harvest (~2 weeks).

Histology and Fluorescent Immunostaining

After sacrifice, kidneys were fixed in 1×PBS buffered formalin (3.7% formaldehyde) for 12 hours, embedded in paraffin, and sectioned at 3 microns. To review histology, slides were stained by hematoxylin & eosin (H&E). Immunostaining of cultured cells was performed following standard protocols. Rabbit anti-E-cadherin was purchased from Cell Signaling Technology (Danvers, MA); Anti-Desmin, anti-Wt1, and anti-THP antibodies were from Santa Cruz Biotechnology. Megalin antibody was a kind gift from Dr. Daniel Biemesderfer (Yale University, New Haven, CT). All fluorescent images were acquired using a Zeiss LCM 710/Duo system.

Acknowledgements

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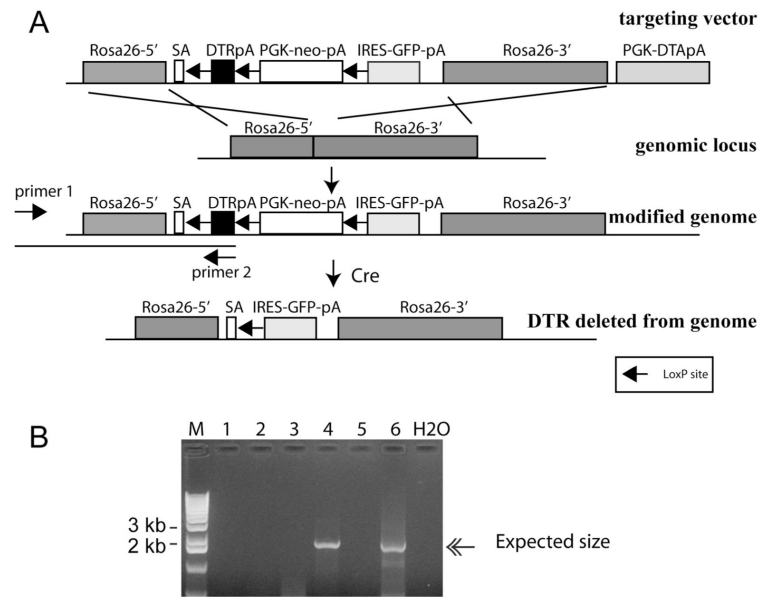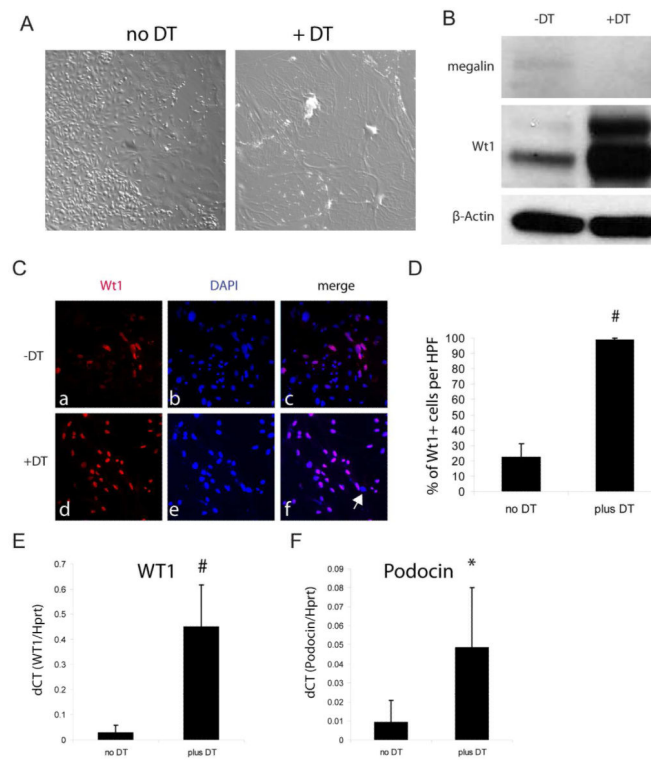
Figure 1. Generation of the Terminator mouse
A. The targeting vector was constructed to contain the DTR-polyA fragment flanked by a pair of loxP sites. PGK-Neo-polyA fragment was placed between the DTR and the IRES-GFP-polyA to serve as a positive selection marker during ES targeting; **B**, long template PCR to screen for clones that have undergone proper homologous recombination. See Panel A for primer location. An example of 6 ES clones is shown, with #4 and 6 exhibiting successful recombination. SA, splicing acceptor sequence.
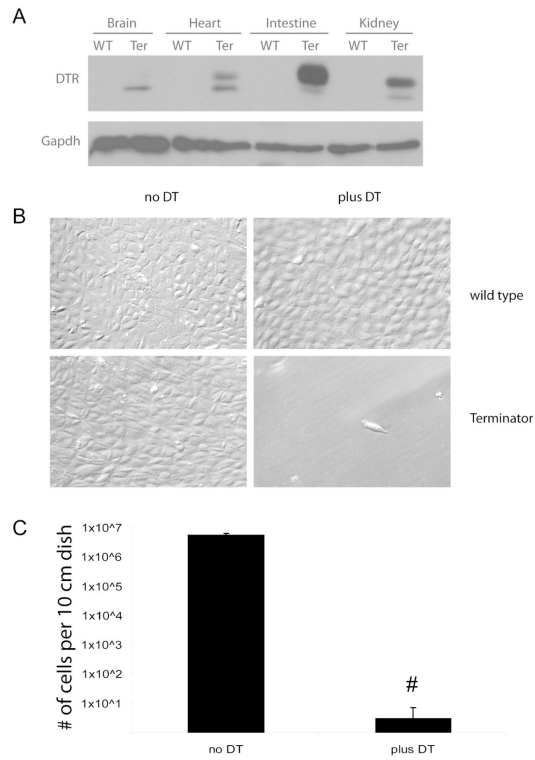
**Figure 2. Functional expression of DTR in the Terminator mouse**
A. Western analysis of tissues from the listed organs immunoblotted with α-DTR. The variation in size of the DTR in different organs is believed to be due to different degrees of O-linked glycosylation[8]. WT, wild type control; Ter, the Terminator mouse.; **B**, Primary cultures of cells derived from whole kidney digest of wild type and Terminator mice treated with DT (100ng/ml) for 72 hours; **C**, quantification of cell numbers of cultures treated as in (B); n=4 for no DT control, and n=6 for plus DT group; # P<0.0001.

Figure 3. Generation of primary podocyte cultures using the Terminator mouse
A. DT treated primary cell cultures derived from *Podocin-Cre;Rosa-DTR^flox* exhibit typical morphology of cultured podocytes as compared to the mixed phenotype seen in control cultures in the absence of DT; **B.** Western analysis of whole cell lysates from control and DT treated primary kidney cultures derived from the *Podocin-Cre;Rosa-DTR^flox* mouse; **C.** Podocyte cultures derived as in (A) were passaged ×1 and immunostained for Wt1 and DAPI. Arrow in panel f points to a single cell that does not have detectable WT1 expression; **D**, quantification of percentage of WT1 positive cells in control vs DT treated cultures as in (C), n=5 each; **E~F**, Quantitative RT-PCR analysis of Wt1 and podocin gene expression in cultured cells normalized to Hprt1. n=5 each; #$P<0.005$, *$P<0.05$
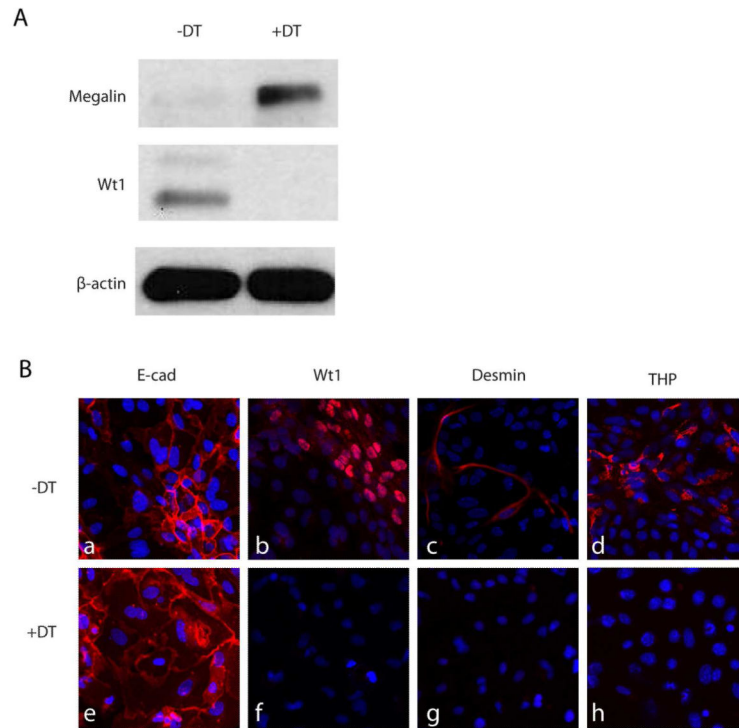
Figure 4. Generation of primary proximal tubular cell culture using the Terminator mouse
A. Primary cultures of proximal tubular cells derived from *Ggt-Cre;Rosa-DTR^flox* mice were treated ± DT for 96 hours, lysed and subjected to Western analysis of megalin and Wt1 expression; B, fluorescent immunostaining of Ecadherin (epithelial marker), Desmin (interstitial/mesangial marker), Wt1 (podocyte marker) and THP protein (thick ascending limb marker) expression by passage zero cells treated as in (A).