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Research Article

A combined artificial chromosome-stem cell therapy method in a model experiment aimed at the treatment of Krabbe's disease in the Twitcher mouse

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Abstract. Mammalian artificial chromosomes (MACs) are safe, stable, non-integrating genetic vectors with almost unlimited therapeutic transgene-carrying capacity. The combination of MAC and stem cell technologies offers a new strategy for stem cell-based therapy, the efficacy of which was confirmed and validated by using a mouse model of a devastating monogenic disease, galactocerebrosidase deficiency (Krabbe's disease). Therapeutic MACs were generated by sequence-specific loading of galactocerebrosidase transgenes into a platform MAC, and stable, pluripotent mouse embryonic stem cell lines were established with these chromosomes. The transgenic stem cells were thoroughly characterized and used to produce chimeric mice on the mutant genetic background. The lifespan of these chimeras was increased twofold, verifying the feasibility of the development of MAC-stem cell systems for the delivery of therapeutic genes in stem cells to treat genetic diseases and cancers, and to produce cell types for cell replacement therapies.

Keywords. Mammalian artificial chromosome, stem cell, Krabbe's disease, transgenic mouse, gene therapy.

Introduction

Globoid cell leukodystrophy (also known as Krabbe's disease) is an autosomal recessively inherited disease caused by a deficiency of galactocerebrosidase (GALC), a lysosomal enzyme that degrades galactosylceramide, a major glycolipid component of myelin and myelin-forming cells. The disease is associated with progressive demyelination, severe gliosis, and the presence in the white matter of characteristic multinucleated cells (globoid cells). These cells contain undegraded galactosylceramide. Accumulation of the toxic compound galactosylsphingosine (psychosine), also a GALC substrate, is believed to result in the apoptosis of oligodendrocytes and the formation of multinucleated globoid cells [1–3].

The Twitcher mouse (*twi/twi*) is a naturally occurring authentic model of human Krabbe's disease, which can result from a mutation in the GALC gene and formation of a protein without enzyme activity. The biochemical and neuropathological findings and the

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clinical course of the disease in the Twitcher mouse closely resemble those observed in humans [4, 5]. *Twi/twi* mice are clinically normal until 20 days of age, after which they grow more slowly and develop typical neurological signs, including tremors and hind leg weakness. The disease progresses rapidly and the mice rarely survive beyond 40 days of age [4, 5]. The Twitcher mouse has become a valuable model that promotes not only an understanding of the pathogenesis of Krabbe's disease, but also the development of strategies to correct the GALC deficiency.

Mammalian artificial chromosomes (MACs) have an almost unlimited therapeutic transgene-carrying capacity and offer a stable, non-integrating vector system without the drawbacks of other gene delivery systems, such as viruses, plasmid vectors and bacterial and yeast artificial chromosomes [6]. The previously described Platform ACE System [7] was developed for the reliable and systematic engineering of MACs with large cDNAs or genomic sequences.

Embryonic stem (ES) cells present opportunities for disease modeling, pharmacological screening, and cell-based therapies. They can generate all somatic cell types and represent a potential and viable target for MAC-based gene therapy. We describe here the successful generation of stable pluripotent mouse ES (mES) cell lines that express the GALC gene from stably maintained therapeutic artificial chromosomes (tACs). We found that these ES cell lines carrying the tACs, which express mouse or human GALC transgenes, significantly increased the lifespan of chimeric *twi/twi* mice.

It was demonstrated earlier that bone marrow transplantation (BMT) can slow down the disease progression and increase the lifespan of recipient Twitcher mice to up to 100 days [8–10]. However, such therapy is unlikely to become successful for the treatment of this disorder, because a toxic milieu develops in this disease through the accumulation of psychosine, and the donor cells would therefore succumb in much the same way as the endogenous cells [11].

Cell replacement therapy could be feasible only if the donor cells are resistant to such a toxin, and are genetically modified to express high levels of the therapeutic GALC gene; this latter could be taken up by the surrounding cells and this could be regarded as local enzyme therapy. Such combined cell replacement and local enzyme therapy should significantly diminish the toxic environment, which could result in a restored neurological function and hence a cure. It seems that stem cells (at least neural stem cells) are rather resistant to psychosine [11]. We present evidence here that a combined artificial chromosome-stem cell therapy approach significantly extended (up to 111 days) the survival of treated mice, even though these mice displayed only 20–30% tAC chimerism. As far as we are aware, this study is the first attempt to use stem cell therapy in combination with a therapeutic artificial chromosome for a genetic disorder in an animal model experiment.

Materials and methods

Tissue culturing, chromosome isolation and transfection experiments. The Platform ACE-carrying Y2913D-SFS Chinese hamster ovary (CHO DG44) cell line was obtained from Chromos Molecular Systems Inc. It was cultured in MEM alpha (Gibco, 22571), 5% FCS, streptomycin-penicillin (Gibco, 15070–063) and 10 μg/ml Puromycin (Sigma, P-7255). Plasmid transfection experiments were performed with the Superfect reagent (Qiagen, 301305) as described by the manufacturer. Mitotic chromosome isolations were performed as reported earlier [12]. Isolated chromosomes were resuspended in KO-DMEM (Gibco, 10829) without serum and supplements. R1 ES cells were cultured on PMEF-N mouse embryonic fibroblasts (Specialty Media) in KO-DMEM, 15% FCS (Specialty Media, ES-010-A), 20 mM Glutamax-I, 20 mM NEAA, 1× Streptomycin-Penicillin (Gibco, 15070–063), 1× β-mercaptoethanol (Chemicon, 800-437-7500), and 1000 unit/ml ESGRO (Chemicon, ESG1107). R1 ES cells were transfected with the isolated chromosomes using the Superfect reagent. Transfection experiments were performed as suggested by the manufacturer, except that we used 50 μl Superfect reagent per 10^7 isolated chromosomes. Transfection was carried out in suspension for 4 h. R1 ES cells were collected by centrifugation at 1000 rpm for 5 min at room temperature and 40 ml fresh ES medium was added to them. R1 ES cells were seeded on four PMEF-N feeder cell-containing 10-cm tissue culture dishes. The next day, the medium was exchanged for fresh ES medium supplemented with 400 μg/ml Geneticin (Gibco, 10131–027). The selective medium was replaced every other day. Picking of drug-resistant colonies started on day 11 of selection.

Chimera production. Chimeras were produced by a standard methodology as described earlier [13]. Briefly, blastocyst-stage embryos were isolated from heterozygous Twitcher females that had previously been mated with heterozygous Twitcher males. Twelve to fifteen R1, RD2 or RG5 ES cells were injected into the blastocoel cavity of these embryos, in separate experiments. Injected embryos were transferred into foster mothers.

Karyotyping. G-banding was performed by a standard protocol. Karyotyping analysis was achieved with Ikaros software (Metasystems Inc.).

Molecular and cell biology methods. Fluorescence *in situ* hybridization (FISH) experiments were performed with a standard protocol. The following DNA sequences were labeled with fluorescent dyes and used as probes: pPur (Clontech, 631601) plasmid for ACE detection, Chinese hamster genomic DNA for the detection of carried-over Chinese hamster genome, human/mouse GALC cDNA, mouse major DNA sequence, and anti-CREST antibody for functional centromere detection [12]. DNA probes were labeled either with Roche DIG Nick Translation Mix (1745816) or with Roche Biotin Nick Translation Mix (1745824). Anti-human-FITC antibody was used for the detection of anti-CREST antibody labeling (Dako, F0202). Genomic DNA samples were isolated with the Wizard Genomic DNA Purification Kit (Promega, A1125). Southern blotting and hybridization was performed *via* a standard protocol. A radioactively labeled neomycin resistance gene sequence was used as a probe. Total RNA was isolated with TRIzol (Invitrogen, 15596–018). Reverse transcription was performed with the iScript Kit (Bio-Rad, 170–8897). The following primers were used in the RT-PCR experiments: mGALC30F (5'-tgttgcggtgcccttattgtgtg) and mGALC436R (5'-tgaagcctttgccccagccatcc) for the detection of transcript expressed from the genomic copy of the GALC gene, mGTGF (5'-ctcggagcggcggcgtattcatt) and mGTGR (5'-cacccacaccagccaccaccttct) for the detection of the TG-mcGALC transcript, hGALC1515F (5'-agaccctggcgagcatcacttc) and hGALC1949R (5'-attgcagccccagccattctttg) for the detection of the TG-hcGALC transcript, and m18SRRNAS (5'-gtaacccgttgaaccccatt) and m18SRRNAAS (5'-ccatccaatcggtagtagcg) for the detection of the mouse ribosomal 18S RNA transcript as an internal control of reverse transcription.

Staining for pluripotency. Alkaline phosphatase (AP) staining was performed with the StemTag Alkaline phosphatase-staining Kit (Cell Biolabs, CBA-300) as suggested by the manufacturer, and Oct3/4, Sox2 and Nanog staining with a human ES cell marker antibody panel plus kit (R&D Systems, SC009) as recommended by the manufacturer.

Clinical evaluation. To follow the course of disease, the onset day of tremor was noted and body weight was measured, and clinical symptoms were recorded as described earlier [14].

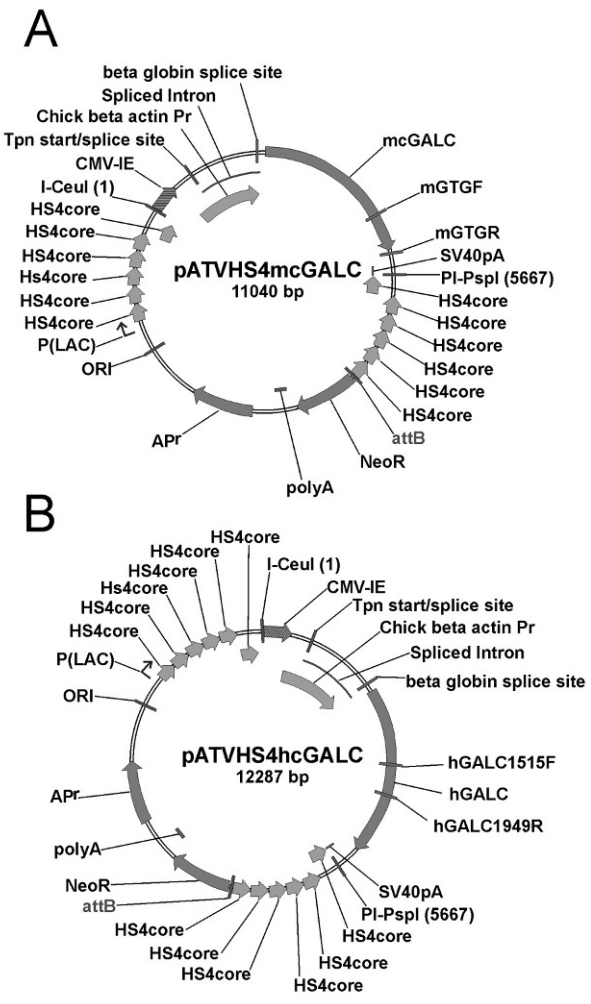


Figure 1. The compositions of the Platform ACE targeting vectors (ATVs) used to deliver transgenes onto the ACE. (*A, B*) The compositions of murine galactocerebrosidase (GALC) cDNA (mcGALC) and human GALC cDNA (hcGALC)-carrying ATV plasmid vectors are shown. The transgenes are expressed from hybrid CMV/β-actin/β-globin promoter. The transgene expression cassette is flanked by six copies of chicken β-globin HS4 core insulator elements on either side, to secure high levels of gene expression. The ATV vectors carry the *attB* recombination site for the site-specific integration of the transgenes onto the ACE by ACE integrase. mGTGF and mGTGR primers are used in PCR and RT-PCR reactions for the detection of mcGALC transgene and its expression. hGALC1515F and hGALC1949R primers are used in PCR and RT-PCR reactions for the detection of hcGALC transgene and its expression.

Results

A platform Chinese hamster cell line (Y2913D-SFS, see Materials and Methods) carrying a previously described, pre-engineered satellite DNA-based MAC (Platform ACE) [7] was used to produce tACs. Platform ACE targeting vectors (ATVs) were engineered as described earlier [7] to encode either murine GALC cDNA (mcGALC) (Fig. 1A) or human GALC

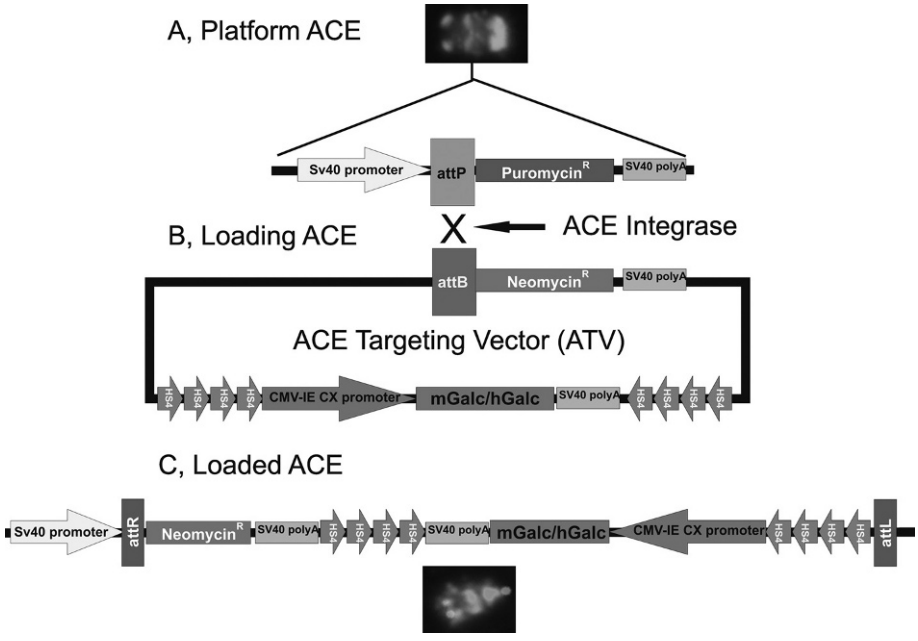


Figure 2. This schematic representation shows the site-specific integration process by which the ACE chromosome is targeted with therapeutic transgenes. (*A*) The Platform ACE contains multiple *attP* recombination acceptor sites for the ACE integrase. The *attP* site is situated between an SV40 promoter and the puromycin resistance gene, which is driven by this promoter. (*B*) The ATV vectors carry the *attB* recombination site for the ACE integrase. Immediately after the *attB* site, a promoterless neomycin resistance gene is cloned. The ACE integrase catalyzes the site-specific recombination between the *attB* and *attP* sites and integrates the ATV into the tAC. (*C*) This event disconnects the puromycin resistance gene from its promoter and replaces it with the promoterless neomycin resistance gene, which in this way acquires the SV40 promoter. Targeted, transgene-carrying cell lines can be selected for neomycin resistance.

cDNA (hcGALC) (Fig. 1B). These cDNA sequences were expressed by a hybrid promoter composed of a cytomegalovirus enhancer and a β-actin/β-globin promoter and were upstream of the SV40 poly(A) sequences. To secure gene expression, the cDNA expression cassettes were flanked on either side with a tandem array of six chicken β-globin HS4-core insulator elements [15]. The ATVs were co-transfected into the Y2913D-SFS cell line with an ACE Integrase-expressing plasmid [7] to achieve site-specific integration into the Platform ACE. A schematic representation of the ATV vector used and the process of site-specific gene targeting into the tAC is shown (Fig. 2). Briefly, the Platform ACE contains multiple *attP* recombination acceptor sites [16, 17] for the ACE Integrase. The *attP* site is situated between an SV40 promoter and the puromycin resistance gene, which is driven by this promoter. The ATV vectors carry the *attB* recombination site for the ACE Integrase. Immediately after the *attB* site, a promoterless neomycin resistance gene is cloned. The ACE Integrase catalyzes the site-specific recombination between the *attB* and *attP* sites, and integrates the ATV into the tAC. This event disconnects the puromycin resistance gene from its promoter and replaces it with the promoterless neomycin resistance gene, which in this way acquires the SV40 promoter. Transgene-carrying cell lines can be selected for neomycin resistance. Antibiotic-resistant, targeted tAC-carrying cell lines were further selected by diagnostic Southern blot hybridizations to separate them from clones with random integration. Southern hybridization patterns show the correct targeting of mcGalc (3D2 cell line) and hcGalc (7G11 cell line) transgene expression cassettes onto tACs. The presence of intact tACs with functional centromeres was verified by anti-centromere immunostaining [12] and FISH (data not shown). The above-mentioned two cell lines were selected for further experiments: 3D2 carrying mcGALC, and 7G11 carrying hcGALC on the tAC. It was demonstrated earlier that Platform ACEs could be delivered into different mammalian cell lines, including stem cell lines, with various techniques [18–22]. These experiments led us to choose to use the Superfect reagent (Qiagen) and we optimized the delivery of isolated metaphase chromosomes, originating from cell lines carrying tACs, into a mES cell line, R1, which was kindly provided by Prof. Andras Nagy [23]. Eighteen tAC-containing mES cell lines were isolated by selection for neomycin resistance that was provided by tACs. Shearing of chromosomes may occur during chromosome isolation and transfer; it was therefore necessary to perform primary quality control of neomycin-resist-

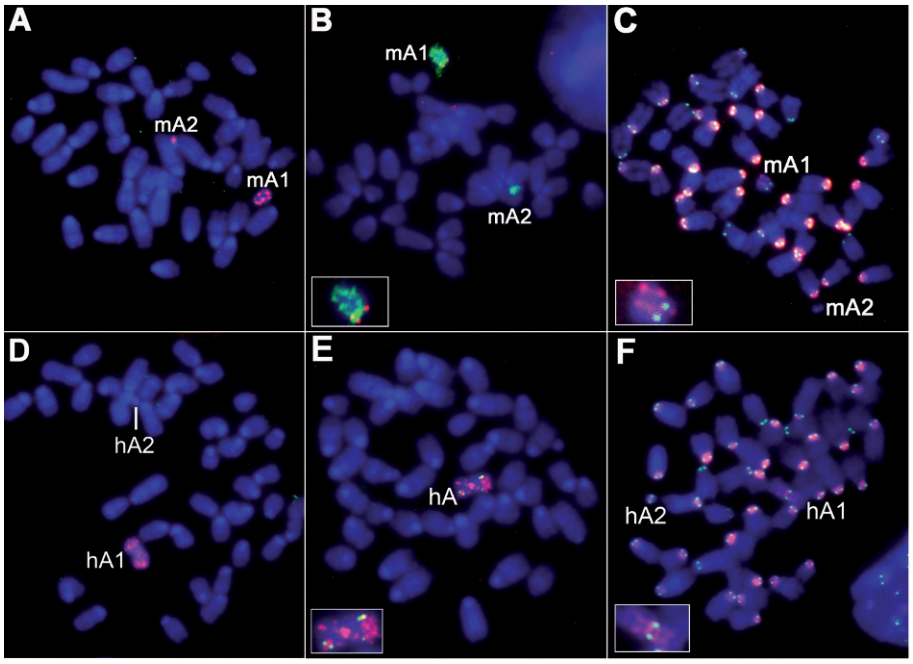


Figure 3. Characterization of the RD2 and RG5 mES cell lines by FISH experiments. (*A, D*) Rhodamine-labeled plasmid backbone sequences were used as probes to detect tACs in the RD2 and RG5 cell lines (red). In the same experiment, hamster genomic DNA was labeled with FITC, and no traces of intact hamster chromosomes or chromosome fragments were found (*A, D*, no green signal). (*B*) GALC transgene sequences were detected as rhodamine red dots in RD2 cell line on tAC. tACs were counterstained with FITC-labeled plasmid backbone sequences (green). (*E*) GALC transgene sequences were detected as FITC (green) dots in the RG5 cell line on tACs. tACs were counterstained with rhodamine-labeled plasmid backbone sequences (red). (*C, F*) The rhodamine-labeled mouse major satellite sequences were found on most of the mouse chromosomes and on tACs (red). (*C, F*) Indirect immunofluorescence staining with an anti-centromere serum specific to the functioning kinetochores [12] revealed that all the mouse chromosomes and tACs had functional centromeres (green dots). mA1, mA2 indicate mouse and hA1, hA2 indicate human GALC transgene-carrying tACs, respectively. Insets: (*B*) 2× further magnification of mA1 tAC chromosome; (*C*) 4× further magnification of mA1 tAC chromosomes; (*E*) 3× further magnification of hA1 tAC chromosome; (*F*) 3× further magnification of hA1 tAC chromosome.

ant mES cell lines by applying FISH analysis. A fluorescently labeled plasmid backbone sequence probe was used to detect tACs (Fig. 3A, D). Twelve cell lines carried one tAC, and six cell lines harbored two tACs or an additional fragment of tAC origin. The RD2 and RG5 cell lines were chosen for further experiments. The RD2 cell line accommodated one full-sized tAC (mA1) and one truncated tAC (mA2) and both of them harbored the mcGALC transgene (Fig. 3A–C). The size of the mA1 tAC is ~40 mega base pairs (MB) and the size of the mA2 tAC is ~10 MB. The RG5 cell line carried two full-sized tACs in the form of an isochromosome (hA1) with the hcGALC transgene on it (Fig. 3D–F), and in ~50 % of the cells a centric fragment of tAC (hA2) with only traces of plasmid sequences was present. The hA1 tAC has an estimated size of about 80 MB and the hA2 tAC is ~5–10 MB long. We used FITC-labeled hamster genomic DNA (CHO-FITC) in FISH painting experiments to investigate the presence of hamster chromosomes coming from the parental cell lines into mES cells. No traces of intact hamster chromosomes or chromosome fragments were detected in mES clones (Fig. 3A, D). We detected GALC transgene sequences on tACs (mA1 and hA1) by FISH (Fig. 3B, E). The mouse major satellite sequence is a prominent component of the mouse pericentromeric heterochromatin and it is also present interstitially on ACEs [24, 25]. The localization of the red rhodamine-labeled major sequences is shown on most of the mouse chromosomes and on tACs (Fig. 3C, F). Using an anti-centromere serum specific to the functioning kinetochores [12], we found that all mouse chromosomes and tACs had functional centromeres (Fig. 3C, F). It is conceivable that tACs undergo structural changes through rearrangements in the process of chromosome isolation, transfection and clonal selection. Such rearrangements could lead to the silencing of transgene expression. To rule out this possibility, we first compared the Southern hybridization pattern of tAC-carrying hamster cell lines (3D2 and 7G11) with the pattern observed in mES clones and found that they were the same (Fig. 4A, D). Regardless of the structural integrity of the tACs, the expression of the GALC transgenes could be turned off by other mechanisms, such as methylation, heterochromatinization, etc., and transgene-specific RT-PCR experiments were therefore performed. Endogenous GALC

gene transcription is undetectable in wild-type R1 ES cells (Fig. 4C). On the other hand, both mcGALC and hcGALC transgenes are expressed in the mES clones (Fig. 4D, E). As an internal control for our RT-PCR experiments, we used mouse 18S ribosomal RNA (m18S) as a target for amplification. Mouse 18S is present in the cells with a high copy number. The intensity of the mcGALC and hcGALC bands relative to that of the m18S bands suggests that both transgenes are overexpressed (Fig. 4D, E, respectively). Our results indicated that the tACs preserved their function and also ensured the overexpression of GALC transgenes in the established mES cell lines. ES cell lines should meet certain quality requirements before they are used in further experiments. We found that both the RD2 and RG5 cell lines had a high-quality karyotype with a modal average of 40 mouse chromosomes in addition to the tACs (data not shown). The markers most commonly used for ES cell pluripotency are the detection of AP activity and the presence of Oct3/4, Nanog and Sox2 proteins [26, 27]. The RD2 and RG5 cells exhibited positive staining for AP activity and were also positively immunostained with antibodies against the protein markers specified above. More than 90 % of the colonies displayed an overall positive staining, and we therefore concluded that these mES cell lines were pluripotent (Fig. 5). The tAC-carrying mouse ES cell lines were maintained under selective culture conditions. However, the stability of tACs in individual ES cell lines of single cell origin may differ significantly. To test this hypothesis, RD2 and RG5 cells were cultured for more than 100 cell generations (24 passages) without selective pressure. In the RD2 cell line, 98.9 % of the ES cells retained the tACs. On the other hand, we found that 53 % of the RG5 ES cells lost the tACs after only 4 passages (~19 cell divisions). This result demonstrates an important advantage of this technology. In a tissue culture dish, we can carefully select for and pick out ES cell lines with the most stable version(s) of tAC(s). Although in a non-selective environment the tACs of the pluripotent RD2 ES cells were as stable as their natural mouse counterparts, tACs might be lost during differentiation. Embryoid bodies (EBs) are formed from ES cell colonies when the leukocyte inhibitory factor is removed from the culture medium and cells are maintained on a non-adhesive substrate. Such EBs contain cell types from all three germ layers [28]. EBs derived from RD2 ES cells were cultured for 8 days, disintegrated and fixed for FISH experiments. RD2 ES cells were also differentiated to cell types that were positively immunostained by the mesoderm-specific marker, anti-smooth muscle actin. tACs were present in ~99 % of the nuclei of both the RD2 EBs and the mesodermal cells. The results of these experiments indicated that *in vitro* differentiation did not affect the stability of the tACs in this cell line. To evaluate the therapeutic value of the tACs, both RD2 and RG5 ES cells were used to produce chimeric mice. ES cells were injected into blastocysts derived from matings between heterozygous Twitcher mice. Ten chimeric mice were obtained on the *twi/twi* mutant host genetic background: 9 with the RD2 and 1 with the RG5 cell line. Chimeric mice were genotyped with a PCR method described earlier [29]. With this method we could not detect the genomic copy of the GALC gene, but we could reveal the presence of the GALC transgenes in the chimeras with transgene-specific PCR reactions. The Twitcher genetic background started to show up as these chimeras got older and the phenotypic characteristics of homozygous Twitcher mutants began to be manifested. In these animals, the only source of GALC enzyme was from the descendants of tAC-carrying mES cells. The chimeras had a 20–40 % lower body weight than that of wild-type mice of similar age. The onset of twitching commenced much later. During the last 2 weeks of their life the chimeras displayed exactly the same frequency/severity of twitching as that for the homozygous mutant Twitcher mice. Chimeric and homozygous Twitcher mice at the moribund condition when they can no longer take in sufficient food and water to support their life displayed the same clinical symptoms. At this stage their abdomen collapsed, they showed severe wasting and incapability of any movement. Mice were euthanized at this stage of the disease. The lifespan of these Twitcher chimeras was compared with that of homozygous mutant control animals: in our hands the control animals lived for an average of 35.2 days (Fig. 6A, blue diamonds), whereas the chimeras survived for an average of 67.9 days (Fig. 6A, pink squares). In fact, one of the chimeras lived for 111 days, which as far as we are aware is the longest survival of a Twitcher mouse receiving stem cell therapy [8–10]. As a control experiment, we used the untransfected wild-type R1 ES cells to produce chimeras on the Twitcher mutant genetic background. The average lifespan of these chimeras was 50 days, *i.e.*, a modest increase compared to the lifespan of Twitcher mutants (35.2 days), which is probably due to the genomic copy of the GALC gene present in the R1 ES cells.

We detected descendant cell types of the RD2 and RG5 ES cells in the chimeras that carried the MACs. Figure 6B illustrates the percentages of these descendants in the various tissues, including brain samples. Cells from these tissue types were smeared on slides and fixed, and FISH experiments were performed with a probe specific to the MACs. We consider that

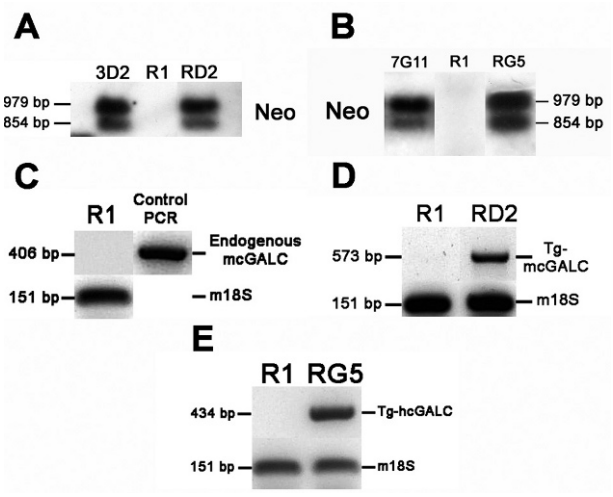


Figure 4. Southern blot hybridization and RT-PCR experiments indicated that the tACs preserved their function in the RD2 and RG5 mES cell lines. (*A*, *B*) The Southern blot hybridization pattern of mES cell lines (RD2 and RG5) did not change relative to the parental cell lines (3D2 and 7G11, respectively). (*C*–*E*) RT-PCR experiments demonstrated that the GALC transgenes were expressed in the RD2 (*D*, Tg-mcGALC) and RG5 (*E*, Tg-hcGALC) mES cell lines. A mouse 18S RNA (m18S) transcript-specific RT-PCR experiment was used as internal control for all of our RT-PCR experiments. A positive control PCR reaction was performed on mouse GALC cDNA (*C*, Control PCR). No GALC transcript was detected in the R1 mES cell line (*C*).

the FISH method is reliable for the detection of transplanted cells, because the MACs are more stably retained than the mouse Y chromosome. We found that only 1.29 % of the RD2 cells lost the MAC after 16 passages under non-selective culture conditions, while 6.92 % of the cultured RD2 cells lost the Y chromosome during the same period of time. Accordingly, the analysis of these various tissues from five chimeras revealed a correlation between the longevity of the tAC-carrying animals and the rate of their overall chimerism (Fig. 6B).

Discussion

We tested the feasibility of a combined stem cell-artificial chromosome therapy using the Twitcher mouse model of Krabbe's disease. In stem cell-based preclinical studies it has been demonstrated that bone marrow transplanted Twitcher mice still died with characteristic pathology, but the life span of the recipient mice was extended from about 40 to 100 days [9, 10, 30, 31]. In our model experiments, chimeric mice carrying tAC on Twitcher genetic background exhibited all the characteristic clinical symptoms of Twitcher mouse, although chimeras showed a significantly delayed onset of twitching, and the lifespan of these chimeras was increased twofold compared to the lifespan of Twitcher mutants. Other preclinical model experiments aimed at achieving an effective treatment of Krabbe's disease have applied BMT, hematopoietic stem cell transplantation and umbilical cord blood transplantation [8–10, 32, 33]. BMT slowed down the disease progression and increased the lifespan of recipient Twitcher mice up to even 100 days [8–10]. Recombinant adenoviruses and lentiviral vectors resulted in the efficient reconstitution of GALC expression in Twitcher neural cells, but did not limit the progression of the disease *in vivo* [34, 35]. Transgenic rescue of the Twitcher mouse was also attempted using human GALC cDNA [36] and a BAC clone containing the entire human GALC gene [37]. Although transgenic Twitcher mice were rescued in the latter approach, this germ-line therapy can not be considered clinically relevant at present. This transgenic

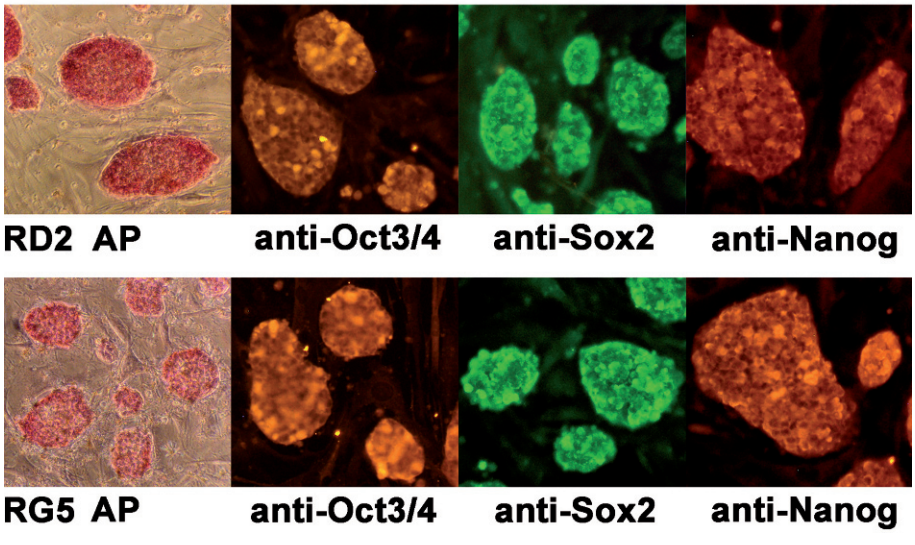
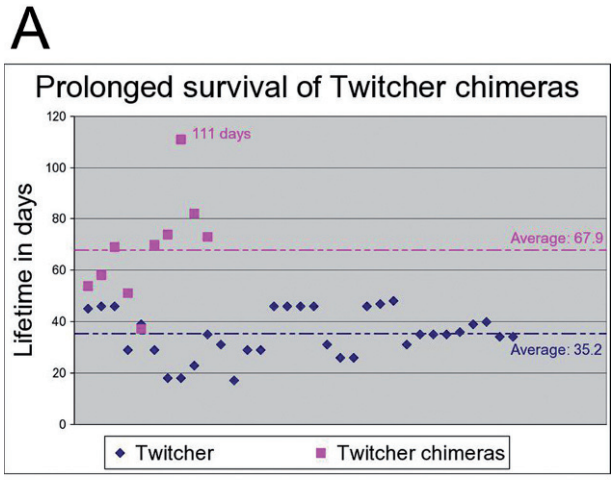


Figure 5. The RD2 and RG5 mES cell lines show pluripotency by four markers: AP, alkaline phosphatase activity (red signal) staining; anti-Oct3/4 (red signal), anti-Sox2 (green signal) and anti-Nanog (red signal) immunofluorescent staining.



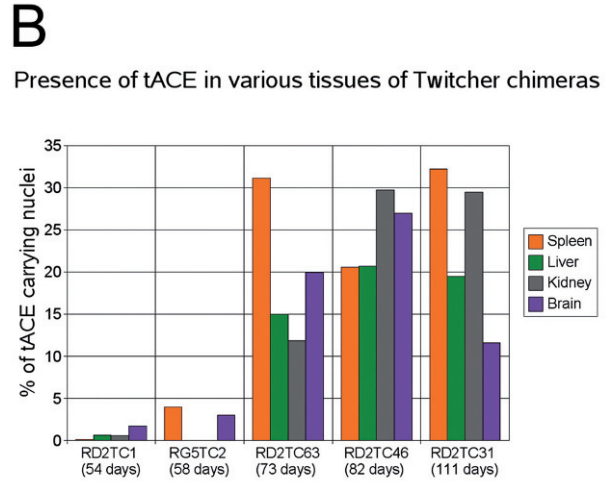


Figure 6. Twitcher chimeras have a significantly increased lifespan as compared with homozygous Twitcher mutant mice. (*A*) The graph shows that the average lifespan of Twitcher chimeras (pink squares) is increased by 93% as compared with Twitcher mutant mice (blue diamonds). One Twitcher chimera lived for an outstanding 111 days. (*B*) Living cells were collected from various tissues of four RD2 Twitcher chimeras (RD2TC) and the one RG5 Twitcher chimera (RG5TC). The cells were fixed and tAC-specific FISH experiments were performed on them. The percentage of tAC-carrying nuclei was determined. The lifespan in days of the Twitcher chimeras is shown in brackets. Less than 5% chimerism was sufficient to increase the lifespan of these mice to 54 and 58 days, respectively. However, there was an even more pronounced increase in lifetime when the level of chimerism reached or exceeded 10%.

approach involves the random integration of the transgene into the genome of the host organism, which raises safety concerns and weakens the therapeutic relevance of the technology in human patients. Cell replacement therapy for Krabbe's disease can be feasible only if the donor cells are resistant to the toxic milieu present, and are genetically modified to express high levels of the therapeutic GALC protein, which could be taken up by the surrounding cells. This combined cell and local enzyme therapy should significantly diminish the toxic environment, which could lead to a restored neurological function and a cure. It seems that stem cells, or at least neural stem cells are rather resistant to psychosine [11]. In our chimeric mice, we delivered a cell type capable of producing cell lineages resistant to the effects of psychosine, and these cells overexpressed the GALC gene (Fig. 4D, E). The untransfected wild-type R1 ES cells only moderately increased the survival of Twitcher chimeras, which was probably due to the genomic copy of the GALC gene present in the R1 ES cells. As mentioned above [11], the genomic copy of the GALC gene does not produce sufficient enzyme to diminish the toxic environment. These results indicate that the combined MAC-stem cell therapy approach we have presented here indeed resulted in cell therapy and enzyme therapy, significantly decreasing the toxic milieu and extending the survival of our treated mice up to even 111 days, although these mice exhibited only ~20–30% tAC chimerism. This approach appears to be of great potential for *ex vivo* therapeutic applications such as the treatment of reversible genetic diseases and cancers, and to produce cell types and genetically modified cell types for cell replacement therapies.

We believe that the work presented here is the first attempt to use stem cell therapy in combination with a tAC for a genetic disorder in an animal model experiment. Our results seem to confirm the feasibility of therapeutic applications of the MAC vector system in *ex vivo* stem cell-based treatments.

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