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Enhanced transthyretin tetramer stability following expression of an amyloid disease transsuppressor variant in mammalian cells

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Abstract

Background—The transthyretin (TTR) amyloidosis is an incurable fatal inherited disease that is characterized by progressive peripheral and autonomic neuropathy. It is caused by missense amyloidogenic mutations in the *TTR* gene that destabilize the native tetrameric state and lead to the cytotoxic misfolded monomeric state. One interesting variant (T119M) stabilizes heterotetramers with amyloidogenic TTR and, in the reported heterozygous individuals, protects the carriers from disease. In the present study, we characterize *in vitro* and *in vivo* the ectopic expression of the human T119M mutant, termed a transsuppressor for TTR amyloid disease.

Methods—Lentiviral vectors encoding wild or mutant forms of human TTR were constructed and transduced to the human hepatocellular carcinoma cell line, HepG2, or mice. Heterooligomerization between T119M TTR and amyloidogenic variants was analysed by immunoprecipitation following western blotting.

Results—T119M TTR was stably expressed in transduced HepG2 cells and was secreted as an oligomer that can interact with its native partner, retinol-binding protein. Importantly, the T119M TTR formed secreted heterooligomers with amyloidogenic TTR variants, V30M, L55P and V122I, in HepG2 cells that were more stable than the homooligomers of the same amyloidogenic TTR variants. Human T119M TTR also formed heterooligomers with V30M TTR in transduced mice.

Conclusions—The results obtained in the present study demonstrate the stabilization of heterotetramers by T119M TTR in human cells and suggest that gene transfer of T119M TTR may have potential as a gene therapy for TTR amyloidosis.

Keywords

amyloidosis; gene therapy; lentiviral vector; neuropathy; transsuppressor; transthyretin (TTR)

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Introduction

Amyloidoses are a vast group of diseases defined by the accumulation of insoluble amyloid fibrils arising from misfolded proteins. The deposition of amyloid fibers in tissue or the extracellular matrix is thought to be the cause of tissue and organ dysfunction [1]. The disease progression is generally severe, although there is currently no cure and palliative care is the only way to relieve symptoms. To date, two dozen unrelated proteins have been reported as amyloid fibril components *in vivo*, including immunoglobulin, transthyretin (TTR), prion protein, amyloid- β -peptide (A β), lysozyme, etc. [2,3]. Amyloidosis can be classified into three major forms according to clinical symptoms and the biochemical type of causative proteins: primary, secondary and hereditary (familial) amyloidoses [3]. Hereditary amyloidosis is rarer than the other two types and can occur in families of almost every ethnic background. This amyloidosis is caused by mutations in specific proteins and the most common mutations occur in TTR.

TTR amyloidosis, which is also known as familial amyloidotic polyneuropathy, is an autosomal dominant neurodegenerative disorder that results from the aggregation of mutated amyloidogenic TTR into amyloid fibrils [4,5]. This disease was originally thought to be restricted to endemic occurrence in certain areas, such as Portugal, Japan and Sweden, but it is now known to occur worldwide [6]. Around 100 different TTR variants have been reported and associated with amyloidosis [7–9]. The amyloid deposition is observed in multiple organs of the body and increased amyloid deposits negatively affect organ function. The typical clinical features are peripheral neuropathy, autonomic dysfunction, cardiac dysfunction, gastrointestinal tract disorders, kidney failure, emaciation and finally death, usually by cardiac failure or sepsis within 10–15 years of disease onset [10]. At the present time, there is no pharmacologic therapy for this disease and orthotopic liver transplantation is the only treatment of proven efficacy. However, liver transplantation does not provide a practical means of treating a large number of patients and other forms of therapy are being pursued.

TTR is a tetrameric protein of four identical subunits of 14 kDa that is synthesized predominantly in the liver and the choroid plexus of the brain as a single polypeptide chain of 127 amino acids [11]. TTR is a secretory protein [12–14] and circulates in plasma and cerebrospinal fluid (CSF) as a 55-kDa tetrameric protein that functions to transport thyroid hormones and retinol/retinal-binding protein (RBP) complex [15]. The concentration is maintained independently in the CSF (5–50 μ g/ml) and blood (200–400 μ g/ml) by secretion primarily from the choroid plexus and liver, respectively, [16].

The mechanisms of TTR amyloid formation and the pathogenesis of TTR amyloid are not well understood. Structural studies by X-ray diffraction indicated that amyloidogenic TTR mutations make TTR more susceptible to dissociation and misfolding [17]. Furthermore, Quintas *et al.* [18] and Hammarström *et al.* [19] reported that the dissociation of the TTR tetramer to its component monomer is required for amyloid fiber formation. Reixach *et al.* [20] showed that dissociated TTR monomer rapidly aggregates and forms complexes smaller than 100 kDa that induce cytotoxicity in the human neuroblastoma cells, IMR-32. These observations suggest that the cytotoxicity of TTR can be inhibited by increasing the stability

of the amyloidogenic transthyretin tetramer. Indeed, rare heterozygotes having the amyloidogenic allele V30M together with the T119M variant, which is called 'transsuppressor TTR', have few manifestations of TTR amyloidosis [21–25]. Almeida *et al.* [26] and Hammarström *et al.* [27] have reported that the T119M variant makes stable heterotetramer with amyloidogenic TTR and the resultant heterotetramer became resistant to dissociation into monomers. Hammarström *et al.* [27] also reported that the incorporation of one T119M TTR subunit into V30M TTR tetramer reduced amyloid fiber formation up to 50% and two or more T119M TTR subunits suppressed amyloid fiber formation by almost 90% *in vitro* conditions. These observations suggest that TTR amyloidosis might be treated by expressing T119M TTR in the patient.

In the present study, we express and characterize transsuppressor T119M TTR using a lentiviral vector pseudotyped with the Ross River virus (RRV) envelope both *in vitro* and *in vivo*. Upon lentiviral vector transduction to HepG2, a human hepatocellular carcinoma cell line, T119M TTR was continuously secreted into the culture supernatant. The lentivirally expressed TTRs form a homotetramer that can be secreted and is competent to interact with RBP. We further demonstrated heterotetramer formation between T119M TTR and the major amyloidogenic TTR mutants (V30M, L55P and V122I) in HepG2 cells. Heterotetramer formation also occurs *in vivo* upon lentiviral vector transduction in mice. These studies provide new insights and potential new gene therapy approaches for the treatment of amyloidogenic diseases.

Materials and methods

Construction and production of lentiviral vectors

The lentiviral plasmids used in this study were derived from the SIN18-RhMLV-E vector [28]. We constructed the vector encoding human TTR expression cassette by replacing enhanced green fluorescent protein (EGFP) with TTR cDNA obtained from mRNA of HepG2 cells using the reverse transcriptase-polymerase chain reaction (PCR) and primers 5'-ACGCGGATCCAGGATGGCTTCTCATCGT-3' (TTR sense) and 5'-TCCGCTCGAGTCATTCCTTGGGATTGGT-3'. Lentiviral vectors encoding variant forms of TTR were prepared by PCR based site-direct mutagenesis using following primer sets; V30M: 5'-CAATGTGGCCATGCATGTGTTTCA-3' and 5'-TCTGAACACATGCATGGCCACATTG-3'; L55P: 5'-GTCTGGAGAGCCGCATGGGCTCACA-3' and 5'-TGTGAGCCCATGCGGCTCTCCAGAC-3'; T119M: 5'-CCTATTCCACCATGGCTGTCGTAC-3' and 5'-GTGACGACAGCCATGGTGAATAGG-3'; V122I: 5'-ACCACGGCTGTCATACCAATCCC-3' and 5'-GGGATTGGTGATGACAGCCGTGGT-3'. We introduced HA tag or Flag tag for each TTR by PCR amplifying each TTR encoding fragment using primers TTR sense and either 5'-GGCGGTCGACTAGTCAAGCGTAGTCTGGGACGTCGTATGGGTATCCTTGGGATTG GTGA-3' for HA tag or 5'-GGCGTTCGACTAGTCACTTATCGTCTCGTCATCCTTGTAATCTTCCTTGGGATTGGTGA-3' for Flag tag. For T119M TTR, we further introduced three tandem repeats of Flag (FLx3)

tag by serially amplifying the T119M fragment using primers TTR sense and 5'-GGCGTCTAGTCACTTATCGTCGTCATCCTTGTAATCTTCCTTGGGATTGGTGA-3', TTR sense and 5'-CTTATCATCGTCGTCCTTTGTAATCCTTATCGTCGTCATC-3' and TTR sense and 5'-CGACTAGTCACTTGTGCATCATCGTCCTTGTAATCCTTATCATCGTCG-3'. All plasmid constructs were confirmed by restriction digestion and DNA sequencing.

We generated lentiviral vector stocks using SIN18-RhMLV encoding TTRs or EGFP, packaging plasmid pCMV R8.2 Vpr and the RRV envelope protein-coding plasmid [29] by calcium phosphate-mediated transient transfection, as described previously [30]. Lentiviral vector particles were concentrated by ultracentrifugation through a 10% sucrose cushion in Hanks' balanced salt solution (HBSS) with 1 mM ethylenediaminetetraacetic acid and resuspended in a 100-fold lower volume of HBSS, filtered through 0.22- μ m filters (Millipore, Billerica, MN, USA) and stored at -80 °C. The infectious titer was determined in 293T cells by infecting with EGFP encoding vector and flow cytometric analysis. We routinely obtained virus titers of approximately 1×10^8 transduction units (TU)/ml.

Cells and *in vitro* lentiviral vector transduction

Human hepatocellular carcinoma HepG2 cells were maintained at 37 °C in α -minimum essential medium containing 10% fetal calf serum, penicillin/streptomycin and glutamine (growth medium). For lentiviral vector transduction, HepG2 cells were plated in 12-well plates at 5×10^4 cells/well and cultured for 24 h before transduction. We infected with 500 ng of viral p24 antigen per 1×10^5 cells in growth medium with 8 μ g/ml of polybrene. After 2 h of incubation at 37 °C, cells were washed twice with phosphate-buffered saline (PBS) and cultured in UltraCULTURE (Cambrex, East Rutherford, NJ, USA) for 2 or 5 days. We routinely transduced approximately 60% of HepG2 cells using these conditions. The culture supernatants were collected and kept at 4 °C.

Animal experiments

All animals were handled in accordance with the statement of the 'Animal Research Committee' in University of California Los Angeles.

TTR expressing lentiviral vectors (20 μ g of viral p24 antigen/mouse) were injected into the tail veins of 4- to 5-week-old female SCID ICR mice (Taconic, Germantown, NY, USA). One week later, whole blood samples were collected and left at 4 °C overnight. The serum was separated by centrifugation and processed for immunoprecipitation.

Immunoprecipitation analysis for TTR heterooligomers

We incubated culture supernatants of lentiviral vector transduced HepG2 cells containing 100 ng of either amyloidogenic or transsuppressor TTR, or 25 μ l of the sera collected from lentiviral vector transduced SCID mice with 2 μ g of either anti-Flag antibody (M2; Sigma-Aldrich, St Louis, MO, USA) or anti-HA antibody (HA.11; Covance, Princeton, NJ, USA) for 4 h at 4 °C, followed by 25 μ l of protein G-4FF beads (50% slurry; GE Healthcare, Piscataway, NJ, USA) for 1 h at 4 °C. We detected heterooligomer formation between TTR and RBP by incubating culture supernatants (1 ml) of T119M TTR encoding lentiviral

vector or mock transduced HepG2 cells with 10 µg of anti-RBP antibody (Clone GE4E; NeoMarkers, Fremont, CA, USA) for 2 h at 4 °C, followed by 30 µl of protein A-4FF beads (50% slurry; GE Healthcare) for 1 h at 4 °C. The beads were then washed four times with PBS and twice with PBS containing 0.5 mg/ml of Z3-14 (Sigma-Aldrich). Immunocomplexes were heated to 100 °C for 5 min in SDS-sample buffer and analysed by western blotting. Membranes were stripped by gently shaking in 100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 62.5 mM Tris-HCl, pH 6.8 at 55 °C for 30 min. As a standard, we used human TTR isolated plasma purchased from Sigma-Aldrich (Prealbumin, P1742). Native gel electrophoresis was performed as described previously [13].

TTR cross-linking

Culture supernatants of lentiviral vector transduced HepG2 cells containing 16 ng T119M TTR or V30M TTR were incubated with glutaraldehyde (0.5% final; Sigma-Aldrich) for 5 min at 25 °C. The proteins were heated to 100 °C for 5 min in SDS-sample buffer and analysed by western blotting using anti-human TTR antibody (Diasorin Inc., Saluggia, Italy).

Results

Lentiviral mediated expression of human TTR and variants in HepG2 cells

We constructed a lentiviral vector encoding human TTR variants cloned into self-inactivating (SIN) vector, SIN18-RhMLV-E [28] (Figure 1A). We first examined the lentiviral mediated expression of wild-type TTR in HepG2 cells. Cells were cultured in a serum-free medium (UltraCULTURE) that supports HepG2 cell growth and does not contain any detectable endogenous transthyretin by western blotting using anti-TTR antibody (Figure 1B, lanes 8 and 12, 'Medium').

Because TTR is a secretory plasma protein, we examined TTR expression in both cell lysates and culture supernatants. TTR levels in the supernatant were approximately 4 µg/ml (Figure 1B). TTR was detected in the cell lysate as well as in the culture supernatant of the transduced HepG2 cells. Higher levels of TTR were observed in culture supernatant than in cell lysate; 460 fg/cell equivalent in culture supernatant and 5 fg/cell equivalent in cell lysate (over 5 days), respectively. Very low levels of endogenous TTR were observed in control cells at higher amounts of supernatant analysed (7.5 µl) and longer exposure times (data not shown). We estimate that lentivirally expressed TTR is expressed at approximately 150-fold higher levels than endogenous TTR expressed in untransduced HepG2 cells. We further examined the expression of variants of TTR (Figure 1C). Both the amyloidogenic (V30M) and transsuppressor (T119M) forms of TTRs were secreted in the culture supernatant as efficiently as wild-type TTR and the expression of those transgenes was maintained for at least 6 months in culture (data not shown). The concentration of V30M TTR secreted in the culture supernatant reached levels as high as 16 µg/ml at 5 days postinfection (Figure 1D). Similar levels of expression and secretion were also observed for T119M TTR and two other amyloidogenic variants, L55P and V122I (data not shown).

Lentivirally expressed TTRs form homotetramers which are secreted in the culture supernatant

TTR circulates in plasma and CSF as a 55-kDa tetrameric protein which is necessary for binding between RBP and thyroid hormones. We examined whether lentivirally expressed TTRs can form tetramers in a similar manner as naturally synthesized TTR. To prevent undesired dissociation during the sample preparation, we treated the culture supernatant of HepG2 cells transduced with lentiviral vector expressing either T119M TTR or V30M TTR with glutaraldehyde as reported by Reixach *et al.* [20]. Broad bands around 70 kDa were observed only in the crosslinked samples (Figure 2A). These bands migrated close to the expected size of TTR tetramer (64 kDa for T119M TTR, 60 kDa for V30M TTR). We did not observe bands greater than 90 kDa, suggesting that our culture condition is insufficient to induce either TTR amyloid fibrils or soluble aggregates which arise by misfolding of TTR monomer as reported by Reixach *et al.* [20].

One TTR tetramer forms a heterohexamer with two RBP molecules [31] and HepG2 cells are competent to secrete TTR-RBP heterooligomer [32]. We examined whether the lentivirally expressed and secreted TTR tetramer from HepG2 cells contain RBP by co-immunoprecipitation followed by western blotting. The culture supernatant of HepG2 cells expressing T119M TTR were immunoprecipitated with an anti-RBP antibody and the immunoprecipitates were analysed by western blotting using anti-TTR antibody (Figure 2B). A band corresponding to TTR was detected in the immunoprecipitate obtained from the culture supernatant of T119M TTR expressing HepG2 cells, indicating that lentivirally expressed TTR exists as a heterooligomer with RBP in the culture supernatant. We concluded that lentivirally expressed TTR maintained at least one functional property of TTR: the ability to associate with RBP.

Lentivirally expressed T119M TTR makes oligomers with amyloidogenic TTRs when co-expressed in HepG2 cells

The amyloidogenic properties of V30M TTR is alleviated by incorporation of a T119M TTR subunit(s) into the heterotetrameric oligomer [27]. We next tested whether lentivirally expressed T119M TTR can form heterotetramers with the amyloidogenic variants under two different conditions: (i) 'super-infection' of T119M TTR expressing vector to HepG2 cells previously transduced and expressing amyloidogenic TTRs and (ii) 'co-culture' of HepG2 cells independently expressing T119M TTR or amyloidogenic TTRs. The culture supernatants of 'super-infected' or 'co-cultured' HepG2 cells were immunoprecipitated with either anti-HA antibody or anti-Flag antibody and analysed by western blotting using either anti-Flag antibody or anti-HA antibody, respectively (Figure 3). T119M TTR and V30M TTR were able to be immunoprecipitated only by the specific antibody against the tag (Figure 3A, lanes 1–4). Coprecipitation of T119M TTR and V30M TTR was observed when the culture supernatants of 'super-infected' HepG2 cells were immunoprecipitated with either anti-Flag antibody or anti-HA antibody (Figure 3A, lanes 5 and 6, 'super-infection'). Heterooligomerization between T119M TTR and amyloidogenic TTR was also confirmed using two other amyloidogenic mutants, L55P and V122I, when both TTRs were co-expressed by 'super-infection' (Figure 3B, lanes 2–4). However, heterooligomerization was not observed when cells independently expressing each TTR mutant were mixed and 'co-

cultured' (Figures 3A, lanes 7 and 8, and 3B, lanes 5–7). We tested various 'co-culture' conditions, but none of the conditions resulted in heterooligomerization between T119M TTR and amyloidogenic TTRs (data not shown), indicating that dissociation of TTR homotetramers and re-association into heterotetramers does not detectably occur in supernatants.

Lentivirally expressed T119M TTR and V30M TTR make heterooligomers *in vivo*

We further confirmed the heterooligomerization between T119M TTR and V30M TTR and secretion into the plasma in a living animal (Figure 4). Similar to what we observed upon 'super-infection' of HepG2 cells (Figure 3), heterooligomerization between T119M TTR and V30M TTR was confirmed when both lentiviral vectors were co-injected into SCID mice (Figure 4, lanes 7 and 8). In the co-injected mouse, 21 ng of V30M TTR was co-immunoprecipitated with 60 ng of T119M TTR when T119M TTR was immunoprecipitated by anti-Flag antibody, whereas 20 ng of T119M TTR was co-immunoprecipitated with 130 ng of V30M TTR when V30M TTR was immunoprecipitated by anti-HA antibody, indicating that 13–26% of lentivirally expressed TTR was secreted as heterooligomer into the serum of co-injected mouse. There was no TTR detected using anti-Flag and anti-HA antibodies in sera from mice receiving single injections of either V30M TTR (Figure 4, lanes 3 and 4) or T119M TTR (Figure 4, lanes 5 and 6) encoding lentiviral vector, respectively, and buffer-injected mouse (Figure 4, lanes 1 and 2, mock). These results show that transsuppressor T119M TTR expressed from a lentiviral vector can be secreted in the blood upon transduction of SCID mice and that the gene products form heterooligomers with amyloidogenic V30M TTR *in vivo*.

Stabilization of amyloidogenic TTR by heterotetramer formation in the presence of T119M TTR

A gene therapy approach using T119M transsuppressor variants would involve ectopic expression of T119M TTR by transduction of cells expressing amyloidogenic forms of TTR, leading to stabilization of secreted heterotetramers that, as a consequence, have reduced amyloidogenic properties. HepG2 cells express low levels of endogenous TTR (see above). However, because there is no facile immunological means of distinguishing endogenous TTR from transsuppressor TTR, we chose to mimic the endogenous situation by first expressing amyloidogenic forms of TTR in HepG2 cells and then transducing those cells with vectors expressing the transsuppressor forms of TTR. The amyloidogenic and transsuppressor forms are distinguished by using distinct epitope tags: HA tags for V30M TTR, L55P TTR and V122I TTR, and Flag tag for T119M TTR (Figure 5A, left panels). In addition, to confirm the results, constructs with reciprocal tags were created: Flag tags for V30M TTR, L55P TTR and V122I TTR, and HA tag for T119M TTR, respectively (Figure 5A, right panels).

HepG2 cells stably expressing V30M TTR, L55P TTR and V122I TTR, respectively, were transduced with a lentiviral vector expressing T119M TTR. The stability of secreted heterotetramers was monitored by native gel electrophoresis. In the absence of Flag-tagged T119M TTR (T119M-FL) transduction, HA-tagged L55P TTR (L55P-HA) and V122I TTR (V122I-HA) were detected as both monomer and tetramer forms (Figure 5A, lower left

panel, lanes 6 and 9, respectively). HA-tagged V30M TTR (V30M-HA) was only detected in tetramer forms (Figure 5A, lower left panel, lane 3). Transduction of T119M-FL into native HepG2 cells yielded a major species migrating at the position of a tetramer (Figure 5A, upper left panel, lane 2). Evidence for stabilization of amyloidogenic TTR was seen following transduction of T119M TTR into cells expressing L55P TTR and V122I TTR. Transduction of T119M-FL resulted in heterotetramerization and significantly decreased levels of monomer in the case of both L55P-HA and that of V122I-HA (Figure 5A, lower left panel, lanes 7 and 10, respectively). Transduction of Flag-tagged L55P TTR (L55P-FL) or V122I TTR (V122I-FL) into cells expressing L55P-HA or V122I-HA, respectively, did not change the mobility of either form of L55P TTR or V122I TTR (Figure 5A; upper and lower left panels, lanes 8 and 11), indicating that the epitope tags alone had no effect on mobility of L55P TTR or V122I TTR. Identical results to the above were seen in reciprocal experiments with HA-tagged T119M TTR and Flag tagged amyloidogenic TTRs (Figure 5A, right panels). These results indicate that the transduction of T119M TTR can significantly reduce the presence of monomer forms of amyloidogenic TTR.

Because monomer forms of V30M TTR were not detected, we were unable to confirm by the above method that the presence of T119M TTR resulted in stable heterotetramer formation with V30M TTR. To demonstrate heterotetramer formation with V30M TTR, we used a version of T119M TTR bearing three tandem repeats of Flag-tag (T119M-FLx3) that resulted in a detectable change in mobility, allowing us to distinguish by size T119M TTR and V30M TTR tetramers (Figure 5B; upper panel, lanes 2 and 5). Transduction of this T119M TTR into V30M TTR expressing cells resulted in a concomitant appearance of higher molecular size oligomers, intermediate in size between T119M TTR and V30M TTR (Figure 5B, upper and lower panels, lane 4). These oligomers presumably represent heterotetramers of V30M TTR and T119M TTR and the heterogeneous mobility in the native gels likely reflects various combinations of V30M TTR and T119M TTR in the heterotetramers [27]. As a control, transduction of V30M-HA expressing cells with a vector expressing Flag-tagged V30M TTR (V30M-FL) did not change the mobility of either form of V30M TTR, indicating that the tags alone had no effect on tetramer formation (Figure 5B, upper and lower panels, lane 5). Thus, consistent with previously published studies using bacterially synthesized TTR [27], these results demonstrate that transsuppressor T119M TTR can form stable heterooligomers with amyloidogenic forms of TTR and prevent the appearance of dissociated monomeric forms of amyloidogenic TTR.

Discussion

TTR amyloidosis causes systemic organ malfunction, which ultimately leads to death. The pathogenesis is closely associated with instability of the TTR tetramer and aggregation of the resulting monomer. The transsuppressor variant, T119M TTR, makes stable heterotetramers with amyloidogenic TTRs, inhibiting its monomerization. In the present proof of concept study, we designed a lentiviral vector encoding T119M TTR and evaluated its activity *in vitro* and *in vivo*. Lentivirally expressed T119M TTR formed oligomers with three major amyloidogenic TTRs (V30M, L55P and V122I) in HepG2 cells. Heterooligomer formation was also observed in the mouse. Similar to previous biochemical studies with bacterially expressed proteins, the heterooligomers containing T119M TTR were more

stable in supernatants. Thus, our results demonstrate that ectopic expression of transsuppressor variants can drive stable heterooligomer formation with amyloidogenic variants.

Heterooligomers between T119M TTR and amyloidogenic TTRs formed only when both TTRs were expressed within the same cell, but not when cells independently expressing either T119M TTR or amyloidogenic TTR were co-cultured. TTR oligomerization occurring primarily inside of the cell emphasizes that efficient vector transduction to the liver, is a major site of TTR production, will be important for heterotetramerization between intrinsic amyloidogenic TTRs and expressed T119M TTR. Our vector has a titer of approximately 1×10^8 TU/ml and transduces approximately 1.5% of total hepatocytes when 1.5×10^7 TU of lentiviral vector was injected into mouse through the tail vein. Other vectors that transduce liver cells more efficiently, such as adeno-associated virus serotype 8 (AAV8) vectors could be used, [33]; however, the expression of transgenes disappears over time due to cell-mediated immunity against the AAV capsid [34]. The present study utilizes integration competent lentiviral vectors for stable long-term expression, which is a desirable trait for hereditary diseases such as TTR amyloidosis.

At present, liver transplantation is the only effective treatment for TTR amyloidosis. However, liver transplantation presents various difficulties, including a shortage of donor livers and costly and stressful therapy. Small molecule inhibitors have also been proposed to prevent TTR amyloid formation by stabilizing the TTR tetramer [35–37] and two candidate drugs, diflunisal and Fx-1006 A, are under investigation in phase II and III clinical trials ([ClinicalTrials.gov](https://clinicaltrials.gov) Identifier numbers: NCT00294671 and NCT00630864, respectively). One gene therapeutic approach was proposed by Nakamura *et al.* [38], who they converted the endogenous TTR gene in the liver of a transgenic mouse (V30M) to wild-type using single-stranded oligonucleotides (SSOs). However, the efficiency of gene conversion by this methodology is highly variable [39] and not yet tested in humans. By contrast, gene transfer of genes for other diseases using a variety of viral vectors has been tested in numerous clinical trials. Heterooligomer formation directed by lentiviral vector gene transfer of transsuppressor variants can be considered as a potential therapeutic modality. Similar gene transfer approaches can be considered as a strategy for other types of amyloidoses.

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References

1. Pepys MB. Amyloidosis. *Annu Rev Med* 2006; 57: 223–241. [PubMed: 16409147]
2. Buxbaum JN. The systemic amyloidoses. *Curr Opin Rheumatol* 2004; 16: 67–75. [PubMed: 14673392]
3. Obici L, Perfetti V, Palladini G, et al. Clinical aspects of systemic amyloid diseases. *Biochim Biophys Acta* 2005; 1753: 11–22. [PubMed: 16198646]
4. Costa PP, Figueira AS, Bravo FR. Amyloid fibril protein related to prealbumin in familial amyloidotic polyneuropathy. *Proc Natl Acad Sci USA* 1978; 75: 4499–4503. [PubMed: 279930]

5. Saraiva MJ, Birken S, Costa PP, et al. Amyloid fibril protein in familial amyloidotic polyneuropathy, Portuguese type. Definition of molecular abnormality in transthyretin (prealbumin). *J Clin Invest* 1984; 74: 104–119. [PubMed: 6736244]
6. Ando Y, Nakamura M, Araki S. Transthyretin-related familial amyloidotic polyneuropathy. *Arch Neurol* 2005; 62: 1057–1062. [PubMed: 16009758]
7. Benson MD, Kincaid JC. The molecular biology and clinical features of amyloid neuropathy. *Muscle Nerve* 2007; 36: 411–423. [PubMed: 17554795]
8. Costa R, Goncalves A, Saraiva MJ, et al. Transthyretin binding to A-Beta peptide – impact on A-Beta fibrillogenesis and toxicity. *FEBS Lett* 2008; 582: 936–942. [PubMed: 18295603]
9. Saraiva MJ. Transthyretin mutations in hyperthyroxinemia and amyloid diseases. *Hum Mutat* 2001; 17: 493–503. [PubMed: 11385707]
10. Andrade C A peculiar form of peripheral neuropathy; familiar atypical generalized amyloidosis with special involvement of the peripheral nerves. *Brain* 1952; 75: 408–427. [PubMed: 12978172]
11. Kanda Y, Goodman DS, Canfield RE, et al. The amino acid sequence of human plasma prealbumin. *J Biol Chem* 1974; 249: 6796–6805. [PubMed: 4607556]
12. Bellovino D, Morimoto T, Pisaniello A, et al. In vitro and in vivo studies on transthyretin oligomerization. *Exp Cell Res* 1998; 243: 101–112. [PubMed: 9716454]
13. Sato T, Susuki S, Suico MA, et al. Endoplasmic reticulum quality control regulates the fate of transthyretin variants in the cell. *EMBO J* 2007; 26: 2501–2512. [PubMed: 17431395]
14. Sekijima Y, Wiseman RL, Matteson J, et al. The biological and chemical basis for tissue-selective amyloid disease. *Cell* 2005; 121: 73–85. [PubMed: 15820680]
15. Monaco HL, Rizzi M, Coda A. Structure of a complex of two plasma proteins: transthyretin and retinol-binding protein. *Science* 1995; 268: 1039–1041. [PubMed: 7754382]
16. Hamilton JA, Benson MD. Transthyretin: a review from a structural perspective. *Cell Mol Life Sci* 2001; 58: 1491–1521. [PubMed: 11693529]
17. Sebastiao MP, Saraiva MJ, Damas AM. The crystal structure of amyloidogenic Leu55 – > Pro transthyretin variant reveals a possible pathway for transthyretin polymerization into amyloid fibrils. *J Biol Chem* 1998; 273: 24715–24722. [PubMed: 9733771]
18. Quintas A, Vaz DC, Cardoso I, et al. Tetramer dissociation and monomer partial unfolding precedes protofibril formation in amyloidogenic transthyretin variants. *J Biol Chem* 2001; 276: 27207–27213. [PubMed: 11306576]
19. Hammarström P, Jiang X, Hurshman AR, et al. Sequence-dependent denaturation energetics: A major determinant in amyloid disease diversity. *Proc Natl Acad Sci USA* 2002; 99(Suppl 4): 16427–16432. [PubMed: 12351683]
20. Reixach N, Deechongkit S, Jiang X, et al. Tissue damage in the amyloidoses: Transthyretin monomers and nonnative oligomers are the major cytotoxic species in tissue culture. *Proc Natl Acad Sci USA* 2004; 101: 2817–2822. [PubMed: 14981241]
21. Alves IL, Divino CM, Schussler GC, et al. Thyroxine binding in a TTR Met 119 kindred. *J Clin Endocrinol Metab* 1993; 77: 484–488. [PubMed: 8102146]
22. Alves IL, Altland K, Almeida MR, et al. Screening and biochemical characterization of transthyretin variants in the Portuguese population. *Hum Mutat* 1997; 9: 226–233. [PubMed: 9090525]
23. Coelho T, Choraó R, Sousa A, et al. Compound heterozygotes of transthyretin Met30 and transthyretin Met119 are protected from the devastating effects of familial amyloid polyneuropathy. *Neuromuscul Disord* 1996; 27: S20.
24. Harrison HH, Gordon ED, Nichols WC, et al. Biochemical and clinical characterization of prealbuminCHICAGO: an apparently benign variant of serum prealbumin (transthyretin) discovered with high-resolution two-dimensional electrophoresis. *Am J Med Genet* 1991; 39: 442–452. [PubMed: 1877623]
25. Longo Alves I, Hays MT, Saraiva MJ. Comparative stability and clearance of [Met30]transthyretin and [Met119]transthyretin. *Eur J Biochem* 1997; 249: 662–668. [PubMed: 9395311]
26. Almeida MR, Alves IL, Terazaki H, et al. Comparative studies of two transthyretin variants with protective effects on familial amyloidotic polyneuropathy: TTR R104H and TTR T119M. *Biochem Biophys Res Commun* 2000; 270: 1024–1028. [PubMed: 10772944]

27. Hammarström P, Schneider F, Kelly JW. Trans-suppression of misfolding in an amyloid disease. *Science* 2001; 293: 2459–2462. [PubMed: 11577236]
28. Kung SK, Bonifacino A, Metzger ME, et al. Lentiviral vector-transduced dendritic cells induce specific T cell response in a nonhuman primate model. *Hum Gene Ther* 2005; 16: 527–532. [PubMed: 15871684]
29. Kang Y, Stein CS, Heth JA, et al. In vivo gene transfer using a nonprimate lentiviral vector pseudotyped with Ross River virus glycoproteins. *J Virol* 2002; 76: 9378–9388. [PubMed: 12186920]
30. Yuan H, Kamata M, Xie YM, et al. Increased levels of Wee-1 kinase in G2 are necessary for Vpr- and gamma irradiation-induced G2 arrest. *J Virol* 2004; 78: 8183–8190. [PubMed: 15254189]
31. Rostom AA, Sunde M, Richardson SJ, et al. Dissection of multi-protein complexes using mass spectrometry: subunit interactions in transthyretin and retinol-binding protein complexes. *Proteins* 1998; (Suppl 2): 3–11.
32. Bellovino D, Morimoto T, Tosetti F, et al. Retinol binding protein and transthyretin are secreted as a complex formed in the endoplasmic reticulum in HepG2 human hepatocarcinoma cells. *Exp Cell Res* 1996; 222: 77–83. [PubMed: 8549676]
33. Nakai H, Fuess S, Storm TA, et al. Unrestricted hepatocyte transduction with adeno-associated virus serotype 8 vectors in mice. *J Virol* 2005; 79: 214–224. [PubMed: 15596817]
34. Manno CS, Pierce GF, Arruda VR, et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* 2006; 12: 342–347. [PubMed: 16474400]
35. Klabunde T, Petrassi HM, Oza VB, et al. Rational design of potent human transthyretin amyloid disease inhibitors. *Nat Struct Biol* 2000; 7: 312–321. [PubMed: 10742177]
36. Almeida MR, Gales L, Damas AM, et al. Small transthyretin (TTR) ligands as possible therapeutic agents in TTR amyloidoses. *Curr Drug Targets CNS Neurol Disord* 2005; 4: 587–596. [PubMed: 16266291]
37. Sacchettini JC, Kelly JW. Therapeutic strategies for human amyloid diseases. *Nat Rev Drug Discov* 2002; 1: 267–275. [PubMed: 12120278]
38. Nakamura M, Ando Y, Nagahara S, et al. Targeted conversion of the transthyretin gene in vitro and in vivo. *Gene Ther* 2004; 11: 838–846. [PubMed: 14961068]
39. Andersen MS, Sorensen CB, Bolund L, et al. Mechanisms underlying targeted gene correction using chimeric RNA/DNA and single-stranded DNA oligonucleotides. *J Mol Med* 2002; 80: 770–781. [PubMed: 12483462]

Figure 1.

Western blotting analyses of TTR expression in lentiviral vector transduced HepG2 cells. (A) Schematic illustration of the lentiviral vectors encoding human TTR and the variants. CPPT, central polypurine tract; RhMLV, murine leukemia virus long-terminal repeat promoter; LTR, long-terminal repeat; SIN, self-inactivated LTR; HA, influenza haemagglutinin tag; FL, Flag epitope tag; V30M, L55P, V122I, amyloidogenic variants; T119M, transsuppressor variant. (B–D) HepG2 cells were infected with lentiviral vector encoding TTR and cultured in UltraCULTURE for 5 days. The cell lysates and culture supernatants of lentiviral vector or mock infected cells were analysed by western blotting using anti-TTR antibody. (B) Expression of wild-type TTR in cell lysate (20 µg) and supernatants (left: 1.5 µl; right: 7.5 µl). (C) Expression of HA-tagged V30M TTR (V30M-HA) and three tandem repeats of Flag-tagged T119M TTR (T119M-FLx3) in supernatant (5 µl) from lentiviral vector infected cells. (D) The culture supernatant (62.5 nl) of V30M-HA

expressing lentiviral vector infected HepG2 cells was loaded on 18% SDS-PAGE gel and analysed by western blotting using anti-TTR antibody. Purified human TTR (0.5, 1, 2, 4 and 8 ng) was analysed in parallel for comparison

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Figure 2.

Homo and heterooligomers formation of TTR secreted from lentiviral vector transduced HepG2 cells. (A) HepG2 cells were infected with lentiviral vector encoding three tandem repeats of Flag-tagged T119M TTR (T119M-FLx3) or HA-tagged V30M TTR (V30M-HA) and cultured in UltraCULTURE for 5 days. 16 ng of TTR containing supernatants, as estimated by comparison with purified human TTR as described in Figure 1D, were crosslinked with glutaraldehyde for 5 min at 25 °C and subsequently analysed by western blotting using anti-TTR antibody. The expected sizes of monomer and tetramer are indicated by respective arrowheads. No treatment: 1 ng of each TTR containing culture supernatant. (B) 1 ml of the culture supernatant of T119M-FLx3 encoding lentiviral vector or mock infected HepG2 cells was incubated with an anti-RBP antibody. The immunocomplexes were collected using protein A conjugated Sepharose beads and analysed by western blotting using anti-TTR antibody. Input: 1 µl of culture supernatant. IP, immunoprecipitation

Figure 3.

In vitro heterooligomer formation between transsuppressor T119M TTR and amyloidogenic variants. (A) HepG2 cells were infected with lentiviral vectors encoding either three tandem repeats of Flag-tagged T119M TTR [T119M-FLx3; (A) lanes 1 and 2] or HA-tagged V30M TTR [V30M-HA; (A) lanes 3 and 4], or ‘super-infected’ T119M-FLx3 expressing lentiviral vector to HepG2 cells already transduced and expressing V30M-HA [super-infection; (A) lanes 5 and 6]. (B) HepG2 cells were infected with lentiviral vectors encoding T119M-FLx3, V30M-HA, HA-tagged L55P TTR (L55P-HA) or V122I TTR (V122I-HA), or ‘super-infected’ T119M-FLx3 expressing lentiviral vector to HepG2 cells already transduced and expressing V30M-HA, L55P-HA, or V122I-HA [super-infection; (B) lanes 2–4]. The cells were cultured for 5 days in UltraCULTURE. For ‘co-culture’ experiment, HepG2 cells expressing T119M-FLx3 and V30M-HA [(A) lanes 7 and 8; (B) lane 5], L55P-HA [(B) lane 6], or V122I-HA [(B) lane 7] were mixed and co-cultured for 5 days. TTRs secreted into the

culture supernatant were monitored by immunoprecipitation (IP) using anti-Flag (Flag) and anti-HA (HA) antibodies, followed by western blotting (WB) using anti-Flag and anti-HA antibodies, respectively

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Figure 4.

In vivo heterooligomer formation between transsuppressor T119M TTR and amyloidogenic V30M TTR. Lentiviral vectors (20 µg of viral p24 each) encoding either HA-tagged V30M TTR (V30M-HA; lanes 3 and 4) or three tandem repeats of Flag-tagged T119M TTR (T119M-FLx3; lanes 5 and 6) were injected into SCID mice or co-injected (lanes 7 and 8) via THE tail vein. At 1 week postinjection, the mice were sacrificed and sera were collected. The presence of TTR in sera was analysed by immunoprecipitation (IP) using anti-Flag (Flag) and anti-HA (HA) antibodies, followed by western blotting (WB) using anti-Flag and anti-HA antibodies to detect T119M TTR and V30M TTR, respectively. The first two lanes indicate TTR variants (100 ng each) purified from supernatants of transduced HepG2 cells. The numbers shown under each band indicate the estimated amount (ng) of immunoprecipitated TTR by densitometry

Figure 5.

Stabilization of amyloidogenic TTR by heterotetramer formation in the presence of T119M TTR. (A) HepG2 cells were mock treated (mock; left and right panels, lanes 1 and 12) or stably infected with lentiviral vectors encoding either single Flag-tagged T119M TTR [T119M-FL; (A) left panel, lane 2] or HA-tagged T119M TTR [T119M-HA; (A) right panel, lane 13], HA-tagged V30M TTR [V30M-HA; (A) left panel, lanes 3–5] or Flag-tagged V30M TTR [V30M-FL; (A) right panel, lanes 14–16], HA-tagged L55P TTR [L55P-HA; (A) left panels, lanes 6–8] or Flag-tagged L55P TTR [L55P-FL; (A) right panel, lanes 17–19] and HA-tagged V122I TTR [V122I-HA; (A) left panels, lanes 9–11] or Flag-tagged V122I TTR [V122I-FL; (A) right panel, lanes 20–22]. HepG2 cells stably expressing V30M-HA, L55P-HA and V122I-HA were subsequently transduced with T119M-FL [(A) left panels, lanes 4, 7 and 10], V30M-FL [(A) left panels, lane 5], L55P-FL [(A) left panels, lane 8] or V122I-FL [(A) left panels, lane 11]. HepG2 cells stably expressing V30M-FL, L55P-FL and V122I-FL were subsequently transduced with T119M-HA [(A) right panels, lanes 15, 18 and 21], V30M-HA [(A) right panels, lane 16], L55P-HA [(A) right panels, lane 19] or V122I-HA [(A) right panels, lane 22]. (B) HepG2 cells were mock treated [mock; (B) lane 1] or stably infected with lentiviral vectors encoding either three tandem repeats of Flag-tagged T119M TTR [T119M-FLx3; (B) lane 2] or V30M-HA [(B) lanes 3–5]. HepG2 cells stably expressing V30M-HA were subsequently transduced with T119M-FLx3 [(B)

lane 4] or V30M-FL [(B) lane 5]. The cells were cultured for 2 days in UltraCULTURE. TTRs secreted into the culture supernatant were monitored by native gel electrophoresis, followed by western blotting using either anti-Flag antibody [(A, B) upper panels] or anti-HA antibody [(A, B) lower panels], as indicated. Molecular size markers were used to determine the approximate mobility of tetramer (T) and monomer (M) forms of TTR. Co-transduction of T119M TTR resulted in heterotetramerization and significantly decreased levels of monomer in cells stably expressing L55P TTR and V122I TTR. The heterogeneous mobility pattern shown in (B) is likely due to differences in charge for tetramers containing different ratios of T119M-FLx3 and V30M-HA