Repeat-Associated Non-ATG (RAN) Translation in Fuchs’ Endothelial Corneal Dystrophy

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PURPOSE. The strongest genetic association with Fuchs’ endothelial corneal dystrophy (FECD) is the presence of an intronic (CTG-CAG)n trinucleotide repeat (TNR) expansion in the transcription factor 4 (TCF4) gene. Repeat-associated non-ATG (RAN) translation, an unconventional protein translation mechanism that does not require an initiating ATG, has been described in many TNR expansion diseases, including myotonic dystrophy type 1 (DM1). Given the similarities between DM1 and FECD, we wished to determine whether RAN translation occurs in FECD.

METHODS. Antibodies against peptides in the C-terminus of putative RAN translation products from TCF4 were raised and validated by Western blotting and immunofluorescence (IF). CTG-CAG repeats of various lengths in the context of the TCF4 gene were cloned in frame with a 3×FLAG tag and transfected in human cells. IF with antipeptide and anti-FLAG antibodies, as well as cytotoxicity and cell proliferation assays, were performed in these transfected cells. Corneal endothelium derived from patients with FECD was probed with validated antibodies by IF.

RESULTS. CTG-CAG repeats in the context of the TCF4 gene are transcribed and translated via non-ATG initiation in transfected cells and confer toxicity to an immortalized corneal endothelial cell line. An antipeptide antibody raised against the C-terminus of the TCF4 poly-cysteine frame recognized RAN translation products by IF in cells transfected with CTG-CAG repeats and in FECD corneal endothelium.

CONCLUSIONS. Expanded CTG-CAG repeats in the context of the third intron of TCF4 are transcribed and translated via non-ATG initiation, providing evidence for RAN translation in corneal endothelium of patients with FECD.

Keywords: Fuchs’ dystrophy, RAN translation, trinucleotide repeat expansion, corneal endothelial cells, microsatellite repeats

Fuchs’ endothelial corneal dystrophy (FECD) is a common, inherited, degenerative disease of the cornea that manifests as attrition of the nonreplicating endothelial cell layer and formation of guttae, which are collagenous excrences of the Descemet membrane. With advanced disease, the fluid-pumping activity of the corneal endothelium is severely impaired, and the resulting vision loss is treatable only by transplantation. The strongest genetic association with FECD is with an intronic (CTG-CAG)n trinucleotide repeat (TNR) expansion in the third intron of the transcription factor 4 (TCF4) gene.1–8 Unaffected individuals typically have 12 to 18 TNR repeats, but up to 79% of individuals with FECD have an unstable CTG-CAG repeat length greater than 50.1 While nearly all TNR expansion diseases to date are directly linked to rare neurologic or neuromuscular disorders, FECD is the first eye disease associated with a TNR expansion. TNR expansions are thought to contribute to disease pathogenesis through several mechanisms: translation of significantly altered proteins,9–12 changes in global alternative RNA splicing patterns due to sequestration of splicing factors,13 and synthesis of repeat-associated non-ATG (RAN) translation products.14–17

We previously demonstrated that, similar to myotonic dystrophy type 1 (DM1), transcription of the noncoding CTG-CAG TNR expansion sequence in the TCF4 gene results in the formation of ribonuclear inclusions (foci) in FECD corneal endothelium. These RNA foci sequester the RNA splicing factor muscleblind-like 1 (MBNL1), reducing its availability and leading to aberrant splicing.18 Key MBNL1-mediated mis-splicing events previously reported for DM1 are also present in FECD corneal endothelium.18,19

In DM1, CUG repeats from the sense RNA transcript and CAG repeats from the antisense RNA transcript initiate protein translation in different reading frames, resulting in homopolymeric polypeptides.14 Repeat-associated non-ATG (RAN) translation has been described in many DNA repeat (microsatellite) expansion disorders, including spinocerebellar ataxia types 8 (SCA8)14 and 31 (SCA31),20 familial forms of amyotrophic lateral sclerosis, frontotemporal dementia,21,22 fragile X tremor/ataxia syndrome (FXTAS),23 Huntington disease (HD),24 and myotonic dystrophy type 2 (DM2).25 These protein species form nuclear and cytoplasmic inclusions and are thought to contribute to disease pathogenesis through a variety of
mechanisms, including proteasome impairment, endoplasmic reticulum stress, nuclear stress, nucleo-lysosomal transport defects, alterations of the nuclear lamina, missplicing, mitochondrial dysfunction, and oxidative stress. Several of these mechanisms have been implicated in FEDC pathogenesis.

Given the genetic and molecular similarity between DM1 and FEDC, it is conceivable that RAN translation is also a hallmark of Fuchs' dystrophy and that RAN translation-related mechanisms could contribute to pathogenesis. Here, we show that expanded CTG-CAG repeats in the context of the third intron of TCF4 are transcribed and translated via non-ATG initiation and provide evidence for RAN translation in corneal endothelium of patients with FEDC.

METHODS

Corneal Tissue, Cell Culture, DNA Isolation, and Conventional PCR

Patient recruitment, corneal endothelium isolation, fibroblast derivation from skin biopsies, DNA isolation, and PCR to determine CTG-CAG repeat length were previously described. Growth conditions for the HCEnc21-T cell line are described in Schmedt et al. Human studies were approved by the Mayo Clinic Institutional Review Board and were conducted in accordance to the Declaration of Helsinki and after informed consent.

CTG-CAG Repeat Cloning

CTG-CAG repeats and TCF4 intron 3-flanking sequences were amplified from genomic DNA extracted from fibroblasts generated from a patient with FEDC (patient 150; Fig. 1A). The poly-alanine (polyA) and poly-cysteine (polyC) open reading frames (ORFs) from the sense strand and poly-glutamine (polyQ) and poly-serine (polyS) ORFs from the antisense strand were cloned into the pcDNA3.1 vector in frame with a FLAG tag by using the Gibson assembly method. Flanking sequences upstream of the repeats were 105 bp for the polyA and polyC ORFs and 133 bp for the polyQ and polyS ORFs. The downstream flanking sequences were chosen to abut the first stop codon of the corresponding ORF. The oligonucleotides used to amplify both genomic DNA and the pcDNA3.1 vector are shown in Supplementary Table S1. The Gibson Assembly master mix was purchased from New England Biolabs, Inc. (Ipswich, MA, USA). A triple (3X) FLAG tag was then added by PCR to all constructs using the oligonucleotides listed in Supplementary Table S1. Methionine to lysine (M to K) and serine to methionine (S to M) mutations in the C218 construct (polyC construct with 218 repeats) were introduced by PCR using the oligonucleotides listed in Supplementary Table S1. The reaction mix for all above-mentioned PCRs was as follows: 40 ng of genomic DNA or 2 ng plasmid DNA, 0.3 μM of each oligonucleotide, 0.2 mM dNTPs, 1 mM MgSO4, and 0.6 U of Platinum Pfx DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) with 1X PCRx enhancer. PCR assays were performed in 50-μl reactions cycled through the following conditions: 94°C denaturation for 20 seconds, annealing for 30 seconds, and 68°C extension for 5 minutes for 40 cycles, with an initial denaturation for 5 minutes and a final extension for 10 minutes. Annealing temperatures were 60°C for cloning CTG-CAG repeats into the pcDNA3.1 vector by Gibson assembly, 55°C for adding the 3X FLAG tag, 58°C for introducing the M to K mutation, and 62°C for the S to M mutation.

Transfection, Western Blotting, and Immunofluorescence (IF)

HEK-293T and HCEnc21-T cells were transfected with Lipofectamine LTX (Thermo Fischer Scientific), following manufacturer's recommendations. IF and Western blotting procedures are described in Soragni et al. Peptide synthesis, antibody production, and affinity purification were performed by GenScript (Piscataway, NJ, USA). Antipeptide antibodies, anti-FLAG tag antibodies (Sigma-Aldrich Corp., St. Louis, MO, USA; Catalog no. F1804) and anti-polyQ antibodies (1C2; EMD Millipore, Temecula, CA, USA; Catalog no. MAB1574) were used at a 1:1000 dilution. Anti ZO-1 antibodies (Thermo Fischer Scientific; Catalog no. 339194) were used at a 1:200 dilution.

Lactate Dehydrogenase (LDH) Assay, Cell Proliferation Assay (MTS Assay), and Quantitative RT-PCR (qRT-PCR)

The LDH cytotoxicity assay (Thermo Fischer Scientific) and cell proliferation (MTS) assay (CellTiter 96 AQueous One Solution
Cell Proliferation Assay; Promega Corporation, Madison, WI, USA) were performed 48 hours after transfection, following the manufacturer's recommendations. qRT-PCR was performed 48 hours after transfection to measure HMOX-1 transcript levels by using the oligonucleotide primers HMOX-1_F and HMOX-1_R (Supplementary Table S1). qRT-PCR methods have been previously described.40

RESULTS

Preliminary Evidence for RAN Translation in FECD Fibroblasts

The detection of MBNL1-positive RNA foci and consequent missplicing in Fuchs' corneal endothelium18,19 indicate that CUG repeat–containing TCF4 RNA transcripts are expressed and can potentially be translated into RAN translation products. The same CTG/C1CAG microsatellite repeats in SCA8, DM1, SCA2, and HD are translated in diseased cells, and in these TNR expansion disorders, both the sense and antisense transcripts can be detected.14,24,41 Hence, if RAN translation occurs in FECD, we expect to find a poly-glutamine-containing protein arising from the antisense CAG repeats. We used a commercially available antibody (1C2)42 that recognizes polyQ-containing polypeptides to detect the presence of RAN translation products in protein extracts derived from healthy and FECD fibroblasts. We derived fibroblast lines from skin biopsies of patients with FECD, with and without CTG repeats in the TCF4 gene and determined CTG repeat size by PCR (Fig. 1A). Considerable heterogeneity was observed in repeat sizes for the expanded TCF4 allele in these cells (with TNR expansions ranging from 90 to over 1000), as has been noted for fibroblasts from patients with other TNR diseases.14 Western blotting using the 1C2 antibody and protein extracts from these fibroblast lines identified a putative polyQ-containing RAN translation product in patient cells that are homozygous for the CTG-CAG repeat expansion (Fig. 1B; patient 101). The band at ~42 kDa that is common to all samples is likely the TATA-box binding protein (TBP), as this antibody was originally raised to human TBP, which contains between 25 and 42 glutamine residues.14

CTG Repeats in the Context of the TCF4 Gene Generate RAN Translation Polypeptides in Transfected Cells

Since the 1C2 antibody likely recognizes all long polyQ-containing proteins, it cannot be used to unequivocally demonstrate that the putative RAN translation polypeptide we identified in the 101 fibroblast cell extract is translated from the expanded TCF4 antisense transcript. To detect the presence of RAN translation polypeptides derived from the TCF4 transcript in vivo, polyclonal antibodies were raised against the C-terminus of each of the predicted homopolymeric proteins (Fig. 2A; Supplementary Figs. S1 and S2). This strategy has been used in previous studies of RAN translation from TNR disease alleles.14 Since both the polyL-containing reading frame from the sense RNA and the polyA-containing reading frame from the antisense RNA each have a stop codon just after the repeats, only peptides derived from 4 out of the 6 ORFs were synthesized (Fig. 2A; Supplementary Fig. S2) (Note that the CTG repeats are followed by a short stretch of CTC repeats, encoding homopolymeric stretches of amino acids to which no specific antibody could be raised). Antibodies against peptide AF (polyA frame), CF1 and CF2 (polyC frame), QF (polyQ frame), and SF (polyS frame) were raised in rabbits by using the synthetic peptides shown in Supplementary Figure S2.
antibodies raised against the AF peptide (AF). (A) Whole-cell extracts from HEK-293T cells expressing the TCF4 polyA ORF containing 99 and 175 alanine residues in frame with a 3X FLAG C-terminal tag (A99 and A175) or an empty vector (pcDNA3.1), were probed by Western blotting with anti-FLAG tag antibodies (FLAG) and antibodies raised against the AF peptide (AF). (B) Whole-cell extracts from HEK-293T cells expressing the TCF4 polyC ORF containing 90 and 112 cysteine residues in frame with a 3X FLAG C-terminal tag (C90 and C112) or an empty vector (pcDNA3.1), were probed by Western blotting with anti-FLAG tag antibodies (FLAG) and antibodies raised against the CF1 peptide (CF1) and the CF2 peptide (CF2). Arrows indicate possible RAN translation products identified by the anti-FLAG and anti-CF2 antibodies. (C) Whole-cell extracts from HEK-293T cells expressing the TCF4 polyQ ORF containing 90 and 93 glutamine residues in frame with a 3X FLAG C-terminal tag (Q90 and Q93) or an empty vector (pcDNA3.1), were probed by Western blotting with anti-FLAG tag antibodies (FLAG) and antibodies raised against the QF peptide (QF). Arrows indicate possible RAN translation products identified by the anti-FLAG antibody. (D) Whole-cell extracts from HEK-293T cells expressing the TCF4 polyS ORF containing 150 and 117 serine residues in frame with a 3X FLAG C-terminal tag (S150 and S117) or an empty vector (pcDNA3.1), were probed by Western blotting with anti-FLAG tag antibodies (FLAG), antibodies raised against the SF peptide (SF), and the 1C2 antibody. Arrows indicate likely RAN translation products identified by the anti-FLAG and 1C2 antibodies. Gray boxes also indicate the presence of putative RAN translation products with aberrant migration (smears).

polyC reading frame contained an ATG codon after the short stretch of CTC repeats (Supplementary Fig. S1) that could be used to initiate the translation of an 88-amino-acid polypeptide (including the C-terminal FLAG tag). To determine if translation of the FLAG-reactive polypeptide was initiated from this codon, we mutated the ATG to AAG (M to K) and also introduced an ATG upstream of the CTG CAG repeats (S to M mutation in the context of a strong Kozak consensus sequence; Fig. 4A). When protein extracts from cells transfected with these constructs (C218 M to K and C218 S to M) were probed with anti-FLAG antibodies, the same polypeptide was identified in all extracts, indicating that its translation is independent of the downstream ATG, and no other polypeptide was identified when an upstream ATG was introduced (Fig. 4C).

Western blotting of cell lysates from cells transfected with the polyQ constructs showed the presence of RAN translation products when lysates were probed with anti-FLAG antibody, but not anti-QF antibody (Fig. 3C). The polyS constructs produced a polypeptide of about 8kDa that is recognized by the anti-FLAG but not the anti-SF antibody (Fig. 3D). The polyS reading frame contained an ATG codon after the short stretch of CTC repeats (Supplementary Fig. S1) that could be translated into a 61-amino-acid polypeptide. Western blotting also revealed the presence of a high molecular weight smear (gray box in Fig. 3D), in agreement with a previous study showing the peculiar migration of these species.14 When these polyS extracts were probed with the 1C2 antibody, polyQ-containing polypeptides were identified, demonstrating that the same transcript can be translated into multiple reading frames, as previously shown.14 Both discrete bands and smears were identified depending on repeat size,
suggesting that longer polyQ-containing RAN translation products become less soluble and/or change their migration properties.

The CF2 antibody was the only antipeptide antibody we generated that could recognize RAN translation peptides by Western blotting, although with very high background (Fig. 3B). Therefore, we tested this antibody in IF experiments with an immortalized human corneal endothelial cell line (HCEnC-21T) and HEK-293T cells transfected with the polyC TCF4 constructs (Fig. 5). Transfected cells were probed by IF with both the anti-FLAG antibody and the anti-CF2 antibody. As shown in Figure 5, there was a clear colocalization of anti-FLAG and antipeptide reactive species, thus making this antibody a valuable tool for the identification of TCF4-derived RAN translation products in FECD-affected tissue. None of the other antibodies showed colocalization of anti-FLAG and antipeptide reactive species in cells transfected with TCF4 constructs (Supplementary Fig. S3). Cells transfected with the A175 construct did not show any anti-FLAG staining, consistent with results obtained by Western blotting (Supplementary Fig. S3; Fig. 3A). IF experiments on HCEnC-21T cells transfected with TCF4 constructs expressing expanded CTG-CAG repeats, both in the presence and in the absence of the ATG codon downstream of the repeats in the polyC frame, indicated that anti-CF2 antibodies recognized species that were also bound by anti-FLAG antibody, regardless of the presence of the downstream ATG (Supplementary Fig. S4).

**FIGURE 5.** Characterization of antibodies raised against the CF2 peptide in the C-terminus of potential RAN translation products by IF. (A) HCEnC-21T cells were transfected with TCF4 constructs containing expanded CTG-CAG repeats of different lengths and probed by IF with anti-FLAG (red) and anti-CF2 antibodies (green). Nuclei were counterstained with DAPI (blue). (B) HEK-293T cells were transfected with the C112 and C220 constructs and probed by IF with anti-FLAG (red) and anti-CF2 antibodies (green). Scale bar: 50 μm.

RAN Translation Polypeptides Confer Cytotoxicity to an Immortalized Corneal Endothelial Cell Line

Extensive evidence exists for RAN translation-induced cytotoxicity. To determine whether any of the TCF4 RAN translation polypeptides would induce stress or cell death, the immortalized corneal endothelial cell line (HCEnC-21T) was transfected with each of the 3× FLAG-tagged constructs, and following incubation for 48 hours, growth medium was assayed for lactate dehydrogenase as a measure of cytotoxicity. As shown in Figure 6A, the S117 construct produced an increase in LDH; notably, the same effect was not observed when HEK-293T cells were transfected with the same construct, suggesting a unique response from corneal endothelial cells. As some studies have linked RAN translation cytotoxicity to oxidative stress, we also measured the levels of HMOX1, a gene known to be induced by oxidative stress and found a considerable increase in transcript levels in cells transfected with the S117 construct (Fig. 6B). Both polyS and polyQ constructs should produce the same transcript and the same RAN translation polypeptides (see Figs. 3C, 3D); however, differences in the size of the homopolymeric stretch could be responsible for the higher toxicity of the S117 construct compared to the Q93 construct. To assess this hypothesis, HCEnC-21T cells were transfected with constructs containing repeats of different lengths, and cell viability was determined using an MTS assay. As shown in Figure 6C, higher repeat numbers corresponded to increased toxicity, at least for
the polyS and polyQ constructs, and a stretch of more than 120 glutamine residues induced a decrease in cell number comparable to the polyS constructs.

Evidence of RAN Translation in Corneal Endothelium of Patients With FECD

Using the anti-CF2 antibody that recognizes the C-terminus of potential RAN translation products initiating from or around the expanded CTG-CAG repeats, we wished to establish whether RAN translation polypeptides could be detected in affected corneal endothelium from FECD patients. FECD endothelial tissue samples collected during endothelial keratoplasty and endothelium from unaffected eye bank corneas were fixed and stained using the anti-CF2 antibody. As shown in Figure 7, we detected clear immunostaining in corneal endothelium of FECD patients with 88 and 55 repeats on the expanded allele, as measured in leukocyte DNA from blood samples from the same patients. Due to the limited amount of corneal explant, we could not measure the repeat size in the endothelial layer of the same patients. IF of unaffected tissue and cornel endothelium from an FECD patient harboring no TNR expansion (FECD 2011-491) revealed some weak, non-specific staining. Four additional FECD endothelial specimens from patients with 57, 93, 74, and 109 repeats on the expanded allele were probed with anti-CF2 antibodies and showed the same nuclear staining (Supplementary Fig. S5). These samples were also probed for Zonula Occludens protein 1 (ZO-1), a known corneal endothelial marker, which showed staining at the cell boundaries (and occasionally some intracellular staining see Figure 7 and Supplementary Fig. S5). We also performed IF with the 1C2 antibody, but, due to the high background of this antibody in corneal tissue (possibly due to high TBP expression), we failed to detect any staining specific to Fuchs’ dystrophy samples (Supplementary Fig. S6).

DISCUSSION

The discovery of RAN translation has revolutionized the field of microsatellite diseases, resulting in a new view of disease mechanisms. The finding that most of these expanded repeats are bidirectionally transcribed and that the corresponding transcripts can be translated in several possible reading frames, producing many potentially toxic expansion proteins, has major implications for disease pathophysiology. As Fuchs’ dystrophy is the newest, non-neurological addition to the list of microsatellite diseases, with striking similarity to DM1, the obvious question is whether, as in DM1 and other TNR disorders, RAN translation also occurs in FECD. Assuming that, like in most microsatellite disorders, repeats in the TCF4 gene are bidirectionally transcribed, we looked for expression of polyQ-containing polypeptides (deriving from the antisense transcript) specific to FECD samples. Using a commercially available antibody that probes the polyQ proteome and protein extracts from FECD fibroblasts, we were able to detect a possible RAN translation product only in patient cells that are homozygous for the CTG-CAG repeat expansion (Fig. 1B; patient 101). It is conceivable that the expression of the CAG-containing transcript and corresponding RAN translation polypeptides is quite low in fibroblasts (an unaffected tissue in FECD), as also indicated by the small size of CUG RNA foci in these cells, and could only be detected in the homozygous line (line 101). Another complication in detecting RAN translation products by Western blotting is the repeat size heterogeneity in fibroblasts, in addition to the aberrant mobility of these protein species. The 1C2 antibody failed to identify a specific staining when it was used in IF experiments with corneal endothelium from patients with FECD (Supplementary Fig. S6).
possibly the low or absent expression of the CAG-containing antisense transcript in corneal endothelium.

The sequence context of microsatellite repeats seems to have a central role in RAN translation; hence, it was essential to show that CTG-CAG repeats embedded in the third intron of TCF4 are also translated via non-ATG initiation. CTG-CAG repeats in the context of the TCF4 gene, cloned under the control of the CMV promoter and in frame with a 3xFLAG C-terminal tag, could be translated in transfected cells without an initiating ATG from the polyC and polyQ frames (Fig. 3). Some RAN translation products are known to have poor solubility and peculiar mobility in SDS-polyacrylamide gels, but we were unable to detect any reactive polypeptides with the anti-FLAG and anti-AF antibodies in cells transfected with the A99 and A175 constructs, in either the soluble or insoluble fraction of these cell lysates (Fig. 3A, data not shown). The polyC frame contains an ATG downstream of the repeats, but we showed that an M to K mutation for this residue did not prevent the translation of a polypeptide identified by anti-FLAG antibodies (Fig. 4C). We tried to force the expression of a polyC-containing polypeptide by introducing an ATG upstream of the repeats, but initiation by RAN translation was preferred, despite a strong Kozak sequence (Fig. 4C). Interestingly, an anti-FLAG-reactive polypeptide was detectable when 29 repeats were present, but at a much lower level (Fig. 4B). This observation, together with the larger size (~15 kDa) of the polypeptide in the C220 extract compared to the C112 extract, points to the size of the translation product being dependent on repeat length and suggests a repeat length threshold in RAN translation, as previously reported. We also detected an anti-FLAG-reactive polypeptide and some high molecular weight species for the polyS frame (Fig. 3D), but due to the presence of an ATG in this ORF downstream of the repeats, we could not unequivocally demonstrate that these

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<tr>
<th>Sample</th>
<th>Repeat Size</th>
<th>ATG Location</th>
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<tr>
<td>FECD 2011-338</td>
<td>18,88 CTG-CAG</td>
<td>polyC</td>
</tr>
<tr>
<td>FECD 2011-506</td>
<td>15,55 CTG-CAG</td>
<td>polyC</td>
</tr>
<tr>
<td>FECD 2011-491</td>
<td>12,12 CTG-CAG</td>
<td>polyC</td>
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<tr>
<td>Unaffected</td>
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**Figure 7.** Evidence for RAN translation in corneal endothelium of patients with FECD. Corneal endothelium from 3 patients with FECD (2 with CTG-CAG repeat expansions) and 1 unaffected individual were collected during corneal transplantation surgery or obtained from the Minnesota Lyons Eye Bank and probed by IF with anti-CF2 antibodies (green) and anti-ZO1 antibodies (red). Nuclei were counterstained with DAPI (blue). Sample repeat size on TCF4 alleles, as measured in blood samples, are indicated when known. Bottom panels show higher magnification images for samples 2011-338, 2011-506, and unaffected. Scale bar: 50 μm.
polypeptides were produced by RAN translation, although their mobility argues for the latter.

In other TNR diseases, RAN translation polypeptides cause toxicity when transfected into human cells,\textsuperscript{1,10,14,20,25} and we found this to also be the case for RAN translation products derived from the TCF4 gene. In fact, the S117 construct induced cytotoxicity (as measured by LDH production) and oxidative stress in a corneal endothelial cell line (HCEnc-21T), but, remarkably, not in HEK-293T cells (Fig. 6). This difference could be attributed to the effect of flanking sequences or promoter strength in the two cell lines or to the presence of compensating mechanisms in one cell line versus the other. As in other microsatellite disorders, FECD pathology is limited to a specific tissue,\textsuperscript{51} and studying the differential effect of RAN translation products in different cell types could further the understanding of FECD pathophysiology. A cell proliferation assay (MTS assay) also showed a length-dependent decrease in cell number for HCEnc-21T cells transfected with all of our TCF4 constructs (Fig. 6C). The MTS assay showed an effect on cell viability for the polyA and polyC constructs that did not emerge from the LDH assay. This represents only an apparent inconsistency, since the LDH and MTS assays are indirect measurements of two related but separate factors, cytotoxicity and cell proliferation, respectively. As is the case for other microsatellite diseases,\textsuperscript{31,52} it is not surprising that not all reading frames are equally expressed and equally toxic.

The detection of a polyQ-containing polypeptide with the IC2 antibody, although identified only in FECD fibroblasts homozygous for the repeat expansion, does not prove that this species is translated from the TCF4 antisense transcript. Previous studies in TNR diseases have used antibodies raised against the C-terminus of putative RAN translation products to demonstrate the presence of such polypeptides in the affected tissue of patients.\textsuperscript{14,21,29} Using this strategy, we identified 1 out of 5 antibodies raised against peptides in the C-terminus of putative TCF4 RAN translation polypeptides as suitable to recognize RAN translation products by IF. When corneal endothelial tissue from patients with FECD were probed with this anti-IC2 antibody, we detected a clear nuclear staining that was specific to cornea from patients with FECD and a CTG-CAG repeat expansion compared to tissue from a patient with FECD with repeat size in the normal range and healthy cornea (Fig. 7, Supplementary Fig. S5).

Evidence for RAN translation in FECD opens the field to numerous lines of investigation. It will be important to demonstrate whether homopolymeric peptides identified in FECD samples are the product of RAN translation or a combination of RAN translation and canonical translation, as is the case for other TNR disorders.\textsuperscript{16,24,31} In FECD, the occurrence of major mis-splicing events\textsuperscript{18,19} raises the question of whether and how RAN translation products may contribute to FECD pathology and whether an interplay exists between RAN translation species, RNA mis-splicing, and quality control of RNA by RNA binding proteins, similar to what happens in DM2 and SCA31.\textsuperscript{20,25} In other microsatellite diseases, there is abundant evidence for RAN translation-mediated pathogenicity,\textsuperscript{17} but also some interesting data hinting at a protective role of some RAN translation products.\textsuperscript{33} Whether this is the case for FECD remains to be determined.

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