SHORT REPORT

A recurrent polyalanine expansion in the transcription factor FOXL2 induces extensive nuclear and cytoplasmic protein aggregation

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Blepharophimosis syndrome is an autosomal dominant disease characterised by eyelid malformations, associated or not with premature ovarian failure. It is caused by mutations in the *FOXL2* gene, which encodes a forkhead transcription factor containing a polyalanine (polyAla) domain of 14 alanines. Expansions of the polyAla tract from 14 to 24 residues account for 30% of the reported mutations and lead mainly to isolated palpebral defects. We have transfected COS-7 cells with DNA constructs driving the expression of the wildtype and mutant *FOXL2* proteins fused to the green fluorescent protein. The polyAla expansion was found to induce the formation of intranuclear aggregates and a mislocalisation of the protein due to extensive cytoplasmic aggregation. These findings were confirmed by immunofluorescence. Co-transfection experiments suggest that the wildtype and mutant proteins can co-aggregate. We propose that the mechanism for the molecular pathogenesis of the polyAla expansions of *FOXL2* may be its mislocalisation concomitant with its inclusion into nuclear aggregates. This may diminish the pool of active protein. Potential effects of aggregation on cell viability are under study.

Mutations of *FOXL2*, a forkhead transcription factor gene, are responsible for the blepharophimosis ptosis epicanthus inversus syndrome (BPES, MIM 110100), an autosomal dominant genetic disease characterised by eyelid malformations, associated or not with premature ovarian failure (BPES type I or II, respectively).^{1–3} This transcription factor contains a polyalanine (polyAla) domain strictly conserved in mammals.^{4,5} Conservation of the length of the polyAla tract suggests the existence of structural or functional constraints that impose a threshold length beyond which deleterious effects may appear.^{4,5} Expansions of the polyAla tract from 14 to 24 alanines account for 30% of the reported *FOXL2* mutations and lead mainly to BPES type II.³ To date, four different *FOXL2* mutations resulting in polyAla expansions have been reported (details at the human *FOXL2* mutation database at <http://medgen.ugent.be/foxl2>; see also Beysen *et al*).⁶ The most frequent of these mutations is the recurrent 30 bp duplication g.909_938dup (c.672_701dup; p.A224_A234dup).³

Apart from *FOXL2*, there are at least eight other genes in which polyAla expansions exceeding a critical threshold have been shown to cause human disease: mutations of *HOXD13* in synpolydactyly, *RUNX2* in cleidocranial dysplasia, *ZIC2* in holoprosencephaly, *HOXA13* in hand foot genital syndrome, *ARX* in X linked mental retardation, *SOX3* in X linked mental retardation with growth hormone deficiency, *PHOX2B* in congenital central hypoventilation syndrome, and *PABPN1* in

oculopharyngeal muscular dystrophy (OPMD). All these genes except the last encode transcription factors. Indeed, OPMD is a dominant disorder characterised by dysphagia and ptosis of the eyelids and caused by polyAla expansions in the poly(A)-binding protein nuclear 1 (PABPN1). In general, the presumed effect of the polyAla expansions varies from complete or partial loss of function to a dominant negative effect (DNE). So far, *HOXD13* is the only transcription factor where genetic evidence suggests that alanine tract expansions may exert a DNE.⁷

Proteinopathies or protein conformational disorders, such as Alzheimer's disease, Huntington's disease, and Parkinson's disease, are associated with protein misfolding and aggregation.⁸ These abnormal protein deposits induce a degenerative process. A typical group consists of inherited neurodegenerative diseases caused by expansions of CAG/glutamine repeats (reviewed in Michalik and Van Broeckhoven⁹) that lead to the formation of intranuclear and cytoplasmic inclusion bodies. A model has been proposed in which polyglutamine expansions confer a toxic gain of function of the protein.¹⁰ Alternatively, but in our opinion not exclusively, aggregates would be pathogenic due to a sequestering of other polyglutamine containing proteins (negative transdominance).¹¹ Bao *et al*¹² and Abu-Baker *et al*¹³ have shown that the polyAla expansions of PABPN1 in OPMD lead to intranuclear inclusions in skeletal muscles.

In previous studies, we have shown that *FOXL2* is strongly expressed in the fetal primordial mesenchyma of developing eyelids and peri-ocular muscles as well as in fetal and adult ovaries.^{4,5} Based on the observations made for polyAla expansions in OPMD, we hypothesised that the polyAla expansion in *FOXL2* may also lead to intracellular protein aggregation that might contribute to abnormal development of eyelids and peri-ocular muscles in BPES patients. To study the effects of the *FOXL2* polyAla expansion, we have undertaken localisation studies in COS-7 cells, a well known cellular model for aggregation of proteins bearing low complexity tracts (that is, polyGlu and polyAla).^{12,13}

METHODS

Expression constructs

The cDNA encoding the *FOXL2* open reading frame (ORF) with 14 alanines (*FOXL2*-Ala14, wildtype) and *FOXL2* with 24 alanines (*FOXL2*-Ala24, mutant g.909_938dup) was amplified by PCR from human normal and mutated genomic DNA, respectively. This was possible as *FOXL2* is intronless.

Abbreviations: BPES, blepharophimosis ptosis epicanthus inversus syndrome; DMEM, Dulbecco's modified Eagle's medium; DNE, dominant negative effect; FCS, fetal calf serum; GFP, green fluorescent protein; OPMD, oculopharyngeal muscular dystrophy; ORF, open reading frame; PABPN1, poly(A)-binding protein nuclear 1; PBS, phosphate buffered saline

The normal and mutant ORFs were cloned into two different pcDNA3.1-topoTA cloning vectors (Invitrogen, CA, USA), one to express FOXL2 ORFs alone and the other to produce fusion proteins with the green fluorescent protein (GFP) on the C terminus of FOXL2. Transcription in both vectors is driven by a CMV promoter. All constructs were sequenced to exclude the presence of Taq polymerase induced mutations. Construct DNA concentrations were determined spectrophotometrically and corroborated by agarose gel electrophoresis.

Cell culture and transfection

African green monkey kidney COS-7 cells were seeded 24 h before transfection in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, CA, USA) containing 10% fetal calf serum (FCS; Gibco) at a concentration of 35 000 cells per well in 24 well plates containing sterile cover slips. The cells were transfected with varying concentrations of plasmid DNA using the calcium phosphate method and rinsed 24 h after transfection.¹⁴ For co-transfections, 500 ng of pcDNA-FOXL2-Ala14-GFP were transfected with 500 ng to 1 µg of FOXL2-Ala14 or FOXL2-Ala24 non-GFP constructs. Then 48 h after transfection, cells were washed with phosphate buffered saline (PBS) and fixed for 15 min with 4% paraformaldehyde. Nuclei were stained with Hoechst reagent (diluted 1/500) and coverslips were mounted on slides using fluorescence mounting medium (DAKO, CA, USA). Transfected cells were visualised using standard (Nikon E600) or confocal (Leica TCS SP2) fluorescence microscopy.

Immunofluorescence

COS-7 cells were transfected as above, using the FOXL2-Ala14 and FOXL2-Ala24 non-GFP constructs. Then 48 h after transfection, cells were washed with PBS, fixed for 15 min with 4% paraformaldehyde, permeabilised for 15 min with PBS/0.1%Triton-X/10%FCS, and then blocked with 5% non-fat milk in PBS/0.1%Tween 20. Cells were incubated overnight in primary anti-FOXL2 antibody (diluted 1/500). This antibody has been produced and characterised in our laboratory.⁴ Cells were washed three times in PBS, and incubated for 1 h in a secondary FITC conjugated anti-rabbit IgG antibody (diluted 1/500). Coverslips were mounted on slides using fluorescence mounting medium (DAKO). Cells were visualised using appropriate filters on a Nikon E600 microscope.

Quantification of intracellular aggregate formation and statistical analysis

Aggregate formation was assessed using standard and confocal fluorescence microscopy. One hundred to 600 GFP positive COS-7 cells were selected from at least three different transfection experiments and the proportion of cells with cytoplasmic or intranuclear aggregates was counted. In the case of co-transfections, to increase the power of the statistical conclusions, more than 1000 cells were scored. Aggregation scores, blind or not, were statistically similar given the striking differences in the appearance of aggregated and non-aggregated GFP fusion proteins. To estimate the significance of the differences between means, two statistical tests were used: (i) the parametric Student's *t* test; and (ii) a randomisation test (distribution free, which is very robust). The randomisation test creates pairs of groups by assigning the original values (from the two real groups in comparison) at random. Then the difference in their means is determined and the process is repeated 10 000 times. The *p* value is estimated as the number of times the difference between the means of the two random groups is equal to or higher than that observed with the original groups, divided by the number of iterations (10 000). We report the *p* values (both

methods in agreement) according to the traditional critical levels of significance.

RESULTS AND DISCUSSION

In single transfection experiments of COS-7 cells with amounts of transfecting DNA between 100 and 500 ng/culture well, almost all transfected cells displayed intranuclear fluorescence for both the normal and mutant constructs. Intranuclear fluorescence due to the Ala14-GFP fusion protein was not homogeneous (for instance, the nucleoli were not stained), but punctate intranuclear aggregates were detected in only 6.9% of the cells. In contrast, expression of the Ala24-GFP fusion protein led to the presence of intranuclear punctate and huge aggregates in more than 30% of the scored nuclei (fig 1A and B). However, the most prominent hallmark of the expression of Ala24-GFP was the strong cytoplasmic fluorescence in more than 85% of cells, most frequently in the form of aggregates (>80%; detailed statistics are given in fig 2). For comparison, less than 3% of cells transfected with the Ala14-GFP construct displayed a cytoplasmic signal. The fluorescence of the Ala24-GFP protein appeared either diffusely spread throughout the cytoplasm, as micro-aggregates punctuating the cytoplasm, and/or as large aggregates (fig 1C, D, and E). This diversity of staining patterns might be explained by differences in DNA uptake in different cell subpopulations. Although homogeneous in principle, cell populations grow asynchronously.

At the highest amount of transfecting DNA tested (1 µg/well), the Ala14-GFP fusion protein still displayed essentially nuclear fluorescence, and intranuclear aggregation was detected in less than 12% of analysed cells. In contrast, the Ala24-GFP FOXL2 induced intranuclear aggregation in about 30% of the nuclei. More remarkably, a dramatic aggregated fluorescence appeared in the cytoplasm of most transfected cells. The proportions of cytoplasmic aggregates (Ala24-GFP) were statistically similar for amounts of DNA ranging from 250 to 1000 ng/well, but the proportion of intensely stained cells increased with the amount of transfecting DNA. This is in contrast with results obtained in PAPBN1, where at high DNA concentrations the intranuclear aggregation level was similar for the normal and mutant GFP fusion proteins.¹²

The aggregation of Ala24-GFP, which is supposed to start in the cytoplasm, was so strong that nuclear import was often hampered and most of the protein concentrated in the cytosol, leaving the nuclei essentially devoid of fluorescence (fig 1C). These data suggest that FOXL2 Ala24 has a much lower concentration threshold for aggregation than the normal Ala14 in both the nucleus and the cytoplasm. Saturation of a nuclear import mechanism, leading to a persistent high cytoplasmic concentration of the recombinant proteins, may also play a role. In such a case, the Ala14-GFP protein remains soluble when being translocated to the nucleus, while Ala24-GFP may reach the aggregation threshold in the cytoplasm. It is known that many transcription factors shuttle between the nucleus and the cytoplasm.¹⁵ One can also consider that at low expression levels, the Ala24 protein may enter the nucleus and aggregate. In such conditions, it might be unable to return to the cytoplasm.

In some instances, detected over the entire range of amounts of Ala24-GFP DNA tested, the nucleus had a green signal but appeared "capped" by aggregates located apparently in the cytoplasm at one side of the nucleus (fig 1E). This suggests the existence of a vectorial protein transport flux along which aggregation occurs preferentially, due to higher local protein concentrations. One may argue that the intense cytoplasmic aggregation in a period of 48 h for the Ala24-GFP construct is an artefact of over-expression. A possible counterargument is that COS7 cells transfected with comparable amounts of DNA of a polyAla expanded PAPBN1 (same

Figure 1 Aggregation of polyAla expanded FOXL2 (Ala24-GFP) in transfected COS-7 cells. Left column: Hoescht staining of nuclei; middle: GFP fusion proteins/FITC; and right: merge. (A) Non-homogeneous, but not aggregated, nuclear fluorescence obtained with Ala14-GFP (insert). Nuclear and cytoplasmic aggregation induced by Ala24-GFP. (B) Punctate nuclear aggregates induced by Ala24-GFP. (C) Examples of large aggregates and near nuclear exclusion (weak nuclear fluorescence). (D) Punctate cytoplasmic aggregates induced by Ala24-GFP. A non-fluorescent region was often observed close to bean shaped nuclei. (E) Example of asymmetric localisation of aggregates ("capped nucleus"). (F) Immunodetection of transfected FOXL2 (Ala14 in the insert) and Ala24 (non-GFP). Panels A, B, D, E: confocal microphotographs. Panels C, F: epifluorescence microphotographs. Scale bars indicate 20 µm in all panels.

promoter) showed only a low level of cytoplasmic aggregation.¹³ It is conceivable that in natural mutants aggregates might form first in the nucleus, as most of the protein will concentrate in this compartment, which corresponds to less than 5% of the cell volume. Cytoplasmic aggregates may appear over time via the accretion of micro-aggregates which cannot be resuspended or digested by the cellular machinery. Unfortunately, peri-ocular tissue excised during surgical correction of the eyelids in BPES patients carrying the polyAla expansion is currently not available to assess in vivo

if there is aggregated FOXL2 in the hypotrophic tissues that remain after the degenerative process.

To rule out a possible artefact of aggregation induced by the GFP in the fusion proteins, we performed similar transfection experiments with constructs driving the expression of the Ala14 and Ala24 ORFs without the GFP. Subsequent detection of FOXL2 was carried out with an antibody directed against the C terminus of FOXL2 which we have described elsewhere.^{4,5} According to our expectations, the immuno-fluorescence results agreed with the results for the fusion proteins (fig 1F). Thus, we conclude that the primary effects of the polyAla expansion of FOXL2 are nuclear aggregation and the mislocalisation of this transcription factor by its aggregation in the cytoplasm. Indeed, a previous study has suggested that the mislocalisation of the forkhead transcription factor FOXC1 is the pathogenic basis of its mutations. Specifically, several missense mutations affecting a putative nuclear localisation signal were shown to induce a cytoplasmic accumulation of the mutant proteins.¹⁶

It is interesting to note that a normal polyAla protein may aggregate in natural conditions. For instance, PABPN1 forms dynamic aggregates in magnocellular neurosecretory neurons of the hypothalamus, which change according to different physiological conditions.¹⁷ We have previously demonstrated that polyAla containing proteins tend to be transcription factors and suggested that the polyAla tract might help concentrate these proteins in nuclear subcompartments.^{18–20} This can also be the case for FOXL2 in spite of the low aggregation level of the normal protein coupled to the GFP. Indeed, the existence of "physiological" micro-aggregates cannot be ruled out, as they might be undetected at the resolution level used in this study (light microscopy).

The reported tendency to aggregation of polyAla containing proteins in pathological conditions could induce: (i) a toxic gain of function; (ii) non-toxic aggregation of the mutant protein (concomitant with its mislocalisation); and/or (iii) a DNE. In addition to the demonstration of FOXL2-Ala24 mislocalisation, we have explored the third scenario, presuming that co-existence in the cell of the normal and the polyAla expanded proteins leads to partial sequestration of the normal protein by the mutant protein. Thus, we co-transfected COS-7 cells with a fixed amount of reporter Ala14-GFP and increasing amounts of either Ala14 (non-GFP) or Ala24 (non-GFP) constructs. At equimolarity of transfecting DNAs, the fluorescent reporter was more frequently present in intranuclear aggregates in the co-transfection with the Ala24 (non-GFP) construct than in the co-transfection with the normal construct (fig 3). A dose-response graph for the same amount of reporter construct and increasing amounts of non-GFP-Ala14 or non-GFP-Ala24 constructs showed that this trend was valid for other ratios (fig 3). This might simply reflect the intrinsically higher capacity of the mutant protein to form aggregates retaining a small fraction of reporter. Even so, it is clear that the Ala24 protein is able to interact with the normal protein.

We carried out linear regression and correlation analyses of the mean intranuclear aggregate percentage over the amount of non-GFP transfecting DNA (everything else being equal). Both correlations were significant (fig 3). Interestingly, the correlations between the amounts of transfecting DNA and the percentage of cells presenting intranuclear aggregates in simple transfections were significant for the Ala14-GFP ($R = 0.6$, $p < 0.01$) but not for Ala24-GFP ($R = 0.3$, $p = 0.3$). This suggests that the significant correlation obtained in co-transfections of Ala14-GFP and Ala24 (non-GFP) is driven by the presence of the normal protein in the aggregates, beyond a mere "colouring" effect. It is conceivable that the mutant protein interferes with the normal protein in other ways, such as retention in the cytoplasm. If so, the effect was not

Figure 2 Fraction (%) of transfected cells displaying intranuclear and cytoplasmic aggregates. The wildtype fusion protein (Ala14-GFP) is represented by grey bars (with standard deviations). The mutant Ala24-GFP is represented by black bars. Statistical significance for pairwise comparisons (Ala14 v Ala24) is represented by * for $p < 0.05$ and by ** for $p < 0.01$.

binary (presence or absence of cytoplasmic fluorescence or aggregates) which renders scoring difficult (that is, amount or intensity of the aggregates), which is why we concentrated our analysis on intranuclear co-aggregation.

Taken together, the aforementioned data suggest the existence of intranuclear interactions between the normal and mutant FOXL2 proteins (that is, the normal protein tends to co-aggregate in a stronger way with the mutant protein). For HOXD13, there is genetic evidence that an alanine tract expansion induces a DNE. Basically, co-existence of a polyAla-expanded Hoxd13 with a normal protein in the mouse leads to a more severe phenotype than that of a heterozygous null mutation (see review in Brown and Brown⁷). In the case of FOXL2, there is no apparent difference in the ocular phenotype resulting from loss of function mutations and polyAla expansions in a heterozygous state. This is compatible with our molecular results if: (i) a heterozygous null allele already leads to the most severe

Figure 3 FOXL2-Ala14 interacts with Ala24 in the nucleus. COS-7 cells were co-transfected with 500 ng/well of reporter Ala14-GFP and increasing amounts of Ala14 or Ala24 non-GFP constructs. Statistically significant differences of the mean percentage of cells with intranuclear aggregates ($p < 0.05$, $***p < 0.001$) were obtained for pair-wise comparisons (that is, 500 ng Ala14-GFP and the same amount of either Ala14 or Ala24 non-GFP). Notice that the condition "500 ng Ala14-GFP+500 ng Ala14 non-GFP" is in principle similar to a single transfection with 1 μ g Ala14-GFP (as displayed in fig 2). Indeed, the mean percentages of intranuclear aggregates in both experiments were statistically similar. The regression lines of the amount of Ala14 and Ala24 non-GFP constructs versus the mean percentage of cell with intranuclear aggregates are represented (Pearson correlation coefficient R, equations, and p values of R are displayed).

eyelid phenotype, so that a DNE due to the polyAla expansion cannot have a higher expressivity; or (ii) there is sequestering of a fraction of the wild type protein by the mutant while a proportion of the latter remains active and partially compensates for the sequestered normal protein. In the latter case, total FOXL2 transcriptional activity may attain a threshold which is not sufficient to ensure normal eyelid development but which does not lead to the most severe phenotype either. In the ovary, the phenotype due to the polyAla expansions is milder. Indeed, of the 48 female BPES cases (including prepubertal cases) in whom polyAla expansions in FOXL2 have been identified so far, only three have a concomitant mild ovarian phenotype with preservation of reproductive capacity as these affected females have offspring. Unfortunately, we cannot estimate the proportion of polyAla mutations associated with an ovarian phenotype because detailed clinical information is lacking for some patients (see supplementary table available at <http://jmg.bmjournals.com/supplemental/>). The differential phenotypic effects of the mutant protein (that is, eyelid versus ovary) may be explained by the existence of different aggregation or functional thresholds in the eyelid mesenchyma and in the follicular cells. This may depend on the presence of different co-aggregation partners according to the tissue. For instance, it is known that nuclear inclusions generated by a polyglutamine expansion in ataxin-3 recruit other glutamine-repeat proteins, which are probably specific to the tissues where aggregation takes place.¹¹ It has also been shown that the polyAla aggregates in OPMD contain chaperones and subunits of the proteasome.^{12,13} The function of chaperones in this context is to properly refold the aggregated polypeptides. A higher chaperone activity in the ovary may explain a decreased tendency of Ala24-FOXL2 to aggregation, leading to a milder disease phenotype. One can not exclude the effect of tissue specific post-translational modifications.

In summary, we demonstrate that the most recurrent polyAla expansion of the transcription factor FOXL2 leads to a shift in protein localisation from the nucleus to the cytoplasm and to cytoplasmic and nuclear aggregation. This study shows a potential mechanism of molecular pathogenesis due to aggregation of a polyAla expanded transcription factor, yet other topics, such as the impact of aggregation on cell death, deserve exploration.^{12,21,22} Further studies, involving other genes in which expansion of alanine tract occurs, are required to demonstrate if the mechanism proposed here is a general one.

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ELECTRONIC-DATABASE INFORMATION

The human *FOXL2* mutation database can be found at <http://medgen.ugent.be/foxl2/>; the supplementary table is available at <http://jmg.bmijournals.com/supplemental/>.

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