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The small co-chaperone p23 overexpressing transgenic mouse

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HIGHLIGHTS

- We report the generation of transgenic mouse lines that overexpress p23 (p23wt) or p23unc (p23D142N) under the control of a ROSA promoter.
- No discernable abnormality in the appearance or behavior was evident in the p23wt or p23unc mice.
- Immunohistochemical analysis revealed protein staining in several areas of the brain.
- The mice will prove useful for studying the role of p23 and/or uncleavable p23 in cellular stress-induced cell death.

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ABSTRACT

Studies from multiple laboratories have identified the roles of several ER stress-induced cell death modulators and effectors. Earlier, we described the role of p23 a small co-chaperone protein in preventing ER stress-induced cell death. p23 is cleaved by caspases at D142 to yield p19 (a 19 kDa product) during ER stress-induced cell death. Mutation of the caspase cleavage site not only blocks formation of the 19 kDa product but also attenuates the cell death process triggered by various ER stressors. Thus, uncleavable p23 (p23D142N) emerges as a reasonable candidate to test for potential inhibition of neurodegenerative disease phenotype that features misfolded proteins and ER stress. In the present work we report the generation of transgenic mouse lines that overexpress wild-type p23 or uncleavable p23 under the control of a ROSA promoter. These mice should prove useful for studying the role of p23 and/or uncleavable p23 in cellular stress-induced cell death.

1. Introduction

Cell death pathways triggered by misfolded proteins and other activators of endoplasmic reticulum (ER) stress display both cytochrome c/Apaf-1 independent and cytochrome c/Apaf-1 dependent activation of programmed cell death (pcd) (Di Sano et al., 2006; Morishima et al., 2002; Rao et al., 2001, 2002; Zhang and Armstrong, 2006). Studies from multiple laboratories have described the roles of several ER stress-induced cell death modulators and effectors (Breckenridge et al., 2003; Bredesen et al., 2006; Rao et al., 2004; Xu et al., 2005) through the use of biochemical, pharmacological and genetic tools. We recently identified p23, a small chaperone protein that participates in ER stress-induced cell death as a key player in the coupling of ER stress to programmed cell death (Chinta et al., 2008; Poksay et al., 2012; Rao et al., 2006). p23 modulates HSP90 chaperone activity by coupling the ATPase activity of HSP90 to polypeptide dissociation. However, we found that a subpopulation of p23 that is not bound to HSP90 plays a key role in ER stress-induced apoptosis (Chinta et al., 2008; Poksay et al., 2012; Rao et al., 2006). In vitro, p23 is able to bind specifically to misfolded proteins and the chaperone activity of p23 prevents aggregation and accumulation of misfolded proteins. Using several complementary approaches including immunodepletion and down-regulating the expression of p23 by RNA interference resulted in enhanced ER stress-induced apoptosis, suggesting a role for p23 as an anti-apoptotic protein (Chinta et al., 2008; Poksay et al., 2012; Rao et al., 2006). p23 is cleaved by caspases to yield a p19 fragment during ER stress-induced cell death and blockage of the caspase cleavage site of p23 is associated with decreased cell death triggered by various ER stressors (Chinta et al., 2008; Poksay et al., 2012; Rao et al.,

Abbreviations: ER, endoplasmic reticulum; pcd, programmed cell death; Tg, transgenic.

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2006). Thus, uncleavable p23 (p23D142N; p23unc) emerges as a reasonable candidate to test for potential inhibition of any cellular stress leading to pcd or neurodegenerative disease phenotype that features misfolded protein and/or ER stress. The potential ameliorative effect may occur because of the inability of p23D142N to be cleaved by caspases during ER stress.

Here we describe the production of transgenic (Tg) mice that overexpress p23wt (wild-type p23) or the p23unc. No gross physical or pathological symptoms have been observed in either of the Tg p23 mice colonies. The availability of genetically modified mice expressing p23wt or p23unc will facilitate studies on their function under normal and pathological conditions.

2. Materials and methods

2.1. Constructs and genotyping

All molecular biology reactions utilized enzymes and reagents from New England BioLabs unless otherwise noted. N-terminal human *Flag-p23* was subcloned into a pcDNA3 expression vector (Invitrogen) by PCR-amplification of *p23* using a 5′-*p23* FLAG BamH1 and a 3′-*p23* Not1 primer pairs (Rao et al., 2006). The amplified N-terminal *FLAG-p23* cDNA (p23wt) was excised using BamH1 and Not 1 restriction enzymes and ligated into the pcDNA3 expression vector. Using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), *FLAG-p23unc* (p23D142N) mutant was generated (Rao et al., 2006). The sequences of all constructs were confirmed by DNA sequencing.

The cDNAs encoding the *p23wt* and *p23unc* were digested with NotI, blunted by the 5′→3′ polymerase activity of Phusion polymerase, digested by BamHI, gel purified (Qiagen) and ligated into a pBroad (Invivogen) vector preparation previously digested with EcoRI, blunted, and digested with BglII in a directional cloning scheme. Resulting clones for both *pBroad-p23wt* and *pBroad-p23unc* were confirmed by DNA sequencing.

Transgenic mice were produced by standard techniques (Hogan B, Costantini F, Lacy E. Manipulating the Mouse Embryo: A Laboratory Manual, 1986) by the Institute's Transgenic Core. Purified linearized DNA (1–2 ng/μl) was injected into the pronuclei of fertilized oocytes derived from C57BL/6 × C57BL/6 mice. 179 *p23wt* and 178 *p23unc* injected oocytes were transferred to oviducts of pseudopregnant female mice that resulted in 26 mice and 29 mice, respectively. Tail DNA from resulting mice was isolated using a DNeasy Tissue Kit (Qiagen). Genotyping of *p23wt* and *p23unc* mice were by PCR and included the primers *p23*-5′ (5′-CAGTTGTCTCGGAGGAAGTGAT-3′) and *BGPA*-3′ (5′-CAGATGCTCAAGGCCCTTCATA-3′). The reaction products were electrophoresed on a 1.5% agarose gel with ethidium bromide. Five *p23wt* and three *p23unc* transgenic founder mice were crossbred with the C57BL/6 mice to obtain transgenic lines.

2.2. Antibodies and Western blotting

Expression of transgene was confirmed by Western blot analysis. Dissected and frozen mouse hemi-brains were homogenized in ice-cold phosphate-buffered saline lysis buffer containing 0.05% Nonidet P-40, 0.25% sodium deoxycholate, 50 mmol/L Tris–HCl (pH 8.5), 100 nmol/L NaCl, 1 mmol/L EDTA (pH 8.0), complete mini cocktail protease inhibitor (Roche), and 2 mg/mL b-glycerol phosphate (Galvan et al., 2006). Samples were then centrifuged at 16,000 × *g* for 10 min at 4 °C and the resulting supernatant assayed for total protein concentration. 50–100 μg of protein from total extracts was used for SDS- PAGE and Western blot analyses as described earlier (Rao et al., 2001, 2002, 2004, 2006). Proteins were detected using FLAG antibody (1:1000, Sigma) to differentiate the p23wt or p23unc from the endogenous mouse p23. Since the ROSA promoter directs the expression of the transgene ubiquitously in all tissues, expression of the transgene was also checked in other tissues including liver and heart. Equal protein loading was confirmed by reprobing blots with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) monoclonal antibody (1:50,000, Research Diagnostics). Protein levels between different lines were quantitated by densitometry.

2.3. Immunohistochemistry

Hemi-brains from transgenic and non-transgenic littermate mice were removed and snap frozen on powdered dry ice. Coronal cryosections (14 μm) were cut, mounted on glass slides, air-dried, and post-fixed for 30 min in 4% paraformaldehyde. A relatively short fixation was necessary to preserve FLAG antigenicity. Sections were then washed in PBS followed by PBST (phosphate buffered saline, 0.05% Tween 20). Non-specific antibody binding was blocked with 5% normal goat serum (NGS) in PBST for 1 h at room temperature. The primary antibody, rabbit anti-FLAG (F7425, Sigma) was used at 1:200 in PBST; sections were incubated overnight at 4 °C. After 2 washes in PBST, sections were incubated with goat anti-rabbit Alexafluor 488 at 1:300 in PBST for 1 h at room temperature. After washes in PBST and PBS, sections were mounted with Prolong Gold (Invitrogen) and glass coverslips, and viewed under a Nikon FM microscope. Images were taken using Act 1 software at 40×. Images were taken in the visual cortex, striatum, hippocampus, and thalamus. As lipofucsin that could be mistaken for FLAG labeling was readily visible using either the red-orange or green filters, only cells with little or no lipofucsin were imaged.

3. Results

3.1. Production of p23wt and p23unc transgenic mice

Initially we generated the *p23wt* mice using a CMV promoter. Despite analyzing several lines, we failed to detect the p23 transgene or the expression of p23 protein by Western blotting. Hence we used the mouse ROSA-26 promoter, a native ubiquitous promoter that directs the expression of the transgene ubiquitously throughout embryonic development and in adult tissues (Fig. 1). The ROSA promoter has been successfully used by a number of laboratories to create transgenic mice (Belteki et al., 2005; Kisseberth

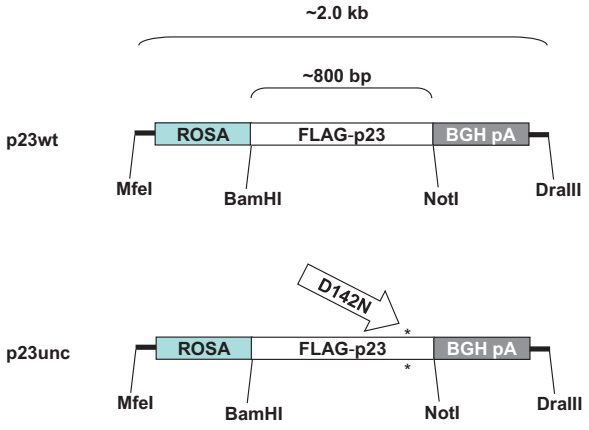

Fig. 1. Schematic diagram of the constructs used for generating the transgenic mice. N-terminal Flag-*p23* (human) and Flag-*p23*D142N were each subcloned into a pcDNA3 expression vector (driven by the ROSA promoter) by PCR-amplification of each construct. Purified linearized DNA (1–2 ng/μl) was injected into the pronuclei of fertilized oocytes derived from C57BL/6 × C57BL/6 mice. WTp23 (*p23wt)* and p23D142N (*p23unc*) transgenic mice were produced by standard techniques as mentioned in Section 2.