



Apoptosis gene profiling reveals spatio-temporal regulated expression of the p53/Mdm2 pathway during lens development[☆]

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ABSTRACT

Evidence is emerging for apoptosis gene expression in the lens during development. Therefore, here we used a filter array to assess expression of 243 apoptosis-related genes in the developing postnatal mouse lens using ³³P labelled cDNA synthesized from p7 and p14 mouse lenses. We demonstrated that 161 apoptosis-related genes were expressed at levels significantly above background and 20 genes were potentially significantly differentially expressed ($P < 0.05$) by at least 2-fold between p7 and p14. We used RT-PCR to confirm expression of these genes in newborn, p7, p14 and 4 wk mouse lens cDNA samples. Expression of 19/20 of the genes examined was confirmed, while 5 genes (*Huntingtin*, *Mdm2*, *Dffa*, *galectin-3* and *Mcl-1*) were confirmed as differentially regulated between p7 and p14. RT-PCR was also used to examine the expression of the chick homologues of the most-highly expressed and/or potentially differentially regulated genes in chick embryo lenses at E6–E16. The majority of genes expressed in the postnatal mouse lens were also expressed in the chick embryo lens. Western blotting confirmed developmentally regulated expression of Axl and Mcl-1 during mouse lens development and of Mdm2, Mdm4/X and p53 during mouse and chick lens development. Western blotting also revealed the presence of p53 and Mdm4/X splice variants and/or proteolytic cleavage products in the developing lens. Since Mdm2 is a regulator of the tumour suppressor gene p53, we chose to thoroughly investigate the spatio-temporal expression patterns of p53, Mdm2 and the functionally related Mdm4/X in mouse lens development at E12.5–E16.5 using immunocytochemistry. We also examined Mdm2 expression patterns during chick lens development at E6–E16 and Mdm4/X and p53 at E14. Expression of Mdm2, Mdm4/X and p53 was spatio-temporally regulated in various compartments of the developing lens in both mouse and chick, including lens epithelial and lens fibre cells, indicating potential roles for these factors in regulation of lens epithelial cell proliferation and/or lens fibre cell differentiation. This study provides a thorough initial analysis of apoptosis gene expression in the postnatal mouse lens and provides a resource for further investigation of the roles in lens development of the apoptosis genes identified. Furthermore, building on the array studies, we present the first spatio-temporal analysis of expression of p53 pathway molecules (p53, Mdm2 and Mdm4/X) in both developing mouse and chick lenses, suggesting a potential role for the p53/Mdm2 pathway in lens development, which merits further functional analysis.

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1. Introduction

Lens development occurs throughout the lifetime of the individual and involves the terminal differentiation of lens epithelial cells into lens fibre cells (Piatigorsky, 1981; Wride, 1996). This process begins during embryogenesis and continues, albeit at a slower rate, into adulthood and old age. A number of characteristic morphological changes are observed in lens fibre cells during differentiation. The cells increase in length by 50- to 100-times, accompanied by an increase in fibre-specific proteins, including intermediate filament proteins CP49 and CP95 (Ireland et al., 2000) and crystallins (Cvekl and Piatigorsky, 1996).

The elimination of potentially light-scattering intracellular organelles, including nuclei and all associated nucleic acid, is a key feature of the differentiation of lens epithelial cells into fibre cells and is thought to involve at least some components of the apoptosis signalling pathway (Dahm, 1999; Wride et al., 1999, 2003; Wride, 2000, 2007; Bassnett, 2002, 2008). However, unlike in 'conventional' apoptosis, the cells from which the organelles have been removed persist throughout life, rather than being destroyed. Additional structural differences have been observed, including the persistence of the cytoskeleton in mature fibre cells, whereas it is completely degraded during apoptosis (Bassnett and Beebe, 1992; Dahm et al., 1998). Also, there is no flipping of phosphatidylserine to the outer membrane of the lens fibre cells as observed in apoptosis (Bassnett and Mataic, 1997; Wride and Sanders, 1998). Finally, in executioner caspase (caspase-3, -6 and -7) knockout mice, lens fibre cell organelle loss proceeds as normal (Zandy et al., 2005).

Cataract occurs when opacities form in the normally transparent lens and is the commonest cause of blindness worldwide (Francis et al., 1999, 2000; Congdon, 2001). Lens opacities can be congenital or appear during ageing and can form as a result of genetic mutations or exposure to toxic insults; e.g. UV radiation. Many of the genetic mutations causing cataract affect structural and/or transparency related components of the lens (e.g. connexins and crystallins, Graw and Loster, 2003). Congenital cataracts are rare in developed countries (30 cases per 100,000 births) (Graw, 2004). Moreover, maternal rubella virus infection causes bilateral congenital cataract (Gregg and Banatvala, 2001; McAlister Gregg, 2001), possibly as a result of defects in lens fibre cell organelle degradation. Accumulation of nuclear and mitochondrial fragments in cortical cataract can occur due to incomplete organelle degradation in the equatorial region of the lens (Pendergrass et al., 2005, 2006). Prevention of DNA degradation in a mouse model, due to DNase II-like acid DNase (DLAD) deficiency leads to DNA accumulation in the lens, thereby causing cataract (Nishimoto et al., 2003). Therefore, DLAD must be the DNase responsible for nuclear degradation during lens cell differentiation (Nishimoto et al., 2003; Nakahara et al., 2007).

Microarray studies have been used to profile gene expression in the lens during early postnatal development in order to compare gene expression therein with non-lens tissues and to compare gene expression profiles in lens compartments at different stages of maturation (Wride et al., 2003; Ivanov et al., 2005; Xiao et al., 2006). This technology has also pinpointed gene expression changes between cataractous and normal age-matched lenses in humans and in mouse models of cataract, including the Sparc and Mimecan knockouts (Hawse et al., 2003; Ruotolo et al., 2003; Hawse et al., 2004; Mansergh et al., 2004; Segev et al., 2004). These studies demonstrated significant differential gene expression between cataractous lenses and age-matched controls. Expression of many unexpected genes has been identified in the lens using arrays, including those encoding the haemoglobin subunits (Wride et al., 2003; Mansergh et al., 2004, 2008). Notably, study of genes

expressed in normal lens development highlighted the presence of many genes associated with apoptotic processes.

Here, we have used nylon arrays comprised of 243 cDNAs representing genes with known roles in apoptosis in order to carry out an initial screen of the expression of these genes at postnatal day 7 (p7) and postnatal day 14 (p14) of mouse lens development. These stages were chosen as the postnatal period before day 14 is a period of rapid lens growth, accompanied by lens fibre cell differentiation and organelle loss involving apoptosis signalling pathways (Wride, 2000). Formation of the organelle free zone (OFZ) is complete at p14 when the eyes open, allowing for clear vision (Kuwabara and Imaizumi, 1974). A number of highly expressed or differentially regulated genes were selected for follow-up using RT-PCR. In order to further select for biological relevance via cross-species comparison, we tested expression of the chick homologues of selected genes during chick embryo lens development (E6–E16) using RT-PCR.

The *mouse double minute 2* (*Mdm2*) gene, the product of which is a regulator of p53, was differentially regulated between the two stages studied in the mouse lens, while p53 itself was also highly expressed. *Mdm4/X*, was not printed on the array used, but is known to be intimately functionally related to both p53 and *Mdm2* (Marine et al., 2006). The p53 pathway is a key component of apoptotic signalling; p53 is possibly the most pivotal tumour suppressor gene and its ablation is a primary cause of cancer (Toledo and Wahl, 2007). Furthermore, it is becoming apparent that p53/*Mdm2* signalling is involved in various developmental processes including osteoblast differentiation (Lengner et al., 2006), nervous system development (Xiong et al., 2006) and in regulating proliferation and progenitor expansion in various cell lineages (Liu et al., 2007). The role of p53 family molecules in embryonic development has recently been reviewed (Danilova et al., 2008a,b) and it was suggested that a significant number of congenital developmental abnormalities may be due to defects in the p53 protein family. Furthermore, there is some evidence that the p53 pathway may be involved in eye and/or lens development. P53 expression has been demonstrated in the normal adult mouse eye in the corneal epithelium (Tendler et al., 2006) and in the lens epithelial cells of the central and pre-equatorial zones and in the lens fibre nuclear bow region (Pokroy et al., 2002), while increased p53 expression in the rat lens epithelium following exposure to UV light has been associated with apoptosis and cataract (Ayala et al., 2007). Furthermore, temporally distinct patterns of p53-dependent apoptosis have been identified during mouse lens development (Pan and Griep, 1995) and overexpression of human wild-type p53 in the mouse lens results in defects in lens fibre cell differentiation (Nakamura et al., 1995). However, there is no prior evidence for *Mdm2* expression in the lens and the spatio-temporal pattern of expression of members of the p53 pathway in lens development remains undetermined. In the latter half of the studies presented here, we therefore focused on *Mdm2*, p53 and *Mdm4/X* in Western blotting and immunocytochemistry studies during mouse and chick lens development.

2. Materials and methods

2.1. Collection of lenses

Mice (129SvEv) were maintained on a 12 h light/12 h dark light cycle with food and water *ad libitum* and were handled according to Home Office UK guidelines. Lenses were extracted from mice at different stages of maturation: newborn (Nb), postnatal day 7 (P7), postnatal day 14 (P14) and 4 wk (4wk). Mice were cervically dislocated and enucleated. The lenses were then removed from a posterior incision in the eyeballs under a research stereo

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