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BRIEF COMMUNICATION

Phenotypic stability of Pro347Leu rhodopsin transgenic pigs as indicated by photoreceptor cell degeneration

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Abstract Rhodopsin (Pro347Leu) transgenic pigs are recognized to be an excellent model for the human disease, retinitis pigmentosa. First published in 1997, the rhodopsin transgenic pigs have been maintained since that time at North Carolina State University by outcrossing hemizygous boars to unrelated sows. Nine generations of outcrossing have been completed. Since the genetic background of these pigs has undoubtedly changed, the question of the current phenotype of the transgenic pigs is relevant for their future use. Age-matched transgenic and non-transgenic eyes were submitted for histological analysis using hematoxylin and eosin staining. Even by 2 weeks of age, significant thinning of the outer nuclear layer of photoreceptors was observed. For ages 3 and 4 weeks, thinning was noted similar to that of 2 weeks of age. By 6 weeks of age outer nuclear layer thinning was greater than that of earlier age. At 11 weeks of age, most of the rods have degenerated leaving only a few layers of cones. In all, the phenotype, based on assessment of photoreceptor degeneration, is similar to that of the first description of the transgenic animals. As such the Pro347Leu rhodopsin transgenic pigs have exhibited phenotypic stability through generations of outcrossing and can be used confidently in future studies of the type of retinal degeneration seen with retinitis pigmentosa.

Keywords Transgenic · Pig · Rhodopsin · Phenotype

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Introduction

Rodent models of human retinal degenerations have been extensively studied and used to test therapeutic interventions (Hafezi et al. 2000). By comparison, the pig is considered a valid model for studies of vision science because of its similarity in size, anatomy and physiology to that of the human (Beauchemin 1974; Chandler et al. 1999; Gerke et al. 1995; Hendrickson and Hicks 2002). With the publication of the production and characterization of Pro347Leu rhodopsin transgenic pigs, a relevant model of retinitis pigmentosa was achieved in this species (Petters et al. 1997; Li et al. 1998; Tso et al. 1997). The recapitulation of human retinitis pigmentosa in this transgenic model was noted to be complete. Retinal degeneration occurred early by extensive rod degeneration and then continued later in life with progressive cone degeneration.

It is possible that the genetic background of a transgenic line of animals can affect the phenotype. This is especially evident in the case of inbred lines

of transgenic mice (Wolfer and Lipp 2000; Wolfer et al. 1997; Cho et al. 1989). Since the availability of inbred lines of pigs is limited, the Pro347Leu rhodopsin transgenic pigs have been maintained on a heterogeneous genetic background. Over time and generations, the genetic background of these pigs may have affected the phenotype of retinal degeneration. The extent of the phenotype of the currently available transgenic pigs is relevant to the design and completion of future studies aimed at therapeutic interventions.

Materials and methods

Transgenic and non-transgenic pigs were bred and maintained according to the NIH Recombinant DNA Guidelines and approved and inspected by the Institutional Animal Care and Use Committee of North Carolina State University. Annual inspections were also carried out by the USDA regional veterinarian. All work was consistent with the Association for Research in Vision and Ophthalmology Policy on the Use of Animals in Research.

Pro347Leu transgenic pigs were identified using standard PCR and gel electrophoresis techniques. Briefly, forward (PIG 1F 5′ ACTGGGTGATGACGAGGC 3′) and reverse (PIG 1R 3′ GGCGTGGACAGTCTTGGT 5′) primers were used to amplify a 170 bp fragment of the rhodopsin gene. Digestion of the wild type fragment with NlaIV results in three bands of 94, 54 and 22 bp. The Pro347Leu fragment is only cut one time with *Nla*IV yielding 148 and 22 bp fragments. Digestion of a transgenic fragment with *Bfa*I results in two bands of 59 and 111 bp. The wild type band is not cut with BfaI. Presence of the transgenic bands can be easily distinguished from wild type after gel electrophoresis.

Each generation one hemizygous transgenic boar was mated to a single unrelated female to continue the lineage. Other offspring were produced at each generation and these were used in experimental studies. Since the founding of this line, nine generations have ensued.

For the present study, one transgenic boar was mated to five non-transgenic sows. Offspring were genotyped and equal numbers of transgenic and non-transgenic pigs were euthanized at 2, 3, 4, 6 and 11 weeks of age. Eyes were removed and a small incision was made in the sclera proximal to the iris. The eyes were then fixed in 4% paraformaldehyde PBS for 24 h and then placed 15% sucrose PBS for 24 h and finally into 30% sucrose PBS until processed for sectioning. Sections were taken bisecting the eye through the optic nerve and stained with hematoxylin and eosin. Sections were viewed at 400× magnification on an Olympus Vanox-S AH2 microscope equipped with a SPOT Insight FireWire 2mp CCD camera and SPOT Advanced imaging software. (Diagnostic Instruments Inc.). Images from three areas of each eye (nasal and temporal periphery and central area) were recorded and used as the basis for comparing transgenic and non-transgenic retina at each age. The nuclei observed in ninety columns of photoreceptor cell bodies of the outer nuclear layer from each animal were counted as a measure for comparison between genotypes. Statistical analysis of the thickness of the outer nuclear layer was accomplished using the Mixed Procedure function of SAS (Cary, NC).

Results and discussion

By inspection of the figure, comparisons between transgenic and non-transgenic animals can be made for each age. At 2 weeks of age, the outer nuclear layer was noticeably thinner than that of controls. The outer nuclear layer was approximately 5 layers thick compared to the control of almost 10 layers thick (Fig. 1). At 3 and 4 weeks of age, the outer nuclear layer was thinned to approximately the same extent as that seen for 2 weeks of age. The outer nuclear layer in the transgenics was about 50% thinner than the controls. At 6 weeks, the outer nuclear layer in the transgenic is noticeably thinner, however, since the control at this age was thinner as well, the transgenic layer was still only about 50% of the control (Fig. 1). By 11 weeks, the photoreceptor layer appears to be composed primarily of cones based on the morphology of the outer segments (Fig. 1). Thinning of the outer nuclear layer is quite pronounced at this age with the outer nuclear layer in the transgenic being roughly 25% of the corresponding control.

In addition to qualitative inspection of the sections, we have completed a statistical analysis of cell counts of the outer nuclear layer in three areas of the retina. Across all ages there were significantly fewer

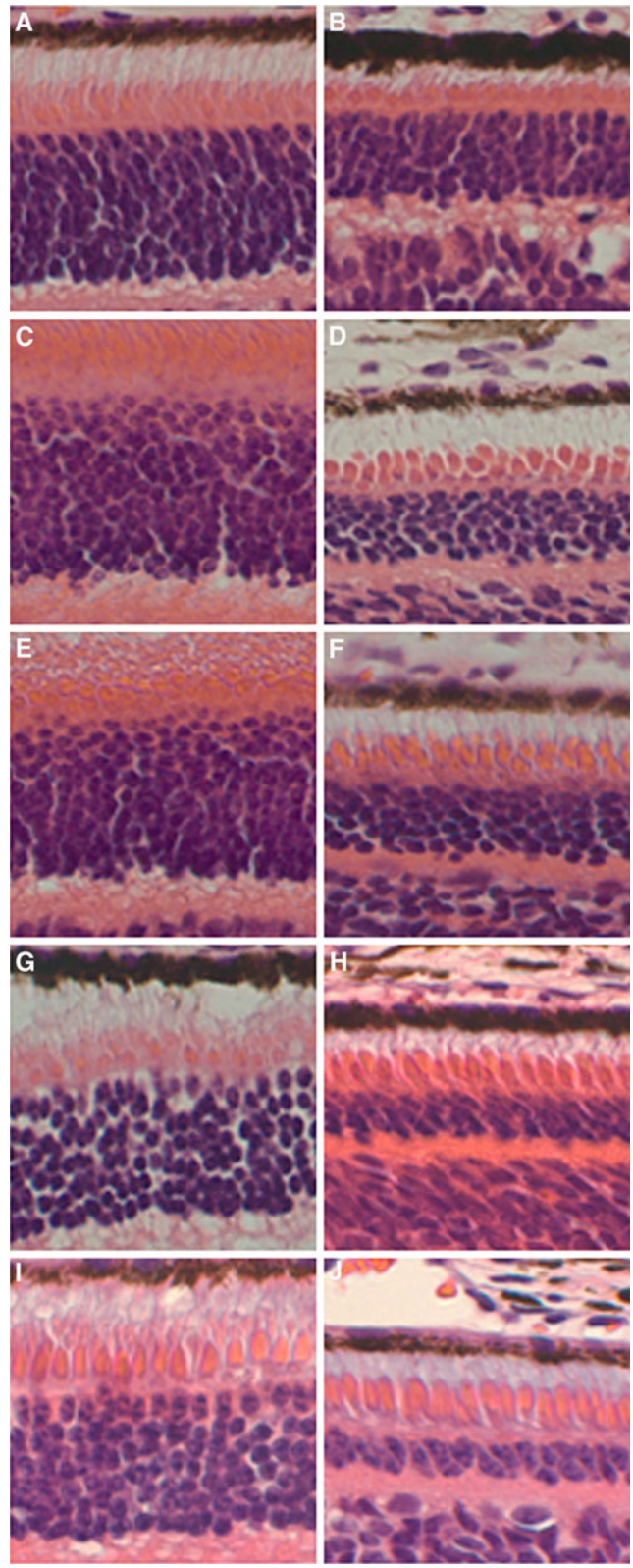

Fig. 1 Outer nuclear layer (ONL) of hematoxylin and eosin stained thin sections of the central portion of pig retina. All images are orientated so that the retinal pigmented epithelium (RPE) is on the *top*. Age matched pigs are found in the *same row*. The *left* column is from non-transgenic pigs and the *right* column is retina from transgenic transgenic pigs. **a** ONL of 2 week old non-transgenic pig. **b** ONL of 2 week old transgenic. **c** ONL of 3 week old non-transgenic pig. **d** ONL of 3 week old transgenic pig. **e** ONL of 4 week old non-transgenic pig. **f** ONL of 4 week old non-transgenic pig. **g** ONL of 6 week old non-transgenic pig. **h** ONL of 6 week old transgenic pig. **i** ONL of 11 week old non-transgenic pig. **j** ONL of 11 week old transgenic pig. The *black bar* on the *bottom* represents 10 microns

cells in the outer nuclear layer of transgenic pigs compared to non-transgenic pigs (4.65 ± 0.66 vs. 9.56 ± 0.66; $P = 0.0008$). Comparisons were made between transgenic and non-transgenic pigs of ages 2–4 weeks and 6–11 weeks. For the non-transgenic pigs, significantly fewer nuclei were noted for the 6–11 week age range than for the 2–4 week range (8.19 ± 0.64 vs. 10.47 ± 0.53; $P = 0.033$). As expected, there was a significant difference between transgenic and non-transgenic pigs at both age ranges. For the 2–4 week age, non-transgenic pigs averaged 10.48 nuclei thick and transgenic were 5.56 ($P = 0.0006$). A similar result was found at 6–11 weeks of age: non-transgenic—8.19 and transgenic—3.29 ($P = 0.0017$). Transgenic pigs were noted to have fewer nuclei at 6–11 weeks compared to 2–4 weeks (3.29 vs. 5.56; $P = 0.0337$).

In summary, a phenotype of retinal degeneration is obvious from the first time point tested, that of 2 weeks of age. The extent of degeneration remained roughly the same for weeks 3 and 4 thus these data were pooled in the analysis. At 6 weeks of age, the degeneration seems to be more extensive (3 cell layers thick), however, still a little over 50% of control. At 11 weeks of age, the degeneration is extreme with only 2 cell layers present in the outer nuclear layer.

Retinal degeneration in the Pro347Leu rhodopsin transgenic pig model is early in onset and progressive in nature. The general sequelae of degeneration seen in this study is in general agreement with that found in the original publication (Petters et al. 1997) based on a comparison of the figures despite the intervening generations of outcrossing. Certainly, by 6 and 11 weeks of age, significant degeneration has occurred with generally only cones surviving at 11 weeks based on the morphology of the outer segments (Fig. 1). In the Petters et al. (1997) publication, it was noted that cones continue to survive until a much older age. This aspect of the phenotype was not addressed in the present study.

The PCR screen used probed the mutant codon so that it was confirmed that each generation inherited the transgene and that it was, indeed, mutant. The Pro347Leu mutation is dominant so only one copy of the transgene is required for the phenotype to be expressed. The transgene is inserted in a permissive region of the genome for expression and thus it is not surprising that the phenotype may be stable. What was not known is whether a changed genetic background might affect either the expression of the transgene or the development of the phenotype.

The sows used to maintain the transgenic lines are composites of the Yorkshire, Landrace, and Large White breeds. While these lines are all white in color, they differ significantly in many other attributes including growth rates and litter size. They are bred to be lean, fast growing lines of pigs that are quite heterogeneous. The use of these pigs helped to minimize inbreeding in the transgenic lines that might have been significant otherwise. The present transgenic lines are very healthy and show no depressive signs of inbreeding.

Currently, the rhodopsin transgenic pig model is maintained at North Carolina State University where they are available at cost to investigators at other universities and research centers. The Pro347Leu rhodopsin transgenic pigs have been used for studies of retinal degeneration (Peng et al. 2000; Shen et al. 2005; Kraft et al. 2005; Ng et al. 2008; Banin et al. 1999; Blackmon et al. 2000; Huang et al. 2000) and for studies aimed at intervening to prevent or treat the retinal degeneration (Ghosh et al. 2004; Mahmoud et al. 2003; Ghosh et al. 2007).

In summary, despite a number of generations of outcrossing, the phenotype of the Pro347Leu rhodopsin transgenic pig has remained similar to the originally published phenotype. In this case, retinal degeneration occurs quickly and progressively making this model excellent for the study of retinal degeneration and therapeutic interventions.

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