Human Papillomavirus E6/E7 Oncogenes Promote Mouse Ear Regeneration by Increasing the Rate of Wound Re-epithelization and Epidermal Growth

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Mammals have limited regeneration capacity. We report here that, in transgenic mice (Tg(bK6-E6/E7)), the expression of the E6/E7 oncogenes of human papilloma virus type 16 (HPV16) under the control of the bovine keratin 6 promoter markedly improves the mouse's capacity to repair portions of the ear after being wounded. Increased repair capacity correlates with an increased number of epidermal proliferating cells. In concordance with the expected effects of the E6 and E7 oncogenes, levels of p53 decreased and those of p16 in epidermal cells increased. In addition, we observed that wound re-epithelization proceeded faster in transgenic than in wild-type animals. After the initial re-epithelization, epidermal cell migration from the intact surrounding tissue appears to be a major contributor to the growing epidermis, especially in the repairing tissue of transgenic mice. We also found that there is a significantly higher number of putative epidermal stem cells in Tg(bK6-E6/E7) than in wild-type mice. Remarkably, hair follicles and cartilage regenerated within the repaired ear tissue, without evidence of tumor formation. We propose that the ability to regenerate ear portions is limited by the capacity of the epidermis to repair itself and grow.

Journal of Investigative Dermatology (2008) **128,** 2894–2903; doi:10.1038/jid.2008.156; published online 12 June 2008

INTRODUCTION

An injured tissue can repair itself by simply healing the wound, a process that usually leaves a scar in the damaged area. Alternatively, upon damage, a tissue can regenerate its original constituents (that is, all cell types of the tissue) without leaving a scar and, in extreme cases, recover the original form and size. In the animal kingdom, wound healing is crucial for organism survival, but regeneration in different degrees is a surplus property retained only by some species. As vertebrates emerged in evolution, the ability to regenerate decreased in most species. Actually, a full regeneration of several organs and tissues can be observed only in certain amphibian species, such as newt and salamander (Brockes and Kumar, 2002). Mammals have limited regeneration capacity and rarely regenerate an organ or tissue to its original form. Rather, regeneration in mammals commonly refers to the individual capacity of some tissues or components of the body to repair upon a ''minor'' damage (Carlson, 2005).

In the absence of regeneration, adult mammals usually repair their tissues by a rapid healing process that involves an immediate immune response. This process has been carefully studied in the skin (Martin, 1997). In this case, many cell types (for example, neutrophils, macrophages, mast cells, platelets, and lymphocytes) infiltrate the wound and are the source of growth factors that promote angiogenesis, proliferation of keratinocytes, and deposition of extracellular matrix components, usually leaving a scar in the damaged area. In general, both regeneration and healing with a scar initiate similarly by covering the wound area with migrating keratinocytes (that is, re-epithelization) followed by their proliferation (Brockes and Kumar, 2005; Santoro and Gaudino, 2005). However, after this point, a regenerating tissue forms what is known as a blastema, a distal structure composed of epithelial and mesenchymal tissues that is essential for the regeneration process (Brockes and Kumar, 2005). Dedifferentiation is considered the mechanism by which most of the different cell types emerge during urodele regeneration (Tsonis *et al.*, 1995). However, few studies have evaluated the contribution of the stem cells used for tissue renewal and repair in regeneration (Echeverri and Tanaka, 2002; Morrison *et al.*, 2006).

Nearly half a century ago, the regeneration of rabbit ear holes after punching was recognized as an example of

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Abbreviations: bK6, bovine keratin 6 promoter; LRC, label-retaining cell; PBS, phosphate-buffered saline; Tg(bK6-E6/E7), transgenic mice expressing E6/E7 oncogenes of human papilloma virus type 16 under the control of the bovine keratin 6 promoter; Wt, wild type

Received 23 November 2007; revised 13 April 2008; accepted 22 April 2008; published online 12 June 2008

mammalian regeneration (Goss and Grimes, 1975). This phenomenon was not observed in other animals such as dogs and sheep. More recently, it was observed that the MRL mouse strain has an increased ability to close ear holes produced by punching (Clark *et al.*, 1998). The reference strain for this study was C57BL6, which is unable to close ear holes. It has been considered that ear-hole closure occurs by regeneration, because a scar is not formed and, more important, because the characteristic ear inter-skin cartilage and hair follicles are regenerated during the repair process. Interestingly, the ears of rabbits and mice undergo similar histological changes early in the regeneration process (Heber-Katz, 1999). Attempts to identify the gene or genes that are responsible for the increased ability to regenerate ear portions in the MRL mouse strain have not been fruitful yet, and the data rather suggest a complex contribution of several genes (McBrearty *et al.*, 1998; Masinde *et al.*, 2006).

Several years ago, we reported on a set of transgenic mouse lines that express in the skin the E6 and E7 oncogenes of the human papillomavirus type 16 (HPV16) under the control of the bovine keratin 6b gene (bK6) promoter (Tg(bK6-E6/E7)) (Escalante-Alcalde *et al.*, 2000). Under normal conditions, these mice do not form tumors; however, they display an evident skin phenotype characterized by low hair density. We now report the remarkable ability of these mice to regenerate an ear portion after a hole is made, which appears to be mostly due to the effect of E6 and E7 oncogenes in re-epithelization and epidermal growth.

RESULTS

Ear-hole closure in Tg(bK6-E6/E7) mice

While studying Tg(bK6-E6/E7) mice, we noticed that the ear punch made to mark the mice frequently closed completely. To determine whether this phenomenon was associated with the bK6-E6/E7 transgene, we systematically followed the closure of ear holes in transgenic compared with wild-type (Wt) mice (Figure 1). When holes closed completely, this occurred within a month after punching, and when the closure was not completed within this time, the hole opening was slightly reduced in the following months but never closed. Hole-closure frequency was determined for two transgene insertions under either the outbred genetic background of the CD1 strain (CD1-Tg(bK6-E6/E7)M8 and CD1-Tg(bK6-E6/E7)H1) or the inbred genetic background of the Fvb/N strain (Fvb/N-Tg(bK6-E6/E7)M8). Ear-hole closure was also compared between females and males. As shown in Figure 2, there is a clear association between the presence of the transgene and the capacity to close the ear hole. For the Tg(bK6-E6/E7)M8 line, in about half of the total animals, the hole measuring less than 1 mm diameter closed. This was accentuated in females, and although the difference between genders might be related to the still unknown cause of death that eliminates most males at a young age, a similar observation was reported for regeneration in the MRL mouse strain (Blankenhorn *et al.*, 2003). In the Fvb/N background, the holes in the ears of Tg(bK6-E6/E7)M8 mice closed markedly better than in Fvb/N Wt mice, although in a very few the hole closed completely. The ability to close the hole

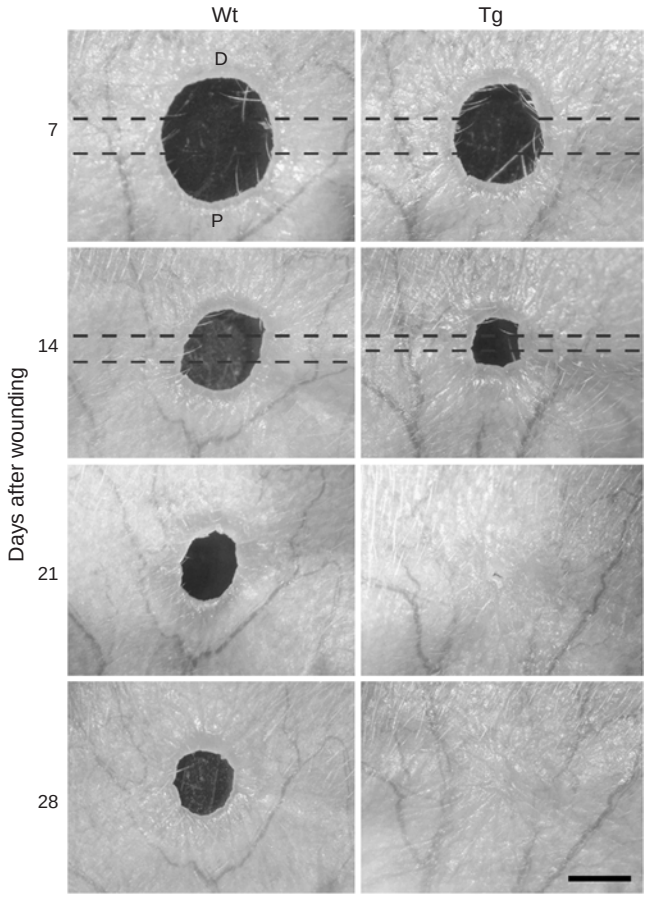

Figure 1. Ear wound healing in mice carrying the bK6-E6/E7 transgene. Pictures of ear holes of mice carrying the bK6-E6/E7 transgene (Tg) and wild-type (Wt) mice taken at 7, 14, 21, and 28 days after wounding (pictures are from the CD1-Tg(bK6-E6/E7)M8 line). Holes in Tg ears were rapidly closed in comparison with holes in Wt ears. The sections for histological analyses were performed perpendicular to the ear proximo- (P) distal (D) axis and taken from the area between the broken lines; pictures in Figures 4–9 show one side of the regenerating ear section. Scale bar = 1 mm.

was barely noticeable in animals belonging to the Tg(bK6-E6/E7)H1 line, which coincided with the less apparent lower hair density phenotype described previously (Escalante-Alcalde *et al.*, 2000). Animals belonging to this line also die at an early age. Despite the fact that transgenic mice clearly have an increased ability to close ear holes, it should be noted that in some Wt animals the hole could close to some degree but rarely completely. From this point forward, the results described were derived from the analysis of the Tg(bK6-E6/E7)M8 mouse line in the CD1 strain (CD1-Tg(bK6-E6/E7)).

Increased epidermal proliferation during repair of ear holes of CD1-Tg(bK6-E6/E7) mice

E6 and E7 are known for their ability to increase cell proliferation (Tommasino and Crawford, 1995). Therefore, we analyzed whether the ability to close the ear hole was related to an increase in cell proliferation. Because in Tg(bK6-E6/E7) mice oncogene expression is directed to the injured