Remodeling of Three-Dimensional Organization of the Nucleus during Terminal Keratinocyte Differentiation in the Epidermis

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The nucleus of epidermal keratinocytes (KCs) is a complex and highly compartmentalized organelle, whose structure is markedly changed during terminal differentiation and transition of the genome from a transcriptionally active state seen in the basal and spinous epidermal cells to a fully inactive state in the keratinized cells of the cornified layer. Here, using multicolor confocal microscopy, followed by computational image analysis and mathematical modeling, we demonstrate that in normal mouse footpad epidermis, transition of KCs from basal epidermal layer to the granular layer is accompanied by marked differences in nuclear architecture and microenvironment including the following: (i) decrease in the nuclear volume; (ii) decrease in expression of the markers of transcriptionally active chromatin; (iii) internalization and decrease in the number of nucleoli; (iv) increase in the number of pericentromeric heterochromatic clusters; and (v) increase in the frequency of associations between the pericentromeric clusters, chromosomal territory 3, and nucleoli. These data suggest a role for nucleoli and pericentromeric heterochromatin clusters as organizers of nuclear microenvironment required for proper execution of gene expression programs in differentiating KCs, and provide important background information for further analyses of alterations in the topological genome organization seen in pathological skin conditions, including disorders of epidermal differentiation and epidermal tumors.

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INTRODUCTION

The cell nucleus is a highly complex organelle that consists of the nuclear membrane, individual chromosomes occupying distinct territories, as well as nuclear bodies (nucleoli, Cajal bodies, promyelocytic leukemia bodies, nuclear speckles, Polycomb bodies) located in inter- and intrachromosomal compartments (Lanctot *et al.*, 2007; Hubner and Spector, 2010). Genetic material (DNA) in the nucleus is compacted up to several thousand folds and organized into chromosomes as a complex, with histones and non-histone proteins that allows the genome to be replicated, transcribed, and repaired (Hemberger *et al.*, 2009; Ho and Crabtree, 2010). The nucleus is also involved in a number of other functions, including the RNA processing, ribosome assembly, and maintenance of the mechanical integrity of the cell (Rippe, 2007; Rowat *et al.*, 2008).

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Abbreviations: EDC, epidermal differentiation complex; FISH, fluorescence in situ hybridization; KC, keratinocyte; 3D, three-dimensional

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Three-dimensional (3D) organization of the nucleus and spatial compartmentalization of the distinct chromatin domains, nuclear bodies, and macromolecular protein complexes are dynamic, and show remarkable changes during cell differentiation (reviewed in Hubner and Spector, 2010; Joffe *et al.*, 2010; Schoenfelder *et al.*, 2010). Activation and silencing of distinct genomic loci during cell differentiation are often accompanied by changes in their positioning relatively to the corresponding chromosomal territories and other nuclear compartments, as well as by changes in the morphology and number of nuclear bodies (Deniaud and Bickmore, 2009). Despite a certain similarity in the nuclear architecture between differentiated versus undifferentiated cells, spatial organization of the genome in the nucleus appears to be unique for each cell type and, together with distinct patterns of nucleosome positioning, DNA methylation, and histone modifications, constitute an ''epigenetic signature'' of the cell (Naumova and Dekker, 2010).

In the epidermis, lineage-committed progenitor cells reside in the basal layer, where they proliferate and differentiate into cells of the suprabasal layers, forming the stratified epithelium (Fuchs, 2007; Koster and Roop, 2007; Blanpain and Fuchs, 2009). Transition of the progenitor cells into keratinocytes (KCs) of suprabasal layers occurs through asymmetric cell division (Lechler and Fuchs, 2005; Poulson and Lechler, 2010). The process of terminal differentiation in epidermal KCs is accompanied by coordinated activation and silencing

of the sets of genes, including those that constitute several lineage-specific loci (keratin type I and II loci, the epidermal differentiation complex (EDC), and so on), and encoding essential structural components of the epidermal barrier (Segre, 2006; Bazzi *et al.*, 2007).

During terminal differentiation of the epidermal KCs, the nucleus undergoes programmed structural and biochemical changes during transition from a highly active status, associated with execution of the genetic programs of epidermal barrier formation, to a fully inactive condition, and finally becomes a part of the keratinized cells of the cornified epidermal layer (Botchkarev *et al.*, 2012). Earlier studies demonstrated that KC transition between the distinct epidermal layers is accompanied by marked morphological and ultrastructural changes in the nucleus, including its size, shape, structure of nucleoli, and so on. (Breathnach, 1971; Karasek *et al.*, 1972; Tsuji and Cox, 1977). As spatial organization of the distinct genomic loci and nuclear bodies are critical for the proper regulation of gene expression (Dundr and Misteli, 2010; Schoenfelder *et al.*, 2010), changes in the size and shape of the KC nucleus associated with terminal differentiation might, on the one hand, influence the 3D genome structure and gene expression programs in differentiating cells. On the other hand, it might reflect reorganization of these programs accompanied by the differentiation-associated accumulation or loss of the distinct cytoplasmic components, such as keratin 14, interacting with nuclear envelope and regulating nuclear shape (Lee *et al.*, 2012).

Recent studies demonstrate that spatial genome organization in KCs is intimately linked to the regulation of gene expression, and that the higher-order chromatin remodeler Satb1, serving as a direct target for p63 transcription factor, controls chromatin folding of the EDC and gene expression during epidermal KC differentiation *in vivo* (Fessing *et al.*, 2011). Furthermore, 3D genome organization also changes during KC differentiation *in vitro*, including the position of selected chromosomes and EDC locus relatively to the corresponding chromosome territory (Williams *et al.*, 2002; Marella *et al.*, 2009).

However, despite an increasing amount of information on epigenetic mechanisms controlling gene expression in KCs (reviewed in Eckert *et al.*, 2011; Wang and Chang, 2011; Botchkarev *et al.*, 2012; Zhang *et al.*, 2012), a systematic analysis of the remodeling of 3D organization of the nucleus during terminal KC differentiation in the epidermis *in situ* has not been done yet. Further, the extent to which nuclear architecture shows preferential changes at defined stages of cell differentiation process in the epidermis *in vivo* still remains unclear.

By using multicolor confocal microscopy, 3D image analysis, and mathematical modeling of the nucleus, we describe here the remodeling of the nuclear architecture during terminal differentiation of the normal mouse epidermal KCs *in vivo.* Our results reveal significant changes in multiple parameters of 3D genome organization in KCs during transition from the basal to the spinous and granular epidermal layers, including changes in spatial associations between the pericentromeric heterochromatin domains, nucleoli, and chromosomal territory 3 bearing the EDC locus. We summarize these data as a model, suggesting that the establishment and silencing of differentiation-associated gene expression programs in epidermal KCs *in vivo* involves marked changes in their nuclear architecture and 3D genome organization.

RESULTS

Terminal KC differentiation in the epidermis is accompanied by changes in the nuclear volume and shape

To define the changes in 3D organization of the KC nucleus during terminal differentiation in the epidermis *in situ*, cryosections of the 10-day-old mouse footpads were stained with DAPI (4′,6-diamidino-2-phenylindole) and analyzed using confocal microscopy, followed by image analysis and 3D computational reconstruction (Supplementary Figure S1 online). Nuclei of the spinous and granular layer cells were determined after immunostaining of the skin cryosections with antibodies against keratin 10 or loricrin, respectively (Supplementary Figure S2 online). Murine footpad epidermis was purposely selected for this study, because it, at least in part, resembles the human palmoplantar epidermis morphologically and, in contrast to other postnatal mouse skin areas, consists of several well-defined layers of KCs (Loomis *et al.*, 1996). Moreover, in newborn mice, basal layer of the footpad epidermis does not contain melanocytes and represents a more homogenous population of epidermal progenitor cells in comparison with the trunk skin (Kunisada *et al.*, 1998; Plikus *et al.*, 2004).

3D reconstructions based on the image-stack analyses, collected using confocal microscopy of 80-μm thick cryosections of the footpad epidermis, confirmed the previously reported observations on changes in the orientation of the long axis of the nucleus from vertical to horizontal during transition of KCs from the basal to suprabasal layer of the epidermis (Figure 1a) (Rowden, 1975; Loomis, 2001; Lechler and Fuchs, 2005; Lee *et al.*, 2012). Analysis of the ratios between the long axis and the average of two shorter axes of the ellipsoid nuclei showed that KC nuclei in the basal and granular epidermal layers were significantly more elongated compared with those in the spinous layer, where nuclei were more round in shape (Figure 1b).

To assess changes in the nuclear volume among KCs of different epidermal layers, skin was immunostained with antibody against proliferative marker Ki-67, and nuclear volume was assessed separately in proliferating and nonproliferating cells. Ki-67 positive cells were seen almost exclusively in the basal epidermal layer (Botchkarev *et al.*, 1999; Sharov *et al.*, 2003; Lechler and Fuchs, 2005), and showed significantly larger nuclear volume compared with nuclei of nonproliferative cells (Figure 1a and c). Both proliferative and nonproliferative basal cells showed significantly larger nuclear volume compared with cells of the spinous or the granular epidermal layers (Figure 1c). These data demonstrate that in addition to the changes in the orientation and shape of the nucleus, terminal differentiation of the epidermal KCs was accompanied by significant decrease of the nuclear volume.