Acta Histochemica 116 (2014) 639–645

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Immunohistochemical demonstration of connexins in the developing feather follicle of the chicken

Wilfried Meyer[a], Anna Oberthuer[a,d], Anaclet Ngezahayo[b], Ulrich Neumann[c], Ralf Jacob[d,*]

[a] Institute for Anatomy, University of Veterinary Medicine Hannover Foundation, 30173 Hannover, Germany
[b] Institute for Biophysics, Leibniz University of Hannover, 30419 Hannover, Germany
[c] Poultry Clinic, University of Veterinary Medicine Hannover Foundation, 30559 Hannover, Germany
[d] Institute for Cytobiology and Cytopathology, University of Marburg, Robert-Koch-Str. 6, 35037 Marburg, Germany

ARTICLE INFO

Article history:
Received 3 September 2013
Received in revised form
26 November 2013
Accepted 27 November 2013

Keywords:
Connexins
Chicken
Feather follicle development
Histochemistry

ABSTRACT

Based on immunohistochemistry, the study demonstrates the varying distribution and reaction intensity of connexins (Cx26 [chicken 31sim], 30 [chicken 31], 31, 32, 43, 45) in the developing feather follicle of the chicken (White Leghorn). The different embryonal stages were identified according to the normal table of Hamburger and Hamilton (1951). The development of the feather follicle complex is closely related to skin layer development, making use of the controlling function of connexins. This was evident during feather follicle differentiation, based on communication between ectomesodermal (fibroblasts) and ectodermal cells (developing epidermis), but also by the subsequent separation of the two cell line types related to their connexin-dependent differentiation degree. With the increase in mesenchymal cell numbers during feather placode development, the multiple connexins Cx26 [chicken 31sim] and 43, supported by Cx30 [chicken 31], 31 and 32, were increasingly activating the fibroblast concentrations as related to epidermal follicle buds, the specific follicle structure, the endothelial cells of capillaries and larger blood vessels, as well as the collagen fiber production and the growing feather musculature shortly before hatching; Cx45 could not be demonstrated. In conclusion, it seems that connexin expression is not only coupled to the origin of embryonic cells, but also connected with tissue formation before the follicle system can be formed.

Introduction

Connexins (Cx) are a family of chordate-specific transmembrane proteins that can form gap junction plaques or aggregates of transmembrane channels that allow direct contact between the cytoplasm of one cell and that of another. Each cell participates with one hemichannel, and in vertebrates this hemichannel is called a connexon, and each connexon is made of six protein subunits named connexins (e.g., Cruciani and Mikalsen, 2006; Abascal and Zardoya, 2013). The number of connexins in cell–cell channels is regulated by controlling transcription, translation, trafficking, and degradation (see e.g., Berthoud et al., 2004; Nielsen et al., 2012). Thus, gap junctional intercellular communication activity is most important, and it shows a wide diversity of tissue functions with multiple regulatory mechanisms including also organ development, and, finally, that its dysfunction can cause severe hereditable diseases (e.g., Arita et al., 2002; Brandner et al., 2004; Kelsell, 2005; Meyer et al., 2005; Goodenough and Paul, 2009; Oyamada et al., 2013).

From an evolutionary point of view there are main lines in the evolution of this protein family from fishes to mammals. Regarding a genetical basis, either of two or more homologous gene sequences have been found in different species or vertebrate groups related by linear descent. This means that orthologs of most connexins can be found throughout the vertebrates. As judged from the connexins in tunicates, the original connexin might be related to the ortholog groups of Cx36, 39.2, 43.4, 45 or 47 (Cruciani and Mikalsen, 2006). In chickens, there are two Cx26/30-like sequences, chicken 31sim and chicken Cx31. In the phylogenetic analysis, they never distribute to Cx26 [chicken 31sim] and Cx30 [chicken Cx31] in a 1:1 fashion, but always group together (Heller et al., 1998; Cruciani and Mikalsen, 2006).

If possible, such evolutionary or genetical information was used in this study to select relevant connexins for our approach to get improved understanding of the development of the structurally very complex feather follicle system. Feathers arise as discrete buds of mesenchyme and epithelium, which are two embryonic

* Corresponding author at: Department of Cell Biology and Cell Pathology, Philipps University of Marburg, Robert-Koch-Str. 6, D-35033 Marburg, Germany.
E-mail address: jacob@staff.uni-marburg.de (R. Jacob).

0065-1281/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.acthis.2013.11.016

tissues that respectively form dermis and epidermis of the integument, generating the buds by epithelial-mesenchymal signaling interactions. In this context, it seems not only of interest that, for example, the developmental steps include branching morphogenesis to create the rachis, barbs or barbules within feather bud, but also that the dermis provides spatiotemporal patterning information to the epidermis, e.g., feather follicle growth of abdominal skin generally shows the slowest progression and that in the feather filaments of the developing wings the most rapid one (Meyer and Baumgaertner, 1998; Widelitz et al., 2003; Eames and Schneider, 2005; Yamazaki et al., 2012). In view of the broad activities of connexins, our study uses immunohistochemistry to demonstrate the role of important representatives of these proteins during feather follicle morphogenesis, as already shown to some extent for developing hair follicles (e.g., Arita et al., 2002; Dhouailly, 2009), realizing, however, that the feather is the most complicated structure of the vertebrate integument (Sawyer et al., 2005; Dhouailly, 2009).

Materials and methods

White Leghorn chick embryos of different age were obtained from the Poultry Clinic of the University of Veterinary Medicine Hannover Foundation. The animals were staged according to the criteria of Hamburger and Hamilton (1951), reaching from 4 to 19 days of incubation (HH stages 23–45; incubation temperature 37.8 °C) until just before hatching (HH stage 46). Always 3 animals of one stage were narcotized and euthanized by chloroform vapor, and then very careful freed from the eggshell and the fetal membranes. Skin material was always taken from the dorsal body region (area of about 0.5–1 cm^2) of each of the embryos and immediately after excision fixed for 48 hours in Bouin's solution (Boeck, 1989). The influences of fixation media on the histological quality were studied and discussed by Hornickel et al. (2011), emphasizing that Bouin's solution produces the best results concerning protein fixation and relevant localization of the substances studied (see also Pearse, 1985). After washing several times in 70% ethanol with the addition of some drops of ammonia (conc.) to remove the picric acid, the skin samples and the related embryos were stored in 80% ethanol. After careful dehydration, the material was embedded via xylene in paraffin wax (Paraplast plus, Covidien, Neustadt, Germany).

For the immunohistochemical demonstration, 8 μm thick paraffin sections were deparaffinized in Histoclear (Biozym Scientific, Hessisch Oldendorf, Germany) and hydrated through descending concentrations of ethanol. Afterwards, these sections were stained for the determination of the different connexins studied (nomenclature according to Cruciani and Mikalsen, 2006): Cx26 [chicken 31sim] (dilution 1:75, anti-mouse, Zymed Medical Products, Vienna, Austria), Cx30 [chicken 31] (dilution 1:50, anti-rabbit, Zymed Medical Products), Cx31 (dilution 1:100, anti-rabbit, BioLogo, Kronshagen, Germany), Cx32 (dilution 1:200, anti-rabbit, Alpha Diagnostic Antibodies, Aachen, Germany), Cx43 (dilution 1:100, anti-rabbit, Zymed Medical Products), Cx45A (dilution 1:50, anti-mouse, Biotrend Chemicals, Cologne, Germany). Most of these connexins were of mammalian origin, and selected based on the information given by Cruciani and Mikalsen (2006) (see Table 2 of this study), who compared the connexin orthologs among vertebrates, because material from birds could not be obtained. Nevertheless, Cx 31, 32, 43, 45 were the same in mammals and birds. Following incubation overnight at 4 °C, the reaction was detected by the EnVision® system (DakoCytomation, Hamburg, Germany), using peroxidase-based very sensitive dextran-polymer visualization. One part of the sections was also incubated for 30 min in TEC buffer at 90 °C prior to the reaction to compare two staining quality types.

Positive controls of the connexins used were performed as follows: for Cx26 [chicken 31sim] and Cx30 [chicken 31] sections of the liver of laboratory mice (Fischer 344, Lewis, from Charles River, Sulzfeld, Germany; all these animals had been controls of research projects) were stained together with those of the chickens, for the control of Cx30 [chicken 31] and Cx31 this was done with sections of mouse skin, and for Cx45 with sections of mouse heart. The mouse samples contained high amounts of these connexins (Fig. 1), so it could be demonstrated that the antigen really bound specifically to the respective antibody. It has to be added that the mouse material had been fixated in the same way as the chicken samples.

To conduct the negative controls, the first antibody was omitted and instead the tissue was incubated with phosphate buffered saline (PBS)/1% bovine serum albumin (BSA). In order to exclude the possibility of non-specific binding of the Fc-part of the primary antibody, isotype controls were conducted (Hornickel et al., 2011). Therefore, the primary antibody was replaced by an IgG mouse antibody (Sigma–Aldrich, Munich, Germany). The respective protein concentration of this antibody was considered and applied in the same concentration as the primary antibody. To evaluate non-specific binding potentially caused by sources other than the primary antibody, tissue sections were also stained with just the secondary antibody followed by application of the visualization system.

Endogenous peroxidase activity was blocked by an incubation of the samples in an 80% ethanol solution with H_2O_2 (197 ml 80% ethanol + 3 ml 30% H_2O_2) for 30 min during the rehydration process (Hornickel et al., 2011).

For control of the antibody qualities and the presence of the related DNA, in situ hybridization was applied. DNA-fragments of about 300 nucleotides with high specificity for each connexin were amplified by polymerase chain reaction and ligated into the pCRII-TOPO vector (Invitrogen, Darmstadt, Germany). Specific sense and antisense digoxygenin-labeled riboprobes were generated by in vitro transcription from linearized vector constructs using the DIG RNA Labeling Kit (SP6/T7) (Roche Applied Science, Mannheim, Germany). Hybridization was carried out with the labeled cRNA probes diluted in hybridization buffer (75% formamide, 10% dextran sulfate, 3× SSC, 50 mM sodium phosphate, pH 7.4, 1× Denhardt's solution, 0.1 mg/ml yeast transfer RNA, and 0.1 mg/ml sheared salmon sperm DNA) with the addition of 1 mM dithiothreitol. Hybridization was performed overnight in a humid chamber at 60 °C. Post-hybridization consisted of an RNase treatment and washing with declining concentrations of SSC (from 2× SSC to 0.2× SSC) and dehydration using increasing concentrations of ethanol. Digoxigenin-labeled RNA molecules were visualized using the HNPP Fluorescent Detection Kit (Roche Applied Science, Mannheim, Germany). The samples were analyzed on a Leica DMI6000 fluorescence microscope.

Results

The positive controls and the in situ hybridization have shown: (a) that the chicken antigens really bound specifically to the respective antibodies of mammalian origin (mouse), and (b) that the matching RNAs were present in the tissue types studied (Fig. 1). Thus, it was possible to achieve relevant connexin staining.

Concerning the developing feather follicle, the first positive reactions of the connexins studied, except for Cx45, appeared between days 9 and 10 (HH stages 35 and 36), whereas in the developing epidermis (periderm) and dermis connexins could already be found beginning with day 4 or 5 (HH stages 25–27) until days 18 and 19 (HH stages 44–45), shortly before hatching. During these processes, fibroblasts, endothelial cells of the blood vessels, and cells of the developing feather musculature generally showed positive reactions for Cx26 [chicken 31sim], 30 [chicken 31], 31, 32, and 43.