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Regulation of Morphogenetic Processes during Postnatal Development and Physiological Regeneration of the Adrenal Medulla

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Regulation of morphogenetic processes during postnatal development of the rat adrenal medulla was studied. Termination of the adrenal medulla growth was found to be associated with decreased chromaffin cell proliferation, activation of canonical Wnt-signaling pathway, and enhanced expression of Sonic Hedgehog ligand. Analysis of transcription factors associated with pluripotency revealed increased percentage of Oct4-expressing cells by the end of medulla growth and no signs of Sox2 expression. All the cells demonstrating activation of Wnt-signaling and expression of Oct4 and Sonic Hedgehog were found to be highly differentiated chromaffin cells actively producing tyrosine hydroxylase. These findings allow considering the formation of the cell pools for dedifferentiation as a putative mechanism for physiological regeneration of the adrenal medulla.

Key Words: *chromaffin cells; adrenal medulla; regeneration; Wnt-signaling; transcriptional regulation*

The exceptional role of the adrenal gland in the protective responses requires its structures to have considerable regenerative potential. The adrenal glands consist of two different tissue types: the medulla, originating from the neural crest and producing catecholamines, and the cortex, originating from the mesoderm and synthesizing steroids. The adrenal medulla mainly consists of chromaffin cells originating from the precursors of Schwann cells [1]. During acute stress reaction, catecholamines such as epinephrine and norepinephrine are produced in the sympathetic nervous system and adrenal medulla within seconds. Instantly, the hypothalamic–pituitary–adrenal axis responds by producing glucocorticoids that support the action of the catecholamines. However, the regenerative capacities of these two structures are different. The medulla is known to have significantly less regenerative potential than the cortex and physiological regeneration of adrenal chromaffin cells is poorly explored. The mechanisms regulating postnatal development of the medulla and self-renewal of chromaffin cells are the least studied issues. To date, there little is known about the transcription factors controlling the growth and proliferation of chromaffin cells in the adrenal glands [2]. Analysis of published reports on the development and differentiation and dedifferentiation of cells in the nervous system showed that mature glial cells can be directly reprogrammed into neural stem cells by a single transcription factor Oct4, and this Oct4-driven reprogramming is enhanced by continuous stimulation of Sonic hedgehog (Shh) [3]. In turn, activation of Shh signaling correlates with the activity of Wnt-signaling pathway during regeneration [4]. In cooperation with Sox2 and other members of the core chain of transcription regulation, Oct4

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activates both protein-coding genes and non-coding RNA necessary for acquisition and maintenance of cell pluripotency [5]. Understanding the general principles of adrenal development and regeneration and their transcriptional control will make it possible to elaborate new approaches to obtaining differentiated adrenal chromaffin cell lines that are considered as a promising method for treating Parkinson's disease and some other pathologies [6].

Here we studied the role of transcription factors and pathways associated with maintenance of the self-renewal capacity of cells during postnatal development of the adrenal medulla.

MATERIALS AND METHODS

The experiment was performed on male Wistar rats with body weight of 240-250 g on day 42 of postnatal development (n=10) corresponding to the pubertal period characterized by active development of the adrenal gland and with body weight of 330-350 g on day 70, *i.e.* in the postpubertal period, when the growth of the rat adrenal gland is completed [7]. The animals were sacrificed by overdosage of Zoletil-100 (Virbac). Histological examination of equatorial sections of the organ stained with hematoxylin and eosin was carried out. Morphometry was performed using Image Scope software (Leica Microsystems). The area of the medulla and the area of chromaffin cells in the medulla were determined.

Immunohistochemical detection of tyrosine hydroxylase with polyclonal rabbit antibodies (Abcam) was used to determine terminal differentiation and functional activity of chromaffin cells. Proliferative activity of chromaffin cells was assessed by immunohistochemical detection of Ki-67 expression. Expression of Shh ligand, transcription factors Oct4 and Sox2 in chromaffin cells was also detected by immunohistochemical method using polyclonal anti-Ki-67, anti-Shh, anti-Oct4, and anti-Sox2 antibodies (Abcam). The percentage of positive cells was counted. Immunohistochemical analysis of β-catenin, an activator of canonical Wnt-signaling, was conducted to determine the percentage of positive cells with membrane, cytoplasmic, and nuclear localization of the protein. Immunohistochemical reactions were performed according to the manufacturer's recommendations and visualized using an Abcam imaging system. Sections without incubation with primary antibodies were used as a negative control. Sections were poststained with Mayer's hematoxylin.

Statistical processing was performed using Statistica 7.0 software (StatSoft, Inc.). Central tendencies and dispersion of quantitative signs having approximate normal distribution were described by $M \pm SEM$. Quantitative parameters in independent groups were compared using the Student's t test with Levene's test for equality of variance and χ^2. The differences were considered statistically significant at $p<0.05$.

RESULTS

In pubertal rats, the adrenal medulla was composed of clusters of chromaffin cells separated by venous sinusoids. The chromaffin cells with round and oval light nuclei and cytoplasm with tinctorial properties ranging from slightly to sharply basophilic occupied about $^3/_4$ of the medulla area. Clusters of chromaffin cells were separated by connective tissue layers and were in contact with venous and capillary sinusoids. The venous sinusoids contained no blood cells and plasma; the capillaries had extremely small diameter. Neurons and zona reticularis cell clusters were also found in the medulla (Fig. 1, *a*).

Immunohistochemical detection of tyrosine hydroxylase, a marker of high degree of differentiation of chromaffin cells, showed very high expression of this enzyme in 100% of chromaffin cells (Fig. 2, *a*). Ki-67 was detected in a small number (~2.5%) of proliferating chromaffin cells (Fig. 3, *a*, *c*).

The expression of β-catenin was detected in 5-6% chromaffin cells. Cells with membrane localization and less often nuclear localization (about 25% of immunopositive cells) of β-catenin were found (Fig. 4, *a*, *c*). Fibroblasts and endotheliocytes were characterized by a significantly higher accumulation of β-catenin in the cytoplasm.

Among chromaffin cells, $Oct4^+$ cells with nuclear localization were extremely rare, and their number did not exceed 1.5% (Fig. 5, *a*, *c*); transcription factor Sox2 was not detected. Solitary cells with nuclear localization of Shh signaling molecule were found (~1.5% of all chromaffin cells) (Fig. 6, *a*, *c*).

After puberty (by day 70 of postnatal development), the size of the rat adrenal gland was significantly higher than on day 42. The medulla was contained large clusters of chromaffin cells separated by thin connective-tissue septa. The cells were in contact with thin capillaries and with venous sinusoids with empty lumens. The total area of the medulla and the area of chromaffin cells increased, while the parenchyma/stroma ratio did not change (Fig. 1, *b*, *c*, *d*). Similar to the previous age period, the content of tyrosine hydroxylase in the cytoplasm was high in all chromaffin cells (Fig. 2, *b*). Proliferative activity of cell was decreased, as was seen from a 20% decrease in number of $Ki\text{-}67^+$-chromaffin cells (Fig. 3, *b*, *c*).

We observed a 3-fold increase in the proportion of chromaffin cells with nuclear localization of β-catenin, unchanged content of cells with its cytoplasmic

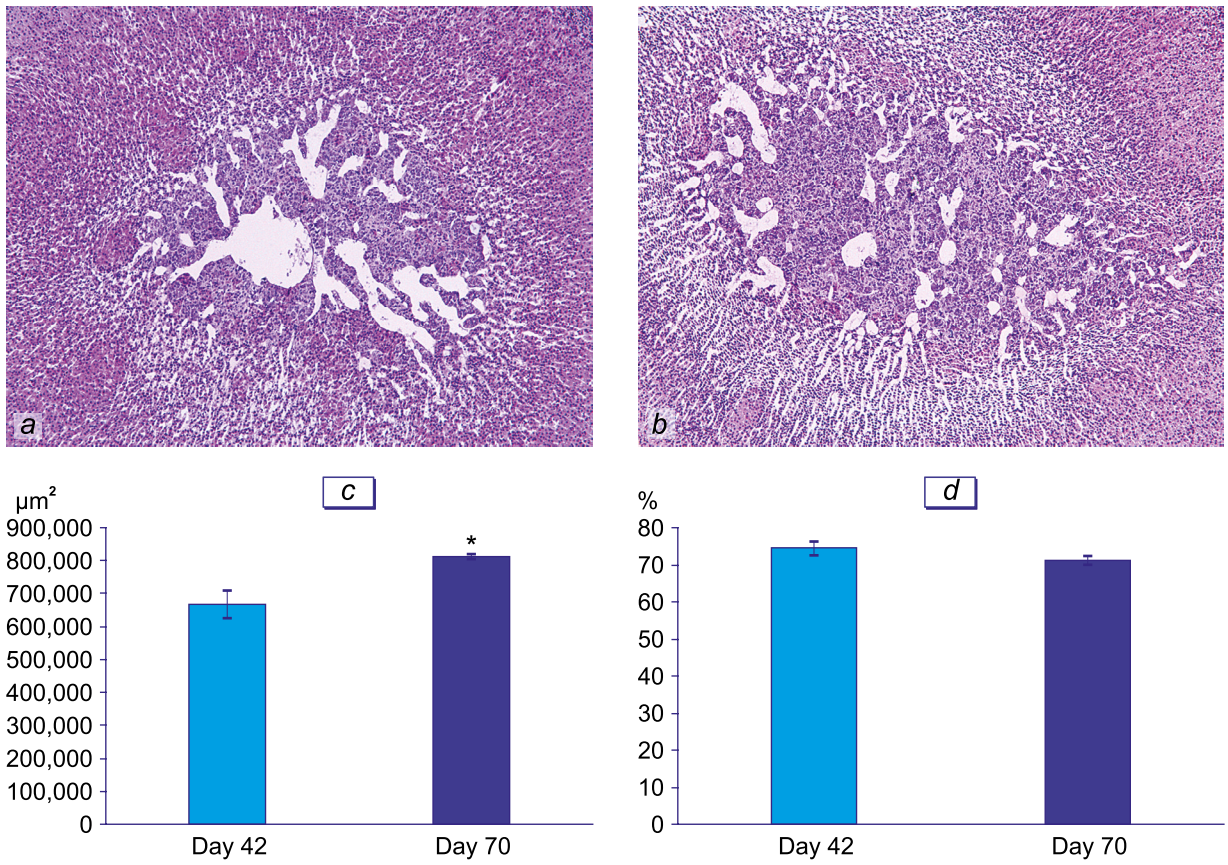

Fig. 1. Parameters of the development of rat adrenal medulla on days 42 (*a*) and 70 (*b*) of postnatal development. Hematoxylin and eosin staining, ×50. *a*, *b*) Structure of adrenal medulla on days 42 and 70, respectively; *c*) area of the medulla in equatorial sections of the adrenal glands; *d*) proportion of chromaffin cells in the area of medulla. *$p<0.05$.

localization, and a 2-fold decrease in the relative number of cells with plasmalemmal β-catenin; the total number of β-catenin$^+$ cells did not change in comparison with the pubertal period (Fig. 4, *b*, *c*). The accumulation of β-catenin in the cytoplasm of capillary endotheliocytes and fibroblasts was also observed.

By the beginning of the postpubertal period, the number of Oct-4$^+$ cells increased by 3 times and they were characterized by the nuclear localization of this factor (Fig. 5, *b*, *c*). The proportion of Shh$^+$ cells increased significantly and reached 5%, similar to the previous age period, Shh ligand was detected

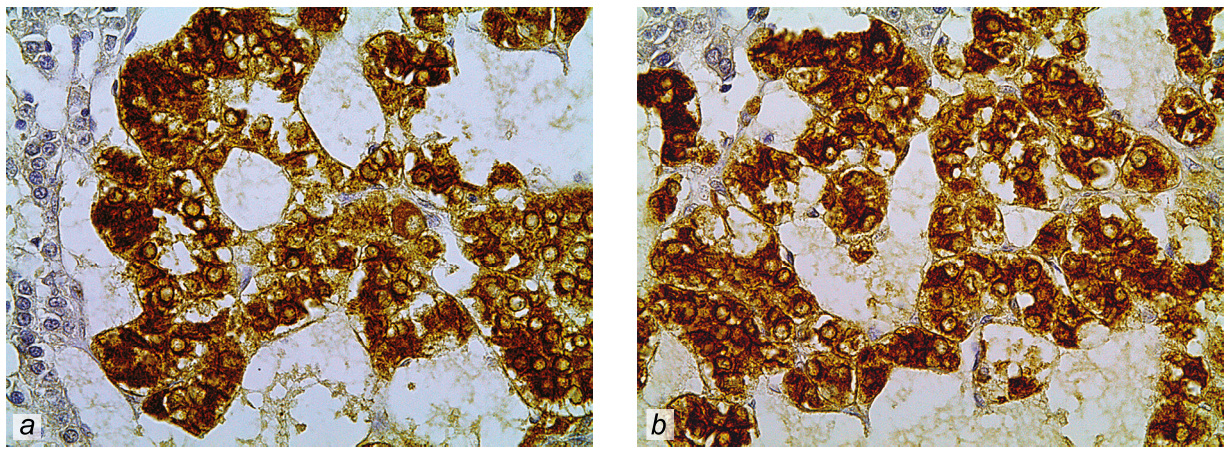

Fig. 2. Expression of tyrosine hydroxylase by chromaffin cells of rat adrenals on days 42 (*a*) and 70 (*b*) of postnatal development. Poststaining with Mayer's hematoxylin, ×400.

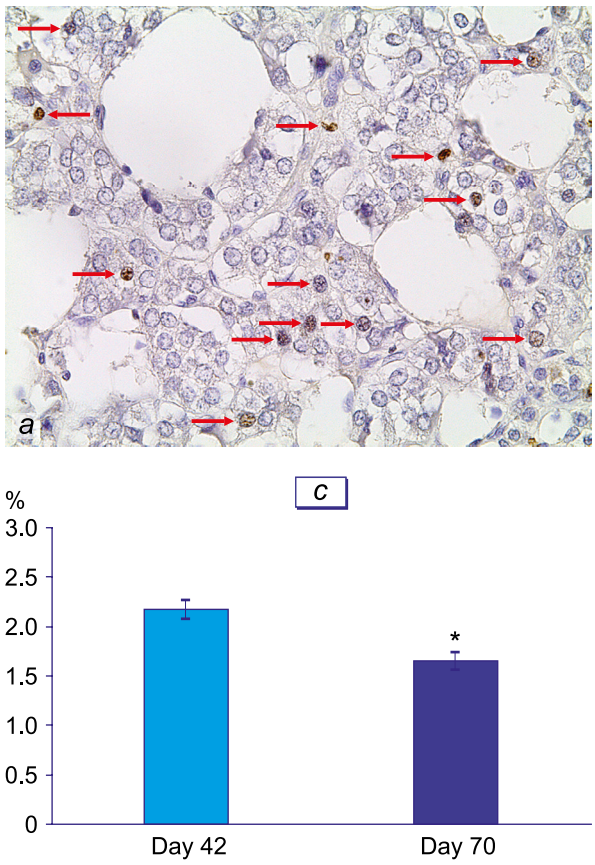

Fig. 3. Proliferative activity of chromaffin cells of rat adrenals on days 42 (*a*) and 70 (*b*) of postnatal development. Poststaining with Mayer's hematoxylin, ×400. *a*, *b*) Ki-67$^+$-chromaffin cells (arrows) in the adrenal medulla on days 42 and 70, respectively; *c*) age-related changes in the number of Ki-67$^+$-chromaffin cells in the adrenal medulla. *p<0.05.

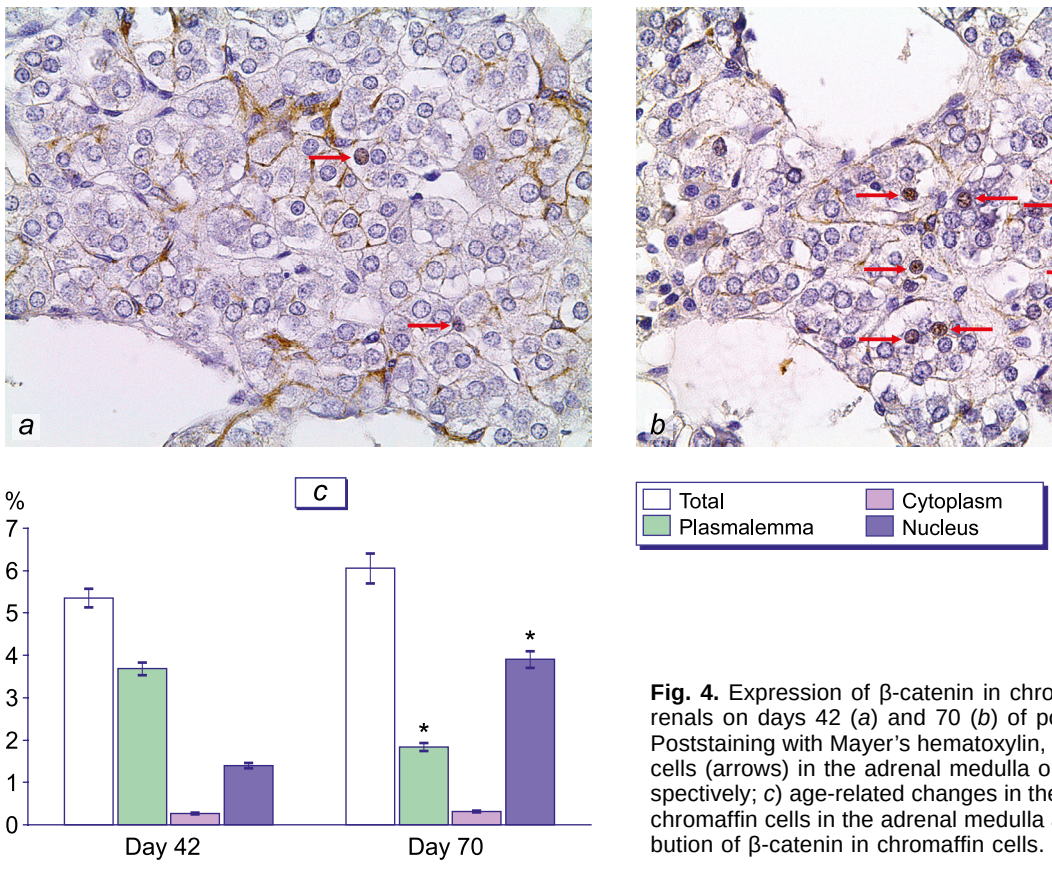

Fig. 4. Expression of β-catenin in chromaffin cells of rat adrenals on days 42 (*a*) and 70 (*b*) of postnatal development. Poststaining with Mayer's hematoxylin, ×400. *a*, *b*) β-Catenin$^+$ cells (arrows) in the adrenal medulla on days 42 and 70, respectively; *c*) age-related changes in the number of β-catenin$^+$ chromaffin cells in the adrenal medulla and intracellular distribution of β-catenin in chromaffin cells. *p<0.05.

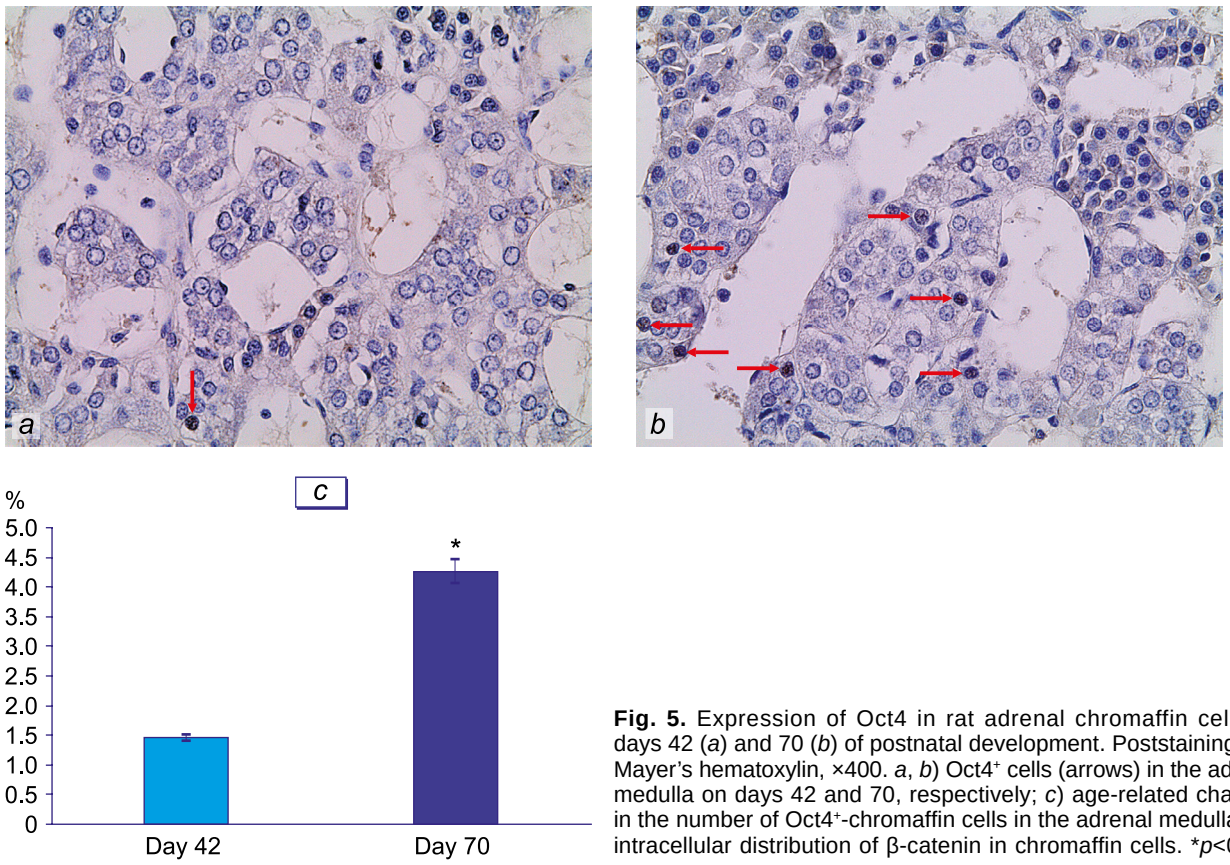

Fig. 5. Expression of Oct4 in rat adrenal chromaffin cells on days 42 (*a*) and 70 (*b*) of postnatal development. Poststaining with Mayer's hematoxylin, ×400. *a*, *b*) Oct4$^+$ cells (arrows) in the adrenal medulla on days 42 and 70, respectively; *c*) age-related changes in the number of Oct4$^+$-chromaffin cells in the adrenal medulla and intracellular distribution of β-catenin in chromaffin cells. *p<0.05.

only in the nucleus (Fig. 6, *b*, *c*). Sox2 was still undetectable.

Embryonic development of the adrenals and regeneration of adult adrenals are known to share common features in terms of regulatory pathways and transcription factors [8]. Proliferation and differentiation of chromaffin cells are the leading processes in postnatal morphogenesis of the medulla. The proliferative activity of chromaffin cells naturally decreases as the adrenal gland grows [9]. Hence, there should be a possibility of induction of cell division and differentiation. Wnt-signaling affects many aspects of nervous tissue development and function. Wnt proteins activate multiple signaling pathways and induce a variety of cellular processes, including cell proliferation and differentiation, changes of gene transcription, cytoskeleton rearrangements, *etc.* [10]. We demonstrated that β-catenin is expressed by adrenal chromaffin cells during postnatal development. It should be noted that the total number of β-catenin$^+$ cells does not change with age, while the number of cells with translocation of β-catenin into the nucleus increases after completion of organ growth. Hence, canonical Wnt-signaling in chromaffin cells is enhanced after growth arrest. Chromaffin cells, like other endocrine cell types, are in close proximity to a well-developed capillary network, which they need to secrete their products. We found that capillary endotheliocytes accumulate β-catenin in the cytoplasm; hence, signals originating from the vascular network may presumably contribute to the differentiation and function of chromaffin cells, because, according to the data obtained in 1980-1990, differentiation of chromaffin cell precursors is affected by signals originating from the adjacent endothelium.

Adrenal chromaffin cells and sympathetic neurons of the autonomic nervous system originate from common neural crest precursor cells [11]. The Shh signaling pathway plays an important role in neurogenesis and neuronal pattern formation during nervous system development [12]. When Shh binds to its receptor Patched (Ptch), it inhibits the G-protein-coupled receptor Smoothened (Smo), which leads to activation of glioma-associated oncogene homologue 1 (Gli-1). Activated Gli-1 promotes the expression of many target genes that regulate the growth, survival, and differentiation of various types of cells, including neurons [13]. Shh expression is activated in neurons during ischemia/hypoxia [14], and inhibition of the Shh pathway has been shown to aggravate ischemic brain neuronal damage in rats, indicating a role of Shh in regeneration of the nervous tissue [15]. In the present study, we demonstrated the presence of chromaffin cells with nuclear localization of Shh ligand and increase of their number with age to the end of

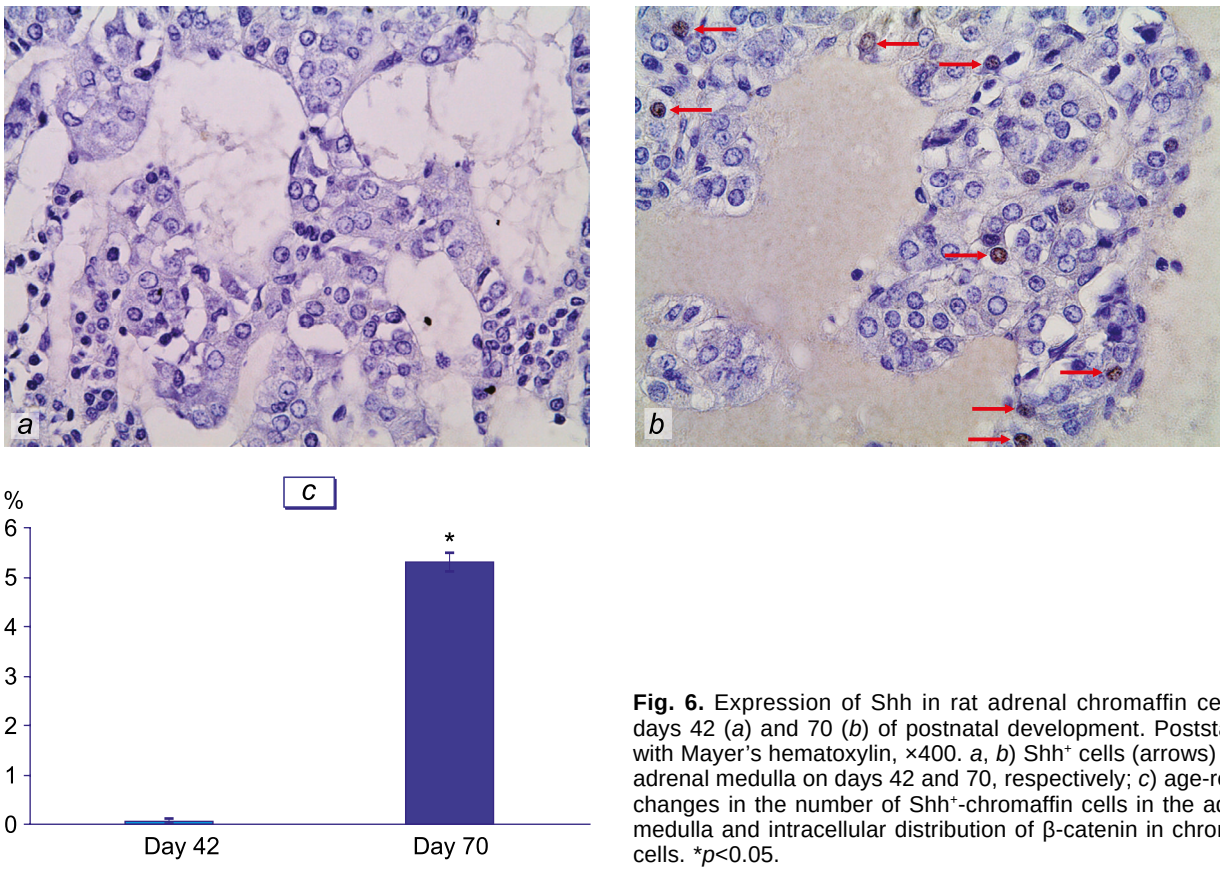

Fig. 6. Expression of Shh in rat adrenal chromaffin cells on days 42 (*a*) and 70 (*b*) of postnatal development. Poststaining with Mayer's hematoxylin, ×400. *a*, *b*) Shh^+ cells (arrows) in the adrenal medulla on days 42 and 70, respectively; *c*) age-related changes in the number of Shh^+-chromaffin cells in the adrenal medulla and intracellular distribution of β-catenin in chromaffin cells. *$p<0.05$.

adrenal growth. It has been previously found that mechanically constitutive activation of Shh signaling correlates with the activity of Wnt-signaling that is activated in the cortical substance during regeneration [4]. On the basis of our data, it can be assumed that similar processes take place in the adrenal medulla, and such readiness for self-renewal of cells increases after completion of organ growth.

The transcription factors Oct4 and Sox2 are expressed in very early stages in mammalian embryogenesis [16,17]. Oct4 is crucial for maintaining pluripotency in the inner cell mass during embryogenesis and has unique structural properties that distinguish it from other members of the POU family [18,19]. Sox2 is also crucial for the maintenance of pluripotency, but is not essential for its induction during embryogenesis, possibly due to overlap with other members of the Sox family [20]. We demonstrated that transcription factor Oct4 is also synthesized in chromaffin cells of the adrenal medulla during postnatal development. Completion of growth was accompanied by a natural decrease in the number of proliferating cells. High levels of tyrosine hydroxylase expression detected in all chromaffin cells indicates their terminal differentiation and significant functional activity both during the period of the medulla growth and after its completion [21]. These data mean that the detected cell expressing Ki-67, nuclear β-catenin, Shh, and Oct4 are highly differentiated chromaffin cells and indicate the absence of a pool of undifferentiated precursors in the medulla. Oct4 expression is not directly related to proliferation as the percentage of $Oct4^+$ cells increases with a decrease in the proportion of $Ki\text{-}67^+$ cells. Activation of Oct4 and Shh expression and their binding to the DNA of chromaffin cells indicates the possibility of their dedifferentiation [3]. Similar phenomena have been detected in the adult adrenal cortex [22,23]. Oct4 and Sox2 directly establish both active and silent transcriptional states in pluripotent cells in a large number of genes [24]. Despite the presence of Oct4 expression in chromaffin cells, we could not immunohistochemically detect the presence of Sox2 expression. Perhaps its presence is not either a prerequisite for maintaining the regenerative potential of adrenal medulla or Sox2 expression is short-lived, as there is evidence that this factor is required for induction rather than maintenance of pluripotency [25]. At the same time, the number of Oct4 and Shh^+ cells and cells with canonical Wnt-signaling activation was similar, indicating a possible equal participation in the self-renewal processes of medulla cells. These data correlate with the findings of other researchers who showed the possibility of obtaining

cells capable of transformation into dopaminergic neurons under certain conditions *in vitro* from adult adrenal medulla [6].

Thus, the completion of rat adrenal development is accompanied by the increased expression and binding to DNA of the transcription factor Oct4 and Shh ligand as well as by the activation of canonical Wnt-signaling in the highly differentiated chromaffin cells, which can be considered as a mechanism of physiological regeneration of the medulla by creating a pool of differentiated cells for further dedifferentiation.

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Conflict of interests. The authors declare no conflict of interests.

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