

Growth of cell populations of the intralobular duct in the submandibular gland of the mouse during postnatal development

Crescimento das populações de células dos ductos intralobulares na glândula submandibular do camundongo durante o desenvolvimento pós-natal

Rumio Taga*
Luiz Carlos Pardini**

ABSTRACT: The growth of cell populations of intercalated ducts (ID), striated ducts (SD) and convoluted granular tubules (CGT) of the submandibular gland of the mouse was analyzed, during the period of 14 to 84 days of postnatal life, by means of morphometric methods. The absolute number of cells in each type of duct was submitted to curve fitting by means of linear regression analysis, and the obtained equations were: $y = 13.22 + 0.87x$ for ID cells in the growth period of 14 to 84 days; $y = -18.82 + 3.69x$ and $y = 176.03 - 1.93x$ for SD cells in the periods of growth and decay of cells, from 14 to 35 and 35 to 84 days, respectively; and $y = 90.59 + 4.60x$ for CGT cells in the growth period of 28 to 84 days. Based on these equations, the daily accumulation (growth rate) or loss (decaying rate) of cells in the period covered by each equation was calculated. Thus, the rate of growth of ID cells was 0.87×10^5 cells/day; the growth and decline rates of SD cells were 3.69×10^5 cells/day and -1.93×10^5 cells/day, respectively; and the growth rate of CGT cells was 4.60×10^5 cells/day. The analysis of the accumulation or loss of cells and the balance of cell populations in the various types of ducts revealed that SD cells were transformed into CGT cells and that the growth of these cells' population also depends on the formation of new cells through proliferative activity, probably in the intercalated ducts.

DESCRIPTORS: Cells; *Corpus striatum*; Submandibular gland; Development.

RESUMO: O crescimento de população de células dos ductos intercalares, ductos estriados e túbulos granulares convolutos de glândulas submandibulares do camundongo durante o período de 14 a 84 dias de vida pós-natal, foi avaliada por métodos morfométricos. Os dados de número absoluto de células em cada tipo de ducto foram submetidos a ajuste de curva por análise de regressão linear e as equações obtidas foram: $y = 13,22 + 0,87x$ para as células dos ductos intercalares no período de crescimento de 14 a 84 dias; $y = 18,82 + 3,69x$ e $y = 176,03 - 1,93x$ para as células dos ductos estriados nos períodos de crescimento e decaimento, respectivamente, de 14 a 35 e 35 a 84 dias; e $y = 90,59 + 4,60x$ para as células dos túbulos granulares convolutos no período de crescimento de 28 a 84 dias. À partir dessas equações, o acúmulo diário (velocidade de crescimento) ou a perda diária (velocidade de decaimento) de células em cada período, foram calculados. Assim, a velocidade de crescimento das células dos ductos intercalares foi $0,87 \times 10^5$ células/dia; as velocidades de crescimento e decaimento de células dos ductos estriados foram, respectivamente, $3,69 \times 10^5$ células/dia e $-1,93 \times 10^5$ células/dia; e a velocidade de crescimento das células dos túbulos convolutos foi $4,60 \times 10^5$ células/dia. Análise do acúmulo ou da perda de células e o balanço entre o crescimento das várias populações celulares ductais, mostraram numericamente que as células dos ductos estriados transformaram-se em células dos túbulos granulares convolutos e que o crescimento total na população dessas últimas células também depende da formação de novas células por atividade proliferativa, provavelmente nos ductos intercalares.

DESCRIPTORES: Células; Corpo estriado; Glândula submandibular; Desenvolvimento.

INTRODUCTION

It is known that the interlobular duct system of rodent submandibular gland is constituted essentially by the intercalated duct (ID), convoluted granular tubule (CGT) and striated duct (SD)^{2,5,8,13,17,18,20,26,27}. Among these three structures, the CGT is the most conspicuous in histological sections due to its enor-

mous total volume and to the fact that its cells are packed with serous secretory granules^{5,17}.

In the mouse, these tubules are much more developed in males than in females, which characterizes a marked secondary sexual dimorphism^{2,3,10,14,16}.

Electron microscopic studies both in rats⁶ and mice⁸ revealed that their secretory cells are prima-

*Chairman, Department of Biological Sciences, School of Dentistry of Bauru; **Associate Professor, Department of Stomatology, School of Dentistry of Ribeirão Preto - University of São Paulo.

rily differentiated from typical SD cells during the postnatal development. Studies on growth kinetics using H3-thymidine labeling have confirmed this event and suggested that IDs also participate in the supply of cells for CGTs^{4,7,22,23,29}.

The objective of the present investigation was to establish the numerical relationship between the growth of these three types of duct cell populations during the postnatal development of the submandibular gland of the mouse, allowing to numerically characterize cell migration from one type of duct to another.

MATERIALS AND METHODS

Male mice (*Mus musculus*, Swiss strain) were obtained from a colony maintained in the Central Animal House, Dentistry School of Bauru, University of São Paulo, Brazil. The animals were assigned to groups of five subjects each, according to their age: 14, 21, 28, 35, 42, 56 and 84 days of postnatal life. The glands were collected always between 8:00 and 10:00 a.m. to avoid circadian variations. The animals were anaesthetized by means of inhalation of ethyl ether, and the body mass of each animal was determined using a Mettler P1000 scale. The submandibular glands of each animal were carefully dissected and rapidly removed, and its fresh mass was determined using a Mettler H-20 precision scale. The glands were fixed in Helly's fluid for 3 h, rinsed overnight in running water, dehydrated in ethyl alcohol, cleared in xylene and embedded in paraffin. Alternate 5-mm thick sections were obtained at intervals of 50 μ m and stained by means of the Masson trichromic method.

Determination of the processed gland volume (Vp)

The processed gland volume (Vp) was calculated based on the fresh gland mass (m), using the formula: $Vp = m/\delta \times Sf$, where d is the average density of the gland and Sf , the correction factor for the observed shrinkage. The density of the gland (δ) was determined in 20 submandibular glands from 10 young-adult mice by means of the method of Scherle, according to indications of Mandarim-de-Lacerda¹⁵. The shrinkage caused by the histological procedures was evaluated in 20 submandibular glands from other 10 young-adult animals by means of the method of Taga and Sesso²⁴. The mean density (δ) obtained was 1.09 g/mm³, with a coefficient of variation of 0.5%,

and the shrinkage factor (Sf) was 0.86, with a coefficient of variation of 17.2%.

Morphometric determination of the absolute number of cells (Ni)

The counting of cells in each type of duct was carried out by means of a Zeiss microscope with an oil-immersion 100 X objective and an 8 X Zeiss Kpl eyepiece containing a Zeiss II integration grid with 10 parallel lines and 100 points distributed within a quadrangular area. The number of cell nuclei (ni) in each type of duct (i) and the crossings (ci) between the nuclear images and grid lines were counted in 50 histological fields per animal, which were selected by means of systematic sampling. The nuclei that fell on the forbidden line were not counted to avoid overestimation of data¹⁰. The absolute number of nuclei for each type of cell (ni) was calculated using the formula of Aherne¹:

$$ni = \frac{2n^2 Vp}{A(i^2 d + 2t)}$$

where: A = total examined area, i = ci/ni, d = distance between the grid lines, and t = thickness of the histological section.

The number of 50 microscopical fields per animal was determined during the pilot work, by multiple χ^2 sample homogeneity test with a probability level²⁵ of 0.05.

It should be pointed out that in the 28-day-old group it was not possible to identify tubules in the paraffin-embedded sections – a very small number of secretory granules was observed, thus, in the counts, these cells were scored in SDs. In borderline instances, i.e., in segments of intralobular ducts containing a mixture of cells with a small number of secretory granules and cells apparently without them, cells were individually recognized and scored in CGTs and SDs, respectively.

During counting, the number of nuclei from cells of other structures, stroma and glandular parenchyma, was also recorded. Thus, the frequency of cells (%) of each type of duct in the gland was calculated.

Statistical analysis

The morphometric data of each age group was compared with that of the other groups by means of ANOVA, using the Sigma Stat-Jadel Scientific Software for Windows version 1.0, with the level of significance set at 0.05 and 0.01. According to recommendations of Russ and Dehoff¹⁹, the data on gland mass and absolute cell number in each

type of duct were submitted to curve fitting in function of the age by means of linear regression analysis using the Arcus Professional Statistical Analysis Software version 2.0XTc. The quality of curve fitting was assessed examining the value of the coefficient of determination (r^2). Based on the equations, the daily gain in mass and the accumulation or loss of cells in each duct type were calculated.

RESULTS

The evolution of fresh gland mass from the 14th to 84th day of postnatal life is presented in Graph 1.

Between 14 and 84 days of age, fresh gland mass increased significantly (621%, $p < 0.01$), from 24.3 mg on day 14 to 175.2 mg on day 84. The equation for gland mass increase was: $y = -0.61 + 2.27x$ ($r^2 = 0.94$), and the calculated growth rate was 2.27 mg/day.

The frequency of cells of each type of duct, estimated as a percentage of the total number of all types of cells, is presented in Graph 2.

Statistical "F" analysis after arcsin transformation of the original data revealed that: the frequency of ID cells decreased from 15.9% to 8.1% ($p < 0.01$), from day 14 to day 35; increased from 8.1% to 10.4% ($p < 0.05$) from day 35 to day 42; and remained stable thereafter ($p > 0.05$); the frequency of SD cells increased from 14.4% to 26.9% ($p < 0.01$) from day 14 to day 28 and noticeably decreased from 26.9% to 2.1% ($p < 0.01$) from day 28 to day 84; CGT cells presented a frequency of 1.3% on day 28, which continually increased up to 39.6% until the 84th day of age ($p < 0.01$). The evolution of the total number of cells of each type of duct is presented in Graph 3.

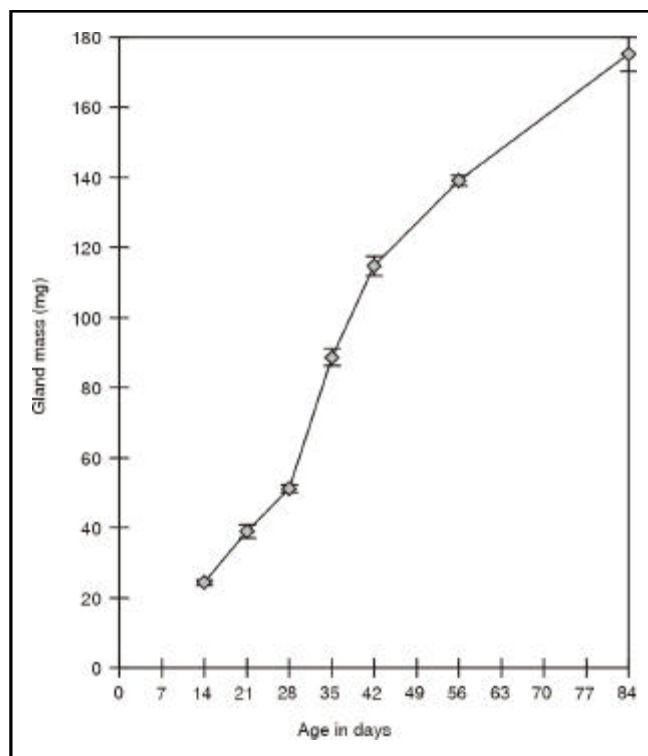
The analysis of data revealed that: the number of ID cells remained stable from day 14 to day 21 ($p > 0.05$). It presented a small increase of 25.3% from day 21 to day 28 ($p < 0.05$), remained stable ($p > 0.05$) between day 28 and day 35 and increased 138.4% ($p < 0.01$) thereafter; the evolution of SD cells was biphasic, i.e., from day 14 to day 35 and from day 35 to day 84. During the first phase they increased 307.5% ($p < 0.01$) - from 26.5×10^5 to 108.0×10^5 cells - and, in the second phase, they decreased 86.8% ($p < 0.01$) - from 108.0×10^5 to 14.3×10^5 cells. The number of CGT cells increased 8,123.5% ($p < 0.01$) from day 28 to day 84 - 3.4×10^5 to 279×10^5 cells.

The equations obtained from the regression analysis to mathematically express the evolution

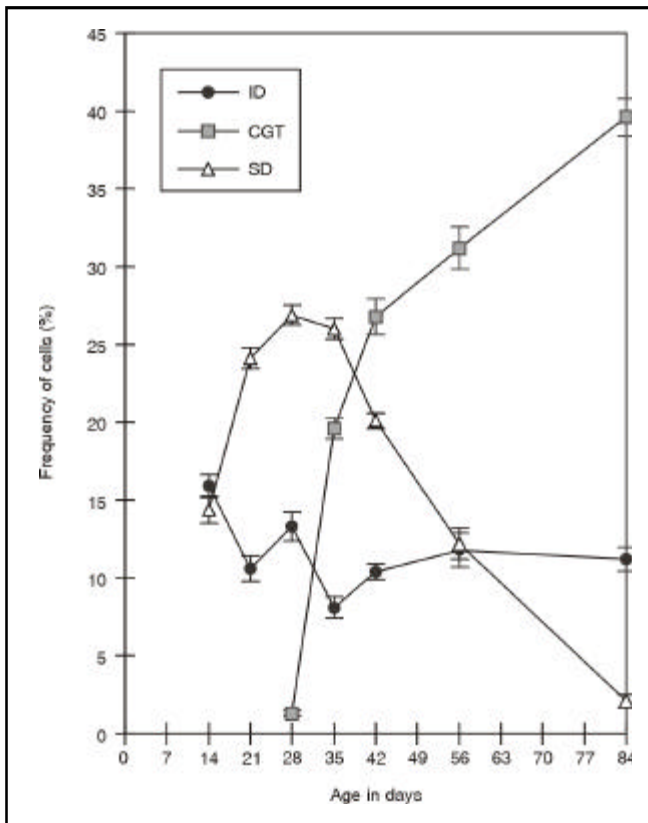
of cells of each type of duct are presented in Table 1. Although the number of intercalated duct cells exhibited a multiphasic growth pattern, it was also submitted to a single linear regression analysis in the entire period under study, with the objective of obtaining a mean value of daily cell accumulation for the period.

With these equations, the daily accumulation and the loss of cells were calculated. Whereby, the growth rate of ID cells during the period of 14 to 84 days was 0.87×10^5 cells/day; the growth and decline rates of SD cells for the periods of 14 to 35 and 35 to 84 days were 3.69×10^5 cells/day and -1.93×10^5 cells/day, respectively; and the growth rate of CGT cells for the period of 28 to 84 days was 4.60×10^5 cells/day.

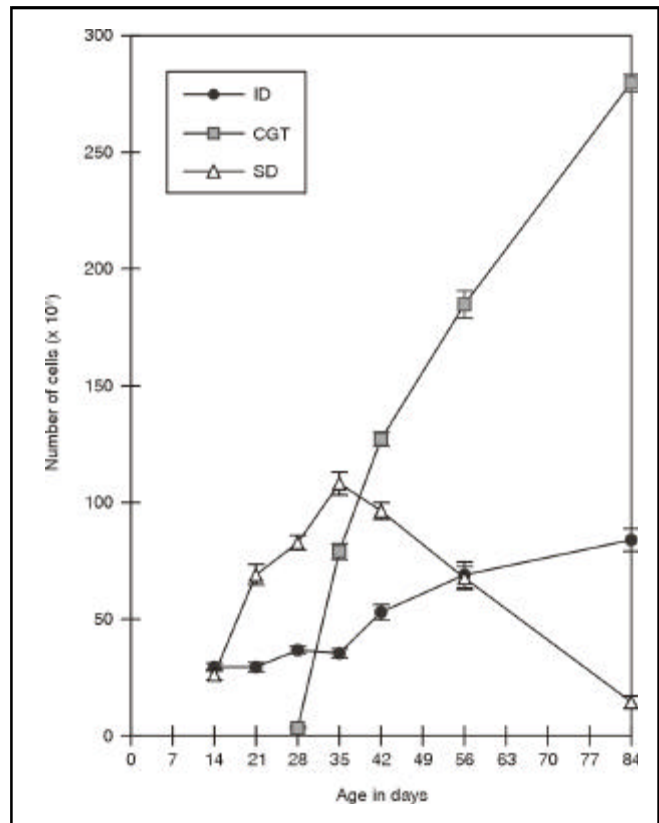
Table 2 presents the relationship between the absolute number of cells in each type of duct and the total number of duct cells in the submandibular glands of mice during the period of 28 to 84 days of postnatal life. In this table, the frequencies in parentheses represent the number of cells per type of duct as a percentage of the total number of duct cells, and the percentages of the last column represent the percental in-



GRAPH 1 - Mass increase in the submandibular glands of mice during postnatal development (bars = standard error of mean).



GRAPH 2 - Evolution of the frequency of each type of duct cell in the submandibular glands of mice during postnatal development (bars = standard error of mean; ID = intercalated duct; SD = striated duct; CGT = convoluted granular tubule).



GRAPH 3 - Evolution of the number of each type of duct cell in the submandibular glands of mice during postnatal development (bars = standard error of mean; ID = intercalated duct; SD = striated duct; CGT = convoluted granular tubule).

crease in the total number of duct cells during each stage of development.

Through the analysis of the data presented in the table, one can observe that: during the period from day 28 to day 35 of postnatal development, the total number of duct cells increased 81%, i.e., 99.3×10^5 new cells were produced by mitotic activity - 75.5×10^5 cells were added to CGTs, and 25.3×10^5 cells, to SDs -; from day 35 to day 42, the total number of cells increased 24%, i.e., there were 54.2×10^5 new cells - 17.8×10^5 cells in IDs and 36.4×10^5 cells in CGTs. Since SDs lost 11.8×10^5 cells during the same period, and since these cells migrate to CGTs, the latter presented a total growth of 48.2×10^5 cells; during the subsequent period, from day 42 to day 56, the total number of duct cells increased 16%, i.e., 45.0×10^5 new cells were observed - 16.5×10^5 cells were added to IDs and 29.0×10^5 cells, to CGTs. Furthermore, CGTs received an additional 28.6×10^5 cells from SDs,

comprising a total of 57.6×10^5 additional cells in the period; and, finally, from day 56 to day 84, the total number of new cells formed was 56.5×10^5 - 14.9×10^5 cells were added to IDs and 41.6×10^5 , to CGTs. Since the latter received more 53.3×10^5 cells previously accumulated in SDs, the total increase for the period was of 94.9×10^5 cells.

DISCUSSION

Despite the fact that studies on growth kinetics with H3-thymidine labeling, which assessed the developing and adult submandibular gland of rats and mice, have shown that the secretory cells of CGTs differentiate from typical SD cells and suggested that ID cells also participate in the supplement of cells for CGTs^{4,7,22,23,29}, there was lack of researches on the numeric characterization of these events.

Therefore, the objective of the present investigation was to determine, morphometrically, the pattern of cell accumulation in each intralobular type of duct of the submandibular gland of mice during

TABLE 1 - Linear regression equations for the growth of cell populations of the intercalated duct (ID), striated duct (SD) and convoluted granular tubule (CGT) in submandibular glands of mice ($\times 10^5$).

Cell type	Analyzed period	Equation	Determination coefficient (r^2)
ID cells	14 to 84 days	$y = 13.22 + 0.87x$	0.94
SD cells	14 to 35 days	$y = -18.82 + 3.69x$	0.96
	35 to 84 days	$y = 176.03 - 1.93x$	0.99
CGT cells	28 to 84 days	$y = -90.59 + 4.60x$	0.94

TABLE 2 - Relationship between the total number of cells in each type of duct and the total number of duct cells.

Period (days)	Cell number ($\times 10^5$) and %*			Total number of duct cells ($\times 10^5$)	Percentage of increase in the total number of duct cells
	ID	SD	CGT		
28	36.7 (29.9%)	82.7 (67.3%)	3.4 (2.8%)	122.8	-
35	35.2 (15.8%)	108.0 (48.8%)	78.9 (35.4%)	222.1	81%
42	53.0 (19.2%)	96.2 (34.8%)	127.1 (46.0%)	276.3	24%
56	69.0 (21.5%)	67.6 (21.0%)	184.7 (57.5%)	321.3	16%
84	83.9 (22.2%)	14.3 (3.8%)	279.6 (74%)	377.8	17%

*Frequency in relation to the total number of duct cells. ID = intercalated duct; SD = striated duct; CGT = convoluted granular tubule.

postnatal development, in order to numerically demonstrate cell migration from one duct to another.

The mass of the submandibular gland of the studied mice increased 621% from the 14th to the 84th day of age. During this growth in mass, the absolute number of ID cells presented a very small increase from day 14 to day 35 and a significant increase of 140% from day 35 to day 84. The number of SD cells underwent a biphasic evolution, with a growth phase from day 14 to day 35 and a distinct decline phase from day 35 to day 84. In the first phase, the number of cells increased 4.1 times and, in the second phase, it declined 7.6 times. In contrast, Srinivasan and Chang²³ observed, for ID cells, an increase of approximately 337% from day 14 to day 42; for SD cells, in the same growth and decline periods, those authors observed values much lower than those obtained in the present study.

As to the number of CGT cells, there was a marked increase of 82.2 times between day 28 and day 84 of postnatal development. It should be pointed out that Srinivassan and Chang²³ detected, in the mouse strain studied in their research, not only a greater rate of differentiation of CGT secretory cells already from day 21 to day 28, but also, a much greater contingent of these cells across all age ranges. Thus, they observed only a 4.14 times increase in the number of CGT cells

from 28 to 84 days of age. The comparison between both reports revealed that this enormous difference was due to the very small number of CGT cells observed in our study, in 28-day-old mice.

Since the beginning of the study, the possibility of underestimation of the number of CGT cells on the 28-day-old group was considered, due to the difficulty in identifying immature CGT cells with a very small number of secretory granules in paraffin-embedded sections. New counts carried out in animals with 28 and 42 days of age, by means of the method of Weibel and Gomes³², using 0.5- μ m-thick plastic embedded sections, revealed results similar to those originally obtained by means of the Aherne's method for the 42-day-old group. However, for the 28-day-old group, the results were 20 to 30% greater. Therefore, in the original counts these cells had been scored in SDs. Nevertheless, even after correcting this underestimation, the differences remained extremely high.

Thereby, the differences mentioned above indicate that the gland of the mouse strain used in the present study exhibits a significantly greater contingent of SD cells and less ID and CGT cells during the analyzed developmental period. It should be noted that the values of body and gland mass, salivary amylase, biologically active factors, and even the histo-architecture of glands can vary between inbred

mouse strains, as well as between some hybrids^{11,12,21,28}. The analysis of daily accumulation and loss of cells revealed that, from day 28 to day 35, there was a clear predominance in the growth of CGT cell population, which accumulated 5.3 times more cells/day than IDs and 1.25 times more cells/day than SDs. Meanwhile, the SD cell population accumulated 4.2 times more cells/day than the IDs. Between 35 and 84 days, SDs lost 1.93×10^5 cells/day through the transformation into CGT cells. Since the latter type of duct accumulated 4.60×10^5 cells/day, a difference of 2.67×10^5 cells/day was lacking to complete this value.

The relationship between the number of cells accumulated in each type of duct and the total number of duct cells produced during the period of 28 to 84 days of age clearly revealed that the majority of the new cells formed by mitotic activity in any of the three types of ducts accumulates in CGTs, and, after day 35, CGTs receive an additional amount of cells which were previously accumulated in SDs.

In the analysis of the data reported by Srinivasan and Chang²³, it was observed that, from 28 to 35 days, the labeling indices for ID cells were approximately 5.1, i.e., these cells, presented the greatest proliferative activity during this period. For CGT cells, the values were 1.7 and 1.4 for each age group, respectively. On the other hand, for SD cells the values were the lowest observed in the period (approximately 0.5). Between 35 and 84 days, a decrease was observed in the labeling indices of all three types of duct cells. Thus, SDs indices became insignificant and those of CGTs and IDs, between day 42 and day 84, decreased from 0.9 to 0.3 and 3.2 to 1.7, respectively.

Assuming that the pattern of evolution of labeling indices for the various duct cells of the mouse strain used in the present study is similar to that

of the above-mentioned work, and that occasional differences are only related to the magnitude of the indices, it is possible to infer that, from day 28 to 35, the new cells added to CGTs and SDs originate mostly from mitotic activity in IDs and, in small quantity, from CGTs and SDs themselves. Between day 35 and day 84, the new cells formed were added to IDs and, especially, to CGTs, where they were approximately half of the added cells and originated mainly from the mitotic activity of ID cells and, to a lesser extent, from mitosis within the own CGTs. The other half of the cells added to the CGTs came from SDs, where they were already accumulated during the previous period.

The analysis carried out in the present study numerically clarified cell migration between the three interlobular ducts in postnatal developing submandibular glands of mice, contributing to a better understanding of how the morphogenesis of the gland in question takes place.

CONCLUSION

The balance between the number of cells accumulated in the various types of intralobular ducts of the submandibular gland of mice during postnatal development numerically confirmed that striated duct cells transform into convoluted granular tubule cells. It also revealed that the growth of the latter cell population depends, substantially, on the production of new cells by means of mitotic activity, probably in intercalated ducts.

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