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# Mathematical Representation and Analysis of the Number of Epithelial Cells in the Intestine of the Mouse Embryo

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The total cell number is the most fundamental data to correctly analyze the development of the tissues and organs. In the present study, we focused on the intestinal epithelium during the period of the intestinal elongation and villi formation. We first measured by using stereology the total number of the intestinal epithelial cells from junction between the stomach and duodenum to the cecum of C57BL/6J mouse embryos at embryonic day（E）11.5, E13.5, and E15.5 during which the intestine elongates rapidly and the villi start to be formed. We then mathematically analyzed the total epithelial cell number data and formulate the proliferation. The results suggest that the intestinal epithelial cell proliferation is exponential and expressed using the same formula with that for rapidly growing *in vitro* cultured cells during the log phase. The mechanism which supports the exponential cell growth and the biological significance are discussed.

Keywords: Intestine, epithelial cells, exponential growth, mouse embryo.

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## INTRODUCTION

Epithelial tubular tissues throughout the body including the intestinal epithelium develop regulating common basic events such as cell proliferation and cell death. Tissue-specific cell differentiation and differential regulation of cell proliferation/death as well as movement/positional rearrangement of the cells lead the formation of various organs. Harmonized regulation of these events realizes the development of these different organs with reproducible sizes and shapes within a normal range, and dysregulation of these events causes various types of morphologic developmental disorders of the organ［1, 2］. In the development of the intestine, multiple signals are involved in the regionalization of the epithelium through the interaction between the epithelium and the underlying mesenchyme（for example, regarding the midgut/duodenum see, ［3］）. During the early development, the intestinal epithelium is pseudostratified columnar and appears homogeneous throughout the entire length of the intestine, however, during the later development, the epithelium becomes stratified/multi-layered in the oral cavity, pharynx, esophagus, and anus, while it becomes single columnar with regionally different cellular components in the stomach and small/large intestine［3, 4］. For understanding these complex developmental phenomena, the most basic information is the exact cell number in each location. Due to lack of information of the cell number, the wrong development-related theories tend to retain for many years in the field and repeatedly mentioned in the world-wide standard textbooks. One of these examples is the temporary occlusion of the duodenal lumen during the organogenetic period. This interesting finding has been interpreted as due to the over-proliferation of the epithelium to occlude the lumen［5, 6］. However, by simply counting the cell number in the transverse

sections of the midgut/duodenum during the period of the temporal occlusion of the lumen, we elucidated that the occlusion occurs due to the convergent extension but not due to the overproduction of the epithelial cells [3, 7].

Further, upon the acquisition of the cell number as a basic information, the mathematical analyses are necessary to elucidate the principle of the developmental events such as how cell proliferation is regulated to reproducibly form the tissues and organs of normal size and shape [2]. Molecular analysis can clarify the precise mechanism at the very localized site, but cannot show the overall view such as how cell numbers are regulated in the organogenesis and histogenesis of the organs such as the entire length of the intestine.

Regarding the principle of cell proliferation, it is well known that the growth of cultured epithelial cells is expressed by an exponential function. When cells are cultured *in vitro*, the cells grow exponentially during the log phase as long as the space and nutrition are available in the container [8]. However, the cultured cells cannot grow beyond their ecological range and eventually stop growing. Under the *in vivo* condition where numerous conditions are related in a complex way, and therefore, the behavior of cell proliferation should be regulated differently from the *in vitro* condition. However, the principle of cell proliferation in the *in vivo* condition has not been addressed due to lack of accurate information of total cell number in the organ such as the intestine.

In the present study, therefore, we first counted the cell number in the total length of the intestine from the beginning of the duodenum to the cecum using the stereology [9], from embryonic day (E) 11.5 to E15.5, during which the intestine elongates more rapidly than the whole body growth rate at least partially due to the above-mentioned convergent extension mechanism and causes a temporary physiological herniation from the abdominal cavity [5, 6]. We then examined whether or not the cell proliferation in the intestine *in vivo* can be expressed by the same principle of *in vitro* condition.

Unexpectedly, we elucidated being based on the change in the counted total cell number that the intestinal epithelial cell proliferation *in vivo* from E11.5 to E15.5 in the mouse embryo is expressed by an exponential function as in the log phase of *in vitro* culture condition.

## MATERIALS AND METHODS

### *Animals*

This study was approved by the Ethics Committee for Animal Experimentation of Shimane University (IZ26-20, IZ30-54), and the animals were handled in accordance with the institutional guidelines. C57BL/6J mice (CLEA Japan, Tokyo) between 8 and 20 weeks of age were used. The mice were housed in the Department of Experimental Animals, Interdisciplinary Center for Science Research, Organization for Research and Academic Information, Shimane University. They were kept under conditions of constant temperature and humidity, and a controlled 12/12 h light/dark cycle. They were given a standard laboratory diet and water, both *ad libitum*. Single potent male and female mice were mated overnight in the same cage, and noon of the day when a vaginal plug was observed was defined as embryonic day 0.5 (E0.5). One embryo for each E11.5, E13.5, E15.5 was obtained under the deep anesthesia (total number of embryos, n = 3, one from each dam). The crown-rump length (CRL) was measured and the intestine was immediately dissected out in the physiological saline. To obtain the positional information and to make transverse sections, the intestine was straightened by removing the mesentery very carefully under a dissection microscope. The intestine was fixed in 4% formaldehyde/70% ethanol at 4°C overnight. The fixed samples were embedded in paraffin and 5 μm-thick serial transverse sections were made from the junction between the stomach and duodenum to the cecum.

### *Caudal-type homeobox protein2 (Cdx2) immunostaining*

The sections were immune-stained with anti-Cdx2 antibody for cell counting. Cdx2 is a transcription factor which is expressed specifically in the intestinal epithelial cells [10]. After deparaffinization, the sections were post-fixed with 4% formaldehyde/70% ethanol and endogenous peroxidase was deacti-

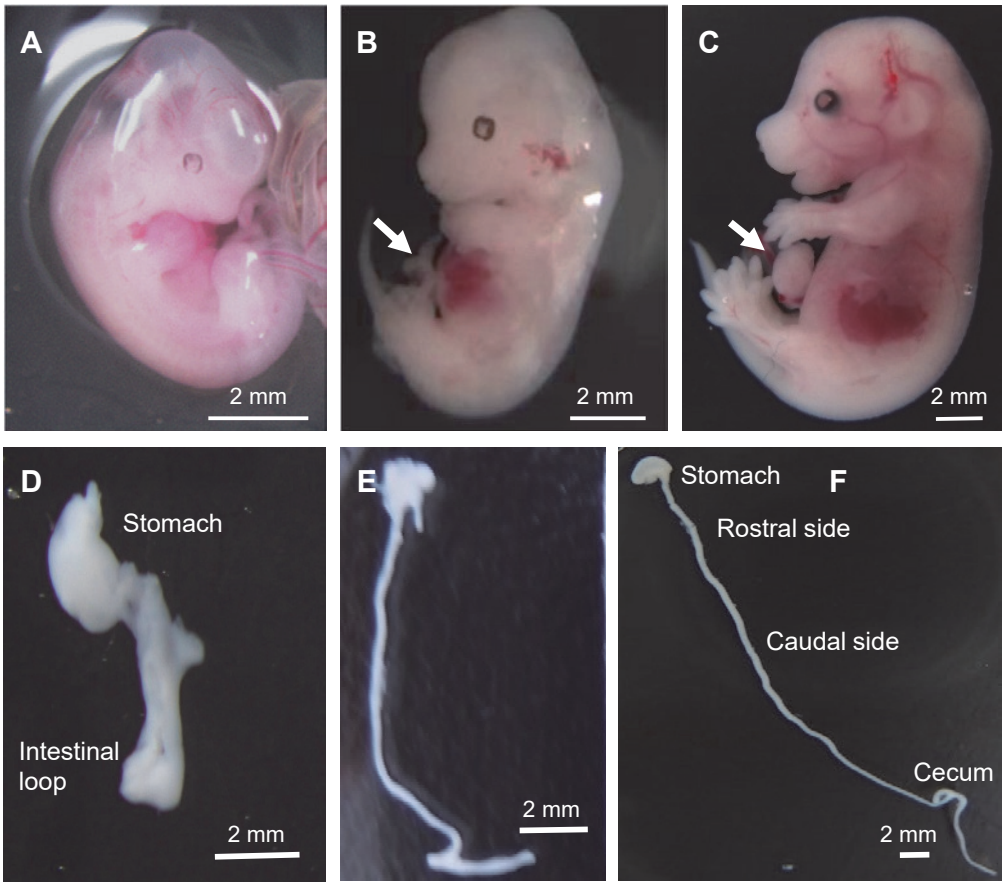


Fig. 1. E11.5, E13.5, E15.5 mouse embryos and the intestines.
Embryos at E11.5 (A), at E13.5 (B), and at E15.5 (C), and the stomach and intestine of the embryos of E11.5 (D), E13.5 (E), and E15.5 (F). The entire gastrointestinal tract was dissected from the mouse embryos and straightened for those at E13.5 and E15.5. Compared to the increase in CRL, the intestine grows more rapidly. Omphalocele/physiological herniation (arrows) is observed in embryos of E13.5 and E15.5. Scale bars: 2 mm.

vated by 0.03% hydrogen peroxide/99% methanol. The sections were treated for antigen activation in 10 mM citric acid buffer (pH 10.0) for 5 min at higher than 90°C heated by microwave. After cooling down lower than 50°C, normal goat serum and M.O.M. mouse Ig blocking reagent (Vector Laboratories, Burlingame, CA) were used for blocking. The primary antibody reaction was performed with monoclonal mouse anti-human clone DAK-CDX2 (1:50, Dako, Glostrup, Denmark), followed by the secondary reaction using M.O.M. biotinylated anti-mouse IgG reagent and Avidin-Biotin Complex reagent (Vector). Sections were then visualized with 0.05% 3,3'-diaminobenzidine-tetrahydrochloride (Dako). Nuclei were counter-stained by hematoxylin.

***Cell number counting by stereology***

Stereology is a branch of science that infers the three-dimensional properties of objects based on two-dimensional views of them, and is used to estimate, for example, the total cell number in the tissue by sampling randomly and systematically from two-dimensional sections of the three-dimensional structure such as the neural tube [9] and the intestinal epithelial tube. Using the Stereo Investigator®, the measurement was performed at E11.5 with 240 μm interval (11 measured / total 249 sections), at E13.5 with 600 μm interval (22 measured / total 2545 sections), and at E15.5 with 1 mm intervals

(36 measured / total 7100 sections).

***Analysis software***

Image J (National Institutes of Health, Bethesda, Maryland, USA) and Excel built-in software (exponential function analysis, multinomial analysis, linear regression analysis) were used.

## RESULTS

***Morphology of the intestine and cell counting***

Mouse embryos (E11.5, CRL: 7.27 mm; E13.5, CRL: 10.57 mm; E15.5, CRL: 16.46 mm, Table 1) examined and the stomach/intestine are shown in Fig. 1. When compared with the growth of the body (Fig. 1A, B, C), the intestine elongates rapidly from E11.5 to E13.5 and from E13.5 to E15.5 (Fig. 1D, E, F). Accordingly, the intestine is protruded from the abdominal cavity and exhibits the physiological herniation at E13.5 and E15.5 (arrows in Fig. 1B, C).

Cdx2 is expressed specifically in the intestinal epithelial cells [10], however, is not expressed in the stomach epithelium. We therefore determined the start point of the intestine (junction between the stomach and intestine) at the first Cdx2-positive section (0 point in the following figures and tables) in the serial sections (not shown), and the end point at the cecum which can be clearly observed macroscopically (Fig. 1). We measured the length of the intestine and counted the cell numbers within the determined length of the intestine. The length of the intestine was 1.25 mm, 12.75 mm, 35.5 mm at E11.5, E13.5, and E15.5, respectively (Table 1). While the CRL increased by 2.27-fold, the intestine increased by 28.4-fold (from E11.5 to E13.5, 10-fold; from E13.5 to E15.5, 2.8-fold). The cell number distribution along the long (rostral/caudal) axis of the intestine is shown in Fig. 2. At E11.5, the cell numbers per section differed regionally and tended to be larger (approximately 100) in the middle part, and smaller at the rostral and caudal parts (Fig. 2). The pseudostratified cell layer number is 1 to 2 in the rostral and caudal parts, and 2 to 3 in the middle part (not shown). At E13.5, the cell number per section tended to be rather constant throughout the entire length (except for the 7800 μm point probably due to the oblique-angle of the sectioning) and nearly the same with the middle part at E11.5 (approximately 100) (Fig. 2). The pseudostratified cell layer number is 3 throughout the entire length (not shown). At E15.5, the cell number per section became much larger than at the earlier dates, and tended to be the larger at the rostral side and gradually decreased toward the caudal end (Fig. 2). The epithelium at the rostral part was simple columnar and villi formation was observed, whereas in the more caudal parts, the epithelium was pseudostratified with the more layer number and villi were the less developed (not shown).

The total cell number counted by stereology was 26,992, 301,770, and 2,746,667 at E11.5, E13.5,

Table 1. CRL, length of the intestine, and the total epithelial cell number in the intestine of the mouse embryo

| Embryonic day | E11.5 | E13.5 | E15.5 |
|---|---|---|---|
| CRL (mm) | 7.25 | 10.57 | 16.46 |
| Length of the intestine (mm) | 1.25 | 12.75 | 35.50 |
| Total cell number | 26,992 | 301,770 | 2,746,667 |

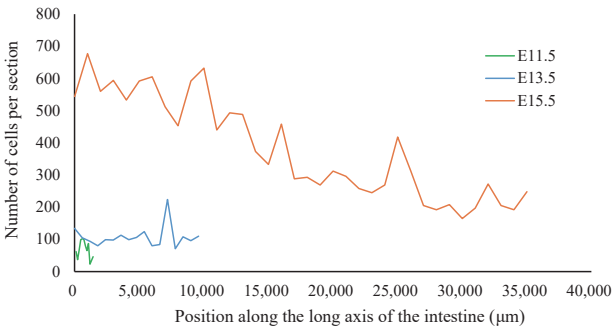


Fig. 2. Distribution of the epithelial cell number per section along the long (rostral/caudal) axis of the intestine at E11.5, E13.5, and E15.5.
The length of the intestine on the abscissa shows a rapid elongation of the intestine during this period. The epithelial cell number per section on the ordinate tended to be larger (approximately 100) in the middle part, and smaller at the rostral and caudal parts at E11.5, rather constant (approximately 100) throughout the entire length (except for the 7800 μm point probably due to the oblique-angle of the sectioning) at E13.5, and became much larger than at the earlier dates, and tended to be the larger at the rostral side and gradually decreased toward the caudal end at E15.5.

and E15.5, respectively (Table 1). The cell number increased from E11.5 to E13.5 by 11.2-fold, and from E13.5 to E15.5 by 9.1-fold.

***Mathematical expression of epithelial cell proliferation***

Based on the obtained total cell number at each date, we tried to make a mathematical model of the epithelial cell proliferation pattern. By using the Excel built-in software, it was revealed that the following formula can approximate the graph of cell proliferation with a high reliability as shown in Fig. 3.

$Y = 0.0472e^{1.1557X}$

Y is the cell number, e is Napier's constant (2.718), X is day number. The reliability of this approximation software is R2 = 0.9993, suggesting that it is almost suitable as an approximate curve of the measured data.

We thus suggested that total cell number increase in the intestinal epithelium is an exponential growth from E11.5 to E15.5 in mouse embryos.

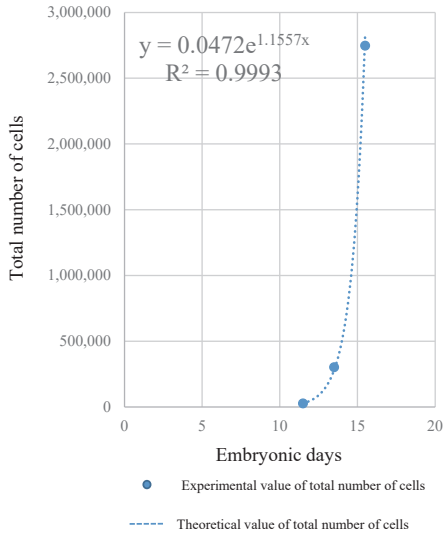


Fig. 3. Comparison of the measured and theoretical values of the epithelial cell number and the approximation by the Excel software.
Total epithelial cell numbers measured and the approximation formula by the Excel software. The formula shown can approximate the graph with a very high reliability.

## DISCUSSION

***Intestinal epithelium cell proliferation from E11.5 to E15.5 in the mouse embryo and the mathematical formulation***

In the present study, we measured the total cell number in the intestinal epithelium of the mouse embryo at E11.5, E13.5, and E15.5 by using stereology, formulated the increase mathematically, and suggested that cell proliferation in the intestinal epithelium during this period can be expressed as an exponential function as in *in vitro* cell growth. There is limitation in our findings, because the obtained data of total cell number in the intestinal epithelium is from different individual embryos, one for each date, and therefore is not continuous nor data from multiple developing embryos with individual variations. We only focused on the cell proliferation, but did not mention about the cell death which also affects the total cell number. However, the apoptotic index is very low (less than 0.5% in humans [7]) and almost constant during the period when the intestine elongates [3, 7], and does not affect significantly the total cell number increase. With these limitations, the high level of approximation and reliability shown in the present findings, even based on the data from one embryo for each date, strongly suggest that the intestinal epithelial cells grow exponentially. We applied the Excel built-in software and showed that there is a high reliability in the cell proliferation graph. The present findings suggest that the intestinal epithelial cells grow exponentially during the observed period in the mouse embryo.

***Biological significance of and the mechanism enabling the exponential growth of the intestinal epithelium from E11.5 to E15.5***

The present finding that cells grow exponentially in *in vivo* tissues as in the log phase *in vitro* cultured cells was unexpected for the present authors, since there are numerous and complex conditions/interactions such as those from the surrounding space-limiting mesenchyme and muscle layers. The present findings and conclusion do not support that cells proliferate exponentially in the intestine at the other developmental dates or stages, nor in the other organs. It is apparent that in adults, for example,

the cells are not growing exponentially, and there should be regulation to harmonize the cell proliferation rate among the tissues and organs in the body and during different stage of the life course. Due to the lack of the total cell number in each organ and tissue at different stages through the life course, it remains unknown how these different proliferation rates are controlled. The exponential growth of the intestinal epithelial cells raises us the questions, i.e., what is the biological significance and what is the mechanism which allows the exponential cell growth during this period.

As observed in the present study, the villi formation occurs during this and the following developmental stages in addition to the intestinal elongation, and a very rapid increase in the surface area surrounding the newly formed villi is urgently needed, which is at least one of the significances to increase the epithelial cell numbers to cover the rapidly increasing surface area. Then, how is the rapid cell growth enabled?

We previously reported the existence of the interkinetic nuclear migration (INM) in the intestinal epithelium throughout the entire length of the intestine during this period [2, 11, 12]. INM is a regulatory mechanism of the epithelial cell proliferation/differentiation of the epithelial tubular tissues and organs of three-germ-layer origin [2, 13, 14]. In INM, the epithelial cell nuclei show oscillatory movement that is synchronized with the progression of the cell cycle, generating the pseudostratified structure of the epithelium. INM has been suggested to maximize the number of mitosis at the limited apical surface by piling up the cell nuclei toward the pial side while maintaining the mono-layered structure (pseudostratified structure) and thus allowing the efficient stem cell expansion [13, 14]. In the present study, at E11.5, the middle part of the intestine exhibited the pseudostratified structure, at E13.5, the epithelium was homogeneously pseudostratified, and at E15.5, the caudal part showed the pseudostratified structure with the increasing layer number toward the caudal end. These findings suggest that INM plays an important role to enable the exponential growth of the epithelial cells in the intestine during this period.

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***Conflict of Interest***

None

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