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Reverse engineering of animal development

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ABSTRACT

In animal development studies, research has been undertaken using various experimental approaches. The embryo of the ascidian *Ciona intestinalis* develops through an invariant cleavage pattern among different embryos. In this study, 73 images from comprehensive whole-mount *in situ* hybridization analyses of *C. intestinalis* embryos were converted into simple character strings and tools for such a conversion from a picture (i.e., two-dimensional) to a character string (i.e., one-dimensional) are provided. It is expected that this approach would change the research style of developmental biologists from laboratory work to *in silico* experimentation similarly to the cases for computer-based researchers such as genome scientists and mathematical chemists, in that they can come up with new hypotheses or examine old ones in their particular research field. The unification of the production of empirical data in the molecular developmental biology area may enable the standardization of input data quality and promote comparative analyses.

KEYWORDS: reverse engineering, *Ciona intestinalis*, comprehensive whole-mount *in situ* hybridization analyses, conversion of images to character strings

INTRODUCTION

"Reverse engineering" involves taking apart a manufactured product in order to see how it works and thereby be able to duplicate it. This could involve understanding how sound is transmitted through radio waves by taking apart an AM (amplitude modulation) radio, for example. In this case, one of the most important circuits is a "detector," which simply consists of a diode. It conducts current in one direction but blocks it in the opposite direction. Therefore, this detector can remove the oscillations on one side of a wave, converting an alternating current (AC) to a varying direct current (DC) (Fig. 1a). This variation corresponds to the amplitude envelope in the original AC.

Basic logic circuits include NOT (denial), AND (logical conjunction), OR (logical disjunction), NAND (denial of logical conjunction), NOR (denial of logical disjunction), and XOR (EOR, exclusive disjunction). Logic IC (integrated circuit) semiconductor packages that are frequently used by amateur electronics enthusiasts contain a number of NAND or NOR gates in the chips (Fig. 1b). NAND and NOR gates are so-called "universal gates" that can be combined to form any other kind of logic gate (Fig. 1c, d). In terms of the behaviors of each gate, their equivalents in a biochemical context can be described as follows (Fig. 1e): In the case of a NOT gate, an input gene encoding a repressor could be involved. In the case of an AND gate, two input transcription factors forming a heterodimer that acts as a functional transcription activator could be involved. For an OR gate, two parallel circuits that activate transcription could be involved. The NAND/NOR gates are the logical negation of the AND/OR gates, respectively. Finally, an XOR gate could be represented by two functional transcription activators that lose their activities when they heterodimerize. All basic logic

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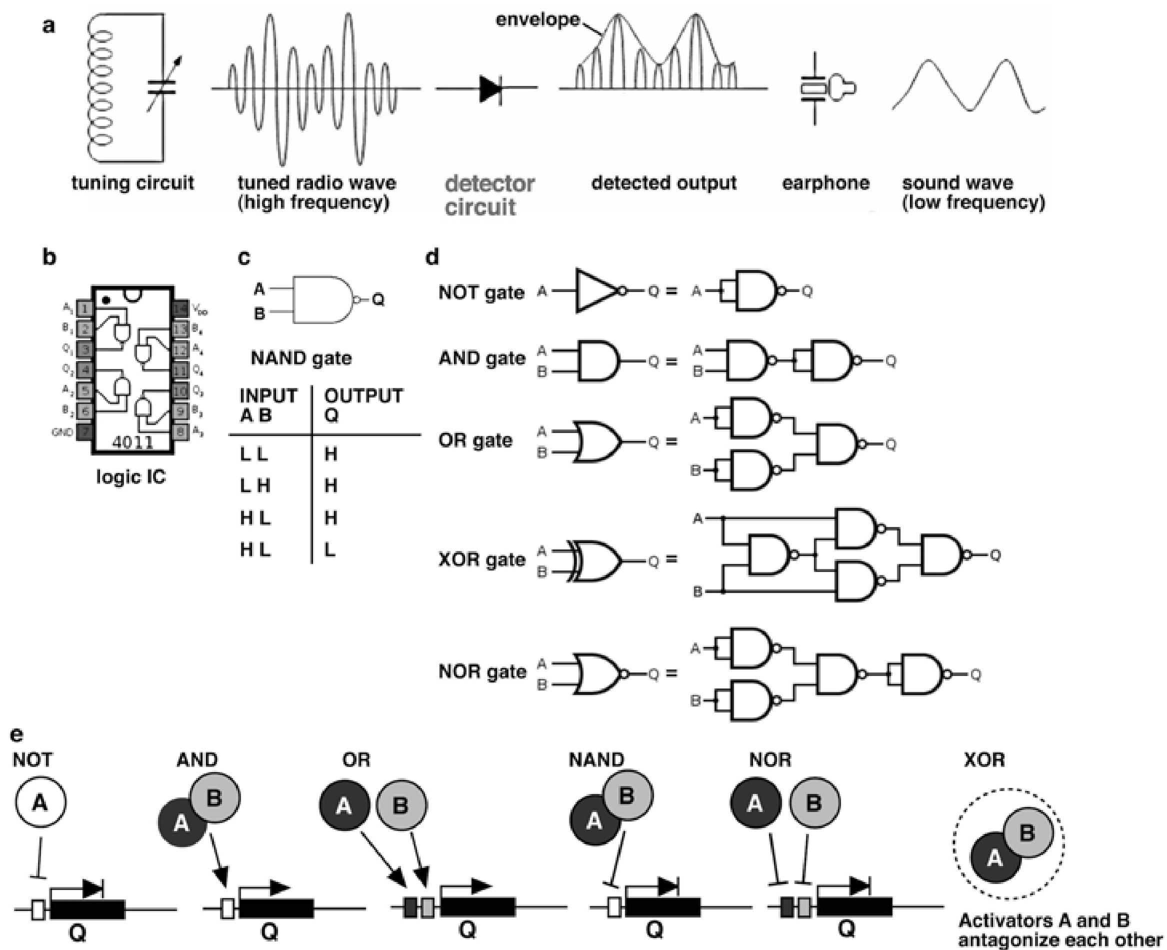

Fig. 1. (**a**) An example of reverse engineering, namely, of an AM radio (see text for details). (**b**) A logic IC. (**c**) The NAND gate. (**d**) The NAND gate can be considered a universal gate since combinations of it can accomplish any of the basic operations. (**e**) Probabilities of biochemical interpretation of the basic logic gates.

gates have recently been shown to be cytologically implementable as combinations of simple NOR universal gates (Fig. 1d, [1]).

Regulatory molecules can be divided into two groups, namely cell-autonomous regulatory molecules (type 1) that function independently of cells and inductive regulatory molecules (type 2) whose function is mediated by cells. The inductive regulatory molecules can be further subdivided into long-range ones such as hormones that are transmitted to remote targets through the blood, and short-range ones such as Wnt cytokines that are stabilized onto the plasma membrane.

This research was conducted to "reverse engineer" a developmental process of the ascidian *C. intestinalis* embryo that looks as if it were a fine art of brickwork consisting of small number of large "block parts" (blastomeres), contrary to the embryos of many other species, which consist of a large number of small blastomeres. This simplicity is a noteworthy brilliant property of the ascidian early development, which should be the primordial form for more complicated developments of other chordates.

In this paper, both an "encoder" that converts a picture (i.e., two-dimensional) to a character string (i.e., one-dimensional) and a "decoder" that returns from the character string to the picture are provided. Forthcoming new and detailed descriptions on the spatial expression in *C. intestinalis* embryos will be equivalent to more refined and more sophisticated substitutes of expression profile graphs seen in current microarray/DNA chip analyses.

MATERIALS AND METHODS

Genome-wide surveys of developmentally regulatory genes in *C. intestinalis* genome

Genome-wide surveys of developmentally regulatory genes in *C. intestinalis* genome [2] have been reported in a series of monumental papers published in 2003 [3]. The subsequent comprehensive whole-mount *in situ* hybridization experiments were carried out and the resultant 6,939 pictures of embryo are freely available on the ANISEED database/ developmental browser [4]. Shown data for this program was cited mainly from [5].

Bioinformatics programs

Three input programs ("encoder": ProgramsS1-32cell.html, ProgramsS1-32cell.html, and Programs S1-110cell.html) and one output program ("decoder": ProgramS2-ciona-expression.html) was written in order to make the best use of the above-mentioned images. They have been uploaded onto the figshare website (https://figshare.com/).

RESULTS AND DISCUSSION

In animal development, there are ultimately two modes for specifying the characteristics of each blastomere [6]. The first is specification by determinants that are precisely localized within the unicellular egg, which are thereafter distributed in a particular way during cleavage and regulate expression of their downstream target genes. This mainly involves type 1 regulatory molecules. The second is specification determined by cell interactions between several blastomeres with different fates. These cell interactions are mediated through type 2 regulatory molecules. In the former case (cell-autonomous signal), information is mainly conveyed via the history of each blastomere through the successive stages of division in the fertilized egg. On the other hand, essential information for the latter (inductive signal) case relies on the geospatial context of each blastomere (contact with other blastomeres). With respect to the former historical and the latter geospatial information [7] on each blastomere of an ascidian *Ciona intestinalis* embryo, two tables (cell lineages and geospatial information) on blastomeres were constructed (Tables 1-2).

In early embryogenesis in animals, there do not seem to be as many precisely localized (choreographed) type 1 determinants as there are differentiated cell types; rather, in *C. intestinalis* embryogenesis, for example, only a limited number of maternal determinants are used [8, 9]. These include the β-catenin vegetally localized transcription factor, the Gata.a animally localized transcription factor, the Macho-1 (or Zic-r.a) zinc finger myogenic determinant transcription factor, the Wnt5 type 2 (short-range)-regulatory molecule in the mesenchyme/ muscle, the *Pem-1* maternal mRNA that plays several developmental roles, including asymmetric cleavage of the posterior pole in embryonic compartmentation, and POPK-1 cytosolic kinase that participates in maternal mRNA distribution to the posterior pole of the embryo after fertilization. Several transcripts share the same "mitochondrion-like" spatial localization (Fig. 2). Consequently, in the vegetal hemisphere, the β-catenin transcription factor activates the *Fgf9/16/20* gene, whereas in the animal hemisphere, the Gata.a transcription factor activates downstream *Ephrina.d*, both of which encode type 2 regulatory molecules (inductive signaling molecules) [8].

It is possible to regard the 16-cell stage as a point of tentative departure of *C. intestinalis* zygotic embryogenesis, and the 110-cell stage as a point of arrival when most of the developmental fates of the blastomeres have been determined (Fig. 3). At the 110-cell stage, the specification of each blastomere depends on a combination of numerous type 1 regulatory molecules (e.g., transcription factors). In the *C. intestinalis* genome, there are at least 670 transcription factors, nearly half of which belong to the group of DNA-binding zinc finger proteins. Interestingly, the zinc finger transcription factors are rarely included in "core" genetic regulatory network (GRN), participating in morphogenesis of tissues such as "mesoderm" that have two or more developmental fates (multipotency); instead, most of them are involved in morphogenesis of tissues that have unipotency [10].

Recently, zinc finger nuclease (ZFN) has been started to be used as a tool for molecular biology experiments [11]. The mechanism behind the specificity of ZFN is surprisingly simple: one zinc finger domain (33-35 amino acids) recognizes two to three nucleotides. Thus, if three zinc finger domains are aligned in a tandem repeat, it is possible to design an artificial nuclease that recognizes nine bases. This suggests that endogenous zinc finger transcription factors would be particularly easy to manipulate when attempting to design any of a wide range of required specificities. The surprising

Table 1. The descendants of blastomeres in 64- (a), 32- (b), and 16-cell (c) stage embryos. The character strings in this table such as "1000000000000000000000000000" represents A7-1, A7-2, A7-5, A7-6, A8-5, A8-6, A8-7, A8-8, A8-13, A8-14, A8-15, A8-16, a8-17, a8-19, B7-1, B7-2, B7-5, B7-6, B7-7, B8-5, B8-6, B8-7, B8-8, B8-15, B8-16, b8-17, b8-19, and b8-27, respectively.

a. 64-cell stage descendants		
A7.1		1000000000000000000000000000
A7.2		0100000000000000000000000000
A7.3	A8.5/A8.6	0000110000000000000000000000
A7.4	A8.7/A8.8	0000001100000000000000000000
A7.5		0010000000000000000000000000
A7.6		0001000000000000000000000000
A7.7	A8.13/A8.14	0000000011000000000000000000
A7.8	A8.15/A8.16	0000000000110000000000000000
B7.1		0000000000000010000000000000
B7.2		0000000000000001000000000000
B7.3	B8.5/B8.6	0000000000000000000110000000
B7.4	B8.7/B8.8	0000000000000000000001100000
B7.5		0000000000000000100000000000
B7.6		0000000000000000010000000000
B7.7		0000000000000000001000000000
B7.8	B8.15/B8.16	0000000000000000000000011000
b. 32-cell stage descendants		
A6.1	A7.1/A7.2	1100000000000000000000000000
A6.2	A7.3/A7.4	0000111100000000000000000000
A6.3	A7.5/A7.6	0011000000000000000000000000
A6.4	A7.7/A7.8	0000000011110000000000000000
B6.1	B7.1/B7.2	0000000000000011000000000000
B6.2	B7.3/B7.4	0000000000000000000111100000
B6.3	B7.5/B7.6	0000000000000000110000000000
B6.4	B7.7/B7.8	0000000000000000001000011000
c. 16-cell stage descendants		
A5.1	A6.1/A6.2	1100111100000000000000000000
A5.2	A6.3/A6.4	0011000011110000000000000000
B5.1	B6.1/B6.2	0000000000000011000111100000
B5.2	B6.3/B6.4	0000000000000000111000011000

finding that should be noted is that there are many alleles for which only a single doublesex (dsx) "zinc finger" transcription factor phenotype a variety of multiple patterns in the mimesis of this species, suggesting that all mutations in this gene do not abolish the functions in transcription regulation that induce coordinated mimetic phenotypes [12].

In order to apply the images from the comprehensive *in situ* hybridization experiments to the computer for processing, it is necessary to digitize these data and enter them into it. The combination of regulatory molecules in *C. intestinalis* is represented by the high-resolution graph of PCP (parallel coordinate plot) expression profiles shown in Fig. 4a, which is frequently used to summarize microarray/DNA chip analysis results [13]. 73 spatial expression patterns [5] from the 32-, 64-, and 110-cell stages of *C. intestinalis* early development [14] ("encoder"

Table 2. The neighboring blastomeres in 110-cell stage embryo. For example, A7-1 (left) blastomere touches A7-1 itself, A7-2, A7-5, B7-1, and A7-1 (right). A7-1"op" represents the "opposite-side" of the A7-1 blastomere.

110-cell stage neighboring blastomeres	
A7-1	1110000000000001000000000000
A7-1op	1000000000000000000000000000
A7-2	1110110000000000000000000000
A7-2op	0100000000000000000000000000
A7-5	1111010011000010000110000000
A7-5op	0000000000000000000000000000
A7-6	0011000001010000000110000110
A7-6op	0000000000000000000000000000
A8-5	0100111000000000000000000000
A8-5op	0000100000000000000000000000
A8-6	0110111110000000000000000000
A8-6op	0000000000000000000000000000
A8-7	0000111100000100000000000000
A8-7op	0000001000000000000000000000
A8-8	0000011110101000000000000000
A8-8op	0000000000000000000000000000
A8-13	0010010111100000000000000000
A8-13op	0000000000000000000000000000
A8-14	0011000011010000000000000000
A8-14op	0000000000000000000000000000
A8-15	0000000110110000000000000000
A8-15op	0000000000000000000000000000
A8-16	0001000001110000000000000010
A8-16op	0000000000000000000000000000
a8-17	0000000100001000000000000000
a8-17op	0000000000000000000000000000
a8-19	0000001000000100000000000000
a8-19op	0000000000000100000000000000
B7-1	1010000000000011000100000000
B7-1op	0000000000000010000000000000
B7-2	0000000000000011101100000000
B7-2op	0000000000000001000000000000
B7-5	0000000000000001111000011000
B7-5op	0000000000000000000000000000
B7-6	0000000000000000110000001001
B7-6op	0000000000000000010000000000
B7-7	0000000000000001101101010000
B7-7op	0000000000000000000000000000
B8-5	0000000000000000000000000000
B8-5op	0000000000000000000000000000
B8-6	0000000000000000110000001000
B8-6op	0000000000000000010000000000
B8-7	0000000000000000001101110000
B8-7op	0000000000000000000000000000
B8-8	0001000000000000000111100100
B8-8op	0000000000000000000000000000
B8-15	0000000000000000101001011000
B8-15op	0000000000000000000000000000
B8-16	0000000000000000110000011001
B8-16op	0000000000000000000000000000
b8-17	0001000000000000000000100100
b8-17op	0000000000000000000000000000
b8-19	0001000000010000000000000110
b8-19op	0000000000000000000000000000
b8-27	0000000000000000010000001001
b8-27op	0000000000000000010000000001

Table 2 continued..

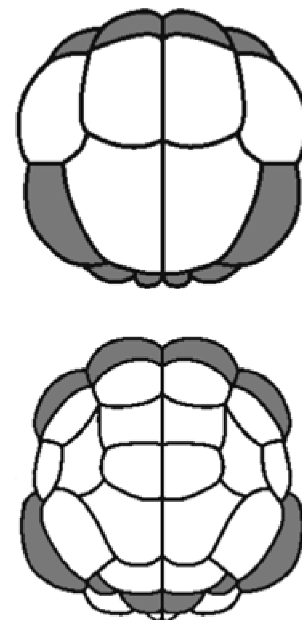

Fig. 2. Mitochondrion-like distribution. Considerably many embryonic genes show the same spatial expression patterns. Together with several maternal mRNAs, these spatial expression patterns may also affect other expression patterns subsequently.

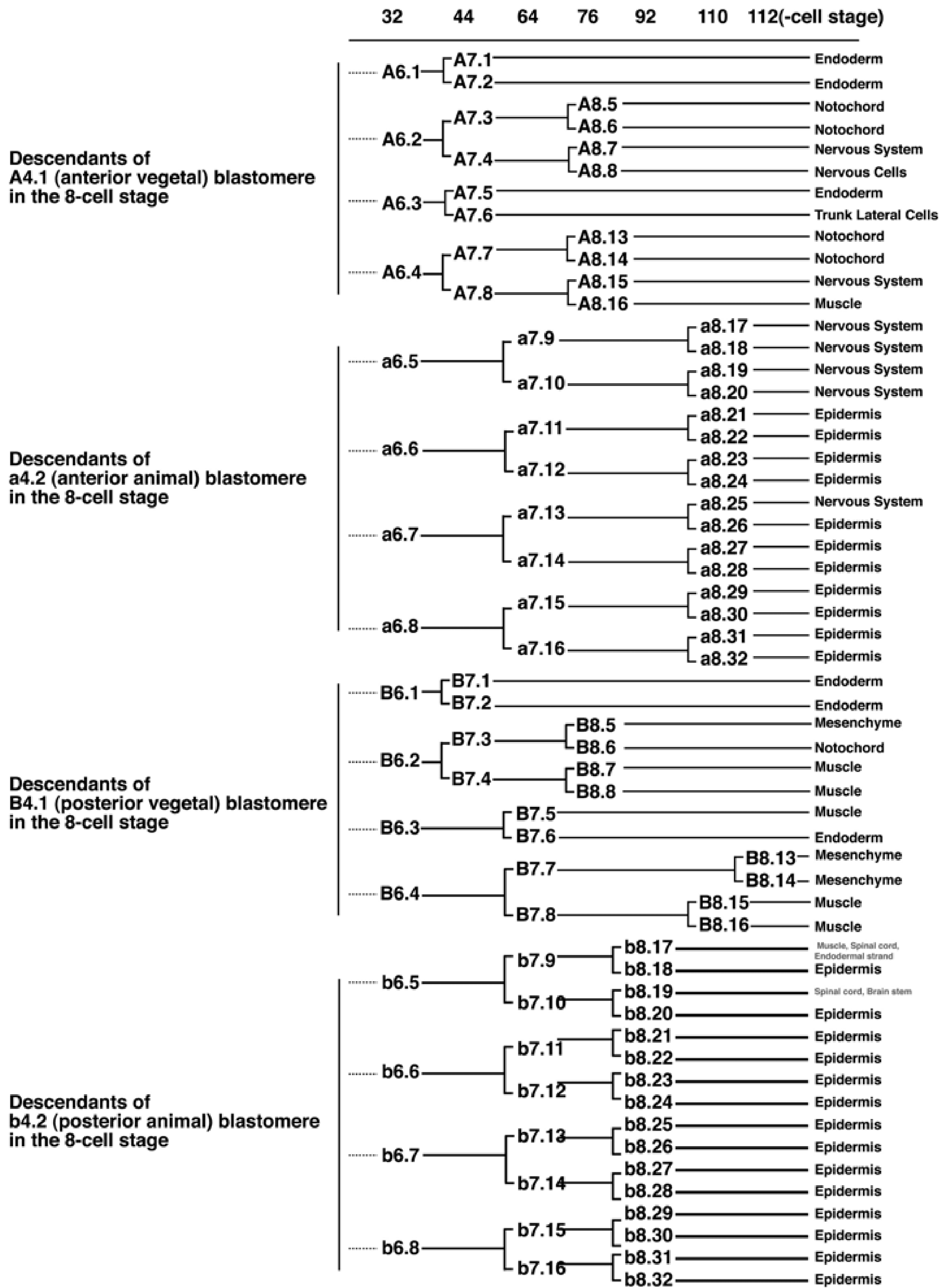

Fig. 3. The embryonic cell lineage of *C. intestinalis* from 32- to 110-cell stage embryos. In the 110-cell stage, the developmental fates of most blastomeres are restricted to a single cell type. Gray letters show the few exceptions to this.

programs in the figshare website: https://figshare.com/) were inputted. The 73 inputs can be divided into several categories, as indicated by the color codes in a table shown on separate sheets of a Microsoft Excel File ("CionaTable.xlsx" in the figshare website). On the other hand, an output tool was also developed, as shown in Fig. 5, which is a simple program to visualize the obtained character strings ("decoder" program in the figshare website).

If the expression of the gene encoding a regulatory molecule is regulated by spatially localized determinants, its pattern may be the same or similar throughout the successive generations within a blastomere cell lineage. A blastomere in the 16-cell stage embryo has approximately eight descendant cells by the time the 110-cell stage is reached. In *C. intestinalis*, for none of the 73 input genes the same pattern of expression is maintained and transmitted down the cell lineage of the blastomere from the 16-cell stage. This implies that, in the 16- to 110-cell stages, the expression of most genes in *C. intestinalis* is regulated not by a maternal determinant alone, but rather by cellular interactions between blastomeres of different origins. In the later developmental stages, the expression of many genes matches between pairs of sister blastomeres (data not shown).

In static analysis, multiple regression method enumerates inputted data and their AND calculations first in order to address a knotty question. The enumerated strings are shown in the "CionaTable.xlsx" spreadsheet in the figshare website (https://figshare.com/). They are simple binary digits; the number of inputted data is 73, and therefore the number of their square becomes 5329 (73^2).

Each string was compared to the input strings. Although numerous matches were identified, most of them could simply be coincidences (Table 3). As examples of such coincidences, cieg022d03 (zinc finger transcription factor) showed the same spatial pattern as cicl002e04 AND citb008i23 (two other zinc finger transcription factors), and cilv006c13 (Lhx3: LIM/homeobox transcription factor) showed the same one as cicl045e03 (Otx transcription factor) AND citb007o19 (putative transcription repressor in the most downstream part of the Notch pathway).

Interestingly, citb002g15 (sFRP1/5 Wnt antagonist) showed an expression pattern horizontally (medio-laterally) complementary to cicl041p23 (Wnt5) (Fig. 4e). Its expression pattern also showed vertical [medio-peripheral (dorsal/ventral lips of the blastopore)] complementarity to those of citb054p04 (Wnt-NAe or Wnttun5) (Fig. 4f). Similarly to this coincidence of the expression patterns of ligand and receptor in the same signaling pathway, cicl031e02 (Dickkopf Wnt antagonist) and cies002j17 (Fz (frizzled)-4 in Wnt signaling) shared the same pattern (Fig. 4i).

Although the author cannot draw any definitive conclusions at present, it seems surprising that the topology of the GRN [15] of development is not well conserved among different organisms. Possibly, comparative evo-devo studies might revert from the current/coming comparison style of GRNs back to the style of comparing spatial expressions of several master genes, as in its early period. For example, in vertebrate endoderm development, not the whole GRN but only several key components of it (e.g., Sox17, GATA5, and multiple zygotic nodal growth factors) have been shown to be conserved between zebrafish and frog [16]. In order to perform a GRN comparison, we must dramatically increase the data input for each organism, and must perform a comparison not between mouse and human data but between rodent and primate data so that a comparison data between mouse/rat (for rodent) and human/macaque (for primate) could be prepared in advance. Of course, a GRN comparison is performed with the purpose of finding such "master genes".

With regard to obtaining more detailed input data of the GRN in each organism, this is associated with major difficulties in all vertebrates. The vertebrate genome has been duplicated twice and contains four overlapping copies of all of the gene paralogues ("ohnologues"), as pointed out by Susumu Ohno in 1970 [17]. If multiple paralogues retain functional redundancy, the construction of a network consisting of them will be a very difficult task. Fortunately, *C. intestinalis* has a simple haploid genome, and the current project has indicated the gaps in the data that need to be filled by further experiments on the early development of ascidians. The 73 input character strings are a kind of sample data for this analysis system. Sorting or phylogenetic

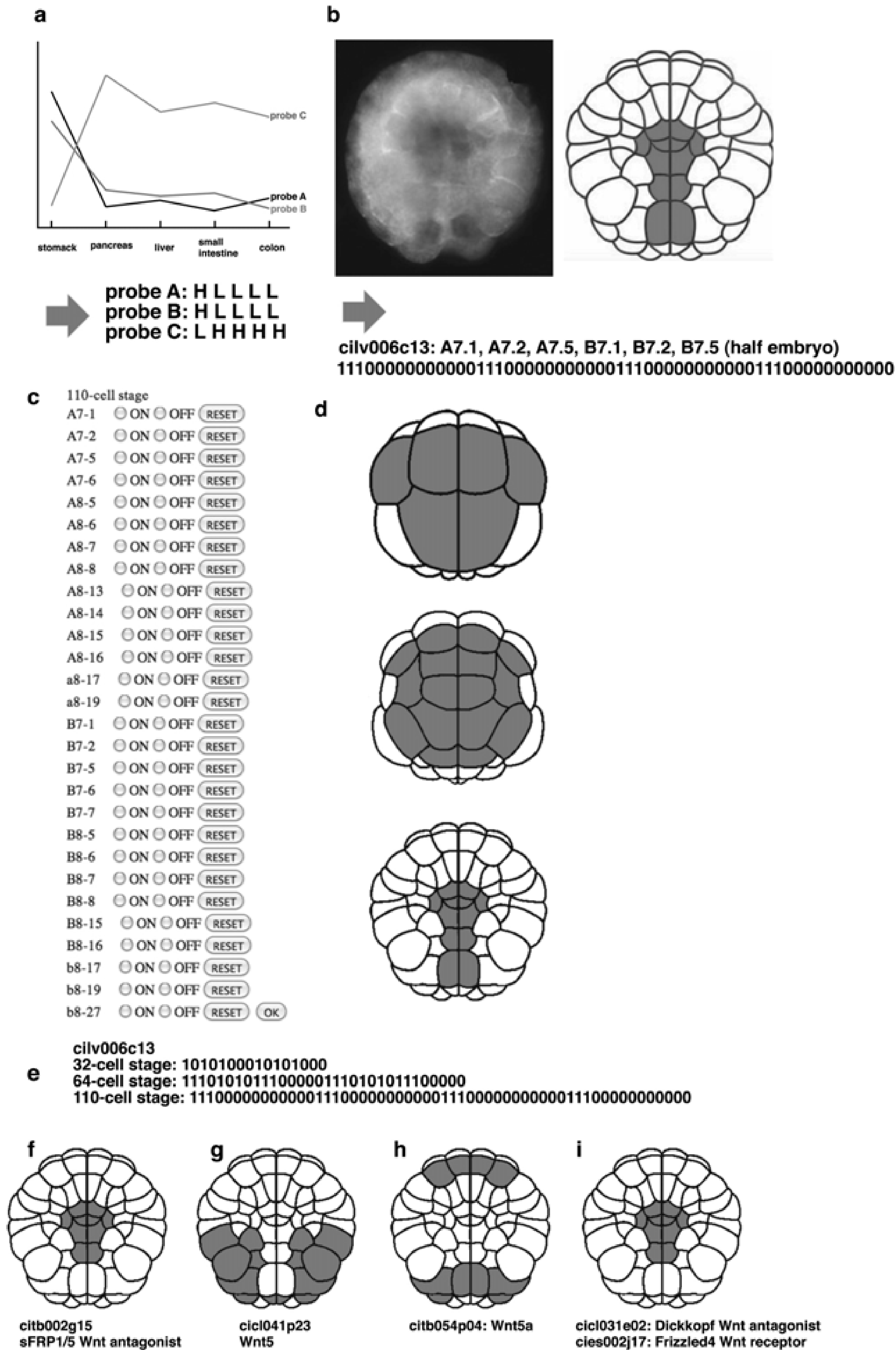

Fig. 4. (**a**) Typical expression profiles of microarray/DNA chip probes. (**b**) Conversion of expression pattern shown in a whole-mount *in situ* hybridization picture (2D) to a character string (1D). (**c**) A 2D to 1D converter ("encoder"). (**d, e**) A 1D to 2D converter ("decoder"). (**f–i**) Examples of visualized regulatory gene expression patterns of interest.

Fig. 5

Fig. 5 continued..

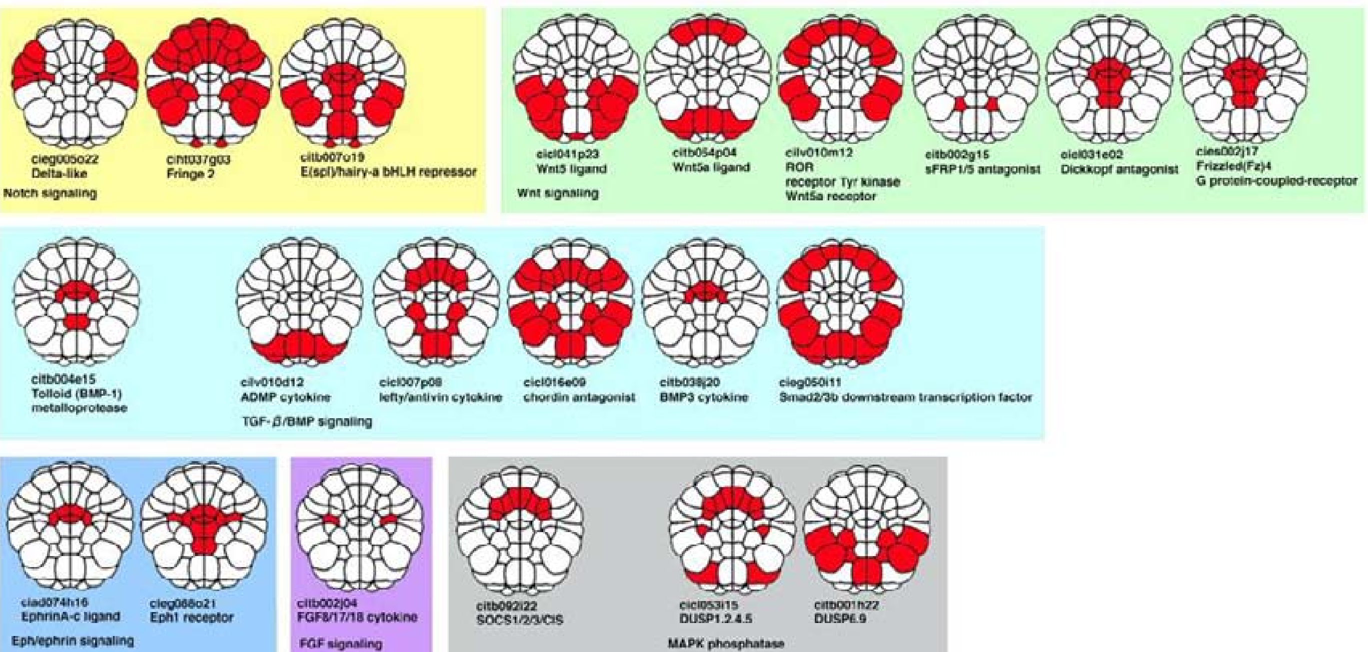

Fig. 5. Formalized spatial expression patterns of 73 regulatory genes in the 110-cell stage. The beige, pink, yellow, green, aqua blue, pale blue, purple and gray genes represent transcription factors (bHLH transcription factors for the first beige rows), zinc finger proteins, proteins involved in the Notch pathway, proteins involved in the Wnt pathway, proteins involved in the TGF-β pathway (except Tolloid (BMP-1) metalloprotease), proteins involved in the Eph/ephrin pathway, proteins involved in the FGF pathway, and other regulatory genes, respectively.

Fig. 6. An unrooted tree diagram of 73 inputs of spatial expressions in the 110-cell stage. See the caption of Fig. 5 for the color code of the 73 regulatory genes.

Table 3. The character strings in this table represents the binary digits of the gene expression (1 : ON, 0 : OFF). Some concordances were identified between encoded expression patterns and logical conjunction of encoded expression patterns of other genes.

cign039e24		Ci-ZF086 transcription factor, zinc finger	000000110011000000000000001
	ciad002g15 AND ciht037g03	Sox transcription factor, HMG-box AND Fringe2 glycosyltransferase involved in Notch signaling pathway	
	ciad002g15 AND citb008i23	Sox transcription factor, HMG-box AND Ci-ZF192 transcription factor, zinc finger	
cilv010m12		ROR receptor Tyr-kinase, Wnt 5a receptor	000000110011000000001100000
citb007k01		transcription factor, Forkhead-type	000000110011000000001100000
	cieg050i11 AND ciht037g03	Smad2/3b transcription factor in TGF-β signaling pathway) AND Fringe2	
	cieg050i11 AND cilv010m12	Smad2/3b AND ROR receptor tyrosine kinase, Wnt5a receptor	
	ciad008k16 AND ciht037g03	transcription factor, ets/pointed2 motif AND Fringe2	
	ciad048l21 AND ciht037g03	Ci-ZF401 transcription factor, zinc finger AND Fringe2	
	cieg056p11 AND ciht037g03	Ci-ZF417 transcription factor, zinc finger AND Fringe2	
	cinc032f17 AND ciht037g03	Ci-ZF136 transcription factor, zinc finger AND Fringe2	
cicl053p06		Fox-1 transcription factor, Forkhead-type	000000110011000010000000000
cieg027d13		E12/E47 transcription factor, bHLH	000000110011000010000000000
	ciad008k16 AND cieg050i11	transcription factor, ets/pointed2 motif AND Smad2/3b	
	cieg056p11 AND cieg050i11	Ci-ZF417 transcription factor, zinc finger AND Smad2/3b	
cicl050d03		Brachyury transcription factor, T-box	000011001100000000010000000
cign044b23		Mnx transcription factor	000011001100000000010000000
	ciad004i10 AND cicl053i15	transcription factor, Forkhead-type AND DUSP1.2.4.5 MAPK phosphatase	
	cicl022a01 AND cicl053i15	Ci-ZF034 transcription factor, zinc finger AND DUSP1.2.4.5 MAPK phosphatase	

Table 3 continued..

	cicl053i15 AND ciht037g03	DUSP1.2.4.5 MAPK phosphatase AND Fringe2	
cicl007p08		lefty/antivin growth factor, TGF-β type	0000110011000000101100000000
	cibd020p17 AND cicl022a01	Snail transcription factor, zinc finger AND Ci-ZF034 transcription factor, zinc finger	
	cicl002e04 AND cicl022a0	transcription factor, zinc finger AND Ci-ZF034 transcription factor, zinc finger	
	cicl016e09A ND Ci-ZF034	chordin, BMP signaling antagonist AND Ci-ZF034 transcription factor, zinc finger	
ciad009i05		SoxC transcription factor	0001001100110000001000000000
	ciad008k16 AND cieg014f19	transcription factor, ets/pointed2 motif AND ERF2 transcription factor	
	ciad048l21 AND cieg014f19	Ci-ZF401 transcription factor, zinc finger AND ERF2 transcription factor	
	cies008l15 AND cieg014f19	Ci-ZF064 transcription factor, zinc finger AND ERF2 transcription factor	

tree construction using 1D data is easier than using 2D data (Fig. 6). As such, the author is expecting that the 6,939 pictures in the ANISEED database [4] will be converted to the strings described in this paper.

CONCLUSION

A magnificent physicist, Richard P. Feynman, satirized the limitations of every analytical science in order to advocate the importance of "reverse engineering": "What I cannot create, I do not understand".

In this paper, the "reverse engineering" approach was introduced into the developmental biology. The ascidian early embryos may turn out to be an ideal subject of research. They consist of a small number of large blastomeres. They undergo invariant pattern of cleavage. A lot of images of comprehensive whole-mount *in situ* hybridization are freely available on the informative ANISEED database [4]. Concretely, two simple tools for handling many images were provided in this paper. They are similar to the ones in the EMBOSS (European Molecular Biology Open Software Suite) package. Some genetic data analysis software (e.g. eBioX, http://malooflab.openwetware.org/eBioX.html) already includes the EMBOSS engines. The simplification of the image was entrusted to the pattern recognition ability of humans, because it remains much more outstanding than the image recognition ability of the computer.

The author believes such tools are necessary for developmental biologists. The unification of empirical data production will standardize the data quality and make analyses in comparative biology much easier and less expensive.

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CONFLICT OF INTEREST STATEMENT

The author declares no competing interests.

ABBREVIATIONS

AC, alternating current; AM, amplitude modulation; DC, direct current; dsx, doublesex; IC, integrated circuit; GRN, gene regulatory network; ZFN, zinc finger nuclease.

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