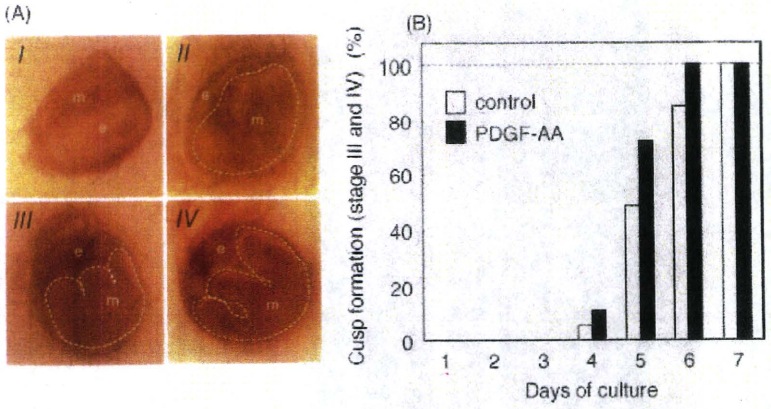

Fig. 5 – PDGF-AA accelerates cusp formation. Tooth germs were cultured with or without 10 ng/ml of PDGF-AA for 7 days. (A) To evaluate the effects of PDGFs, cusp formation was classified into 4 stages: SI, the initial stage; S-II, during which the tooth germ grew and become round; S-III, during which the initial signs of tooth cusp development could be identified; and S-IV, during which the tooth cusp could be clearly identified and became sharp. (B) The total percentage of S-III and S-IV tooth germs following treatment with PDGF-AA was greater than that of untreated tooth germs on days 5 and 6, indicating that cusp formation was accelerated by PDGF-AA.

indicate that cusp formation is accelerated by PDGF-AA. In addition, our results suggest that, in contrast to PDGF-BB, exogenous PDGF-AA via PDGFRα signalling is important for cusp formation.

3.5. *PDGF-AA regulates Fgf2 and ameloblastin expression in dental epithelium*

In the present experiments, AG17 inhibited the proliferation of tooth germ epithelium and SF2 cells, whereas PDGF-AA and -BB did not have an effect on proliferation of those cells. Furthermore, PDGF-AA via PDGFRα signalling was found to have an effect on cusp formation. The presence of these complimentary functions suggests that other key factors also take part in PDGF signalling. In our previous study, we clearly demonstrated that PDGF signalling regulates the expression of Fgfs in submandibular glands,[12] thus it is possible that a similar system functions in teeth and we investigated the effects of AG17 on the expressions of Fgfs, Bmps and Shh in tooth germ cultures. AG17 partially decreased the expressions

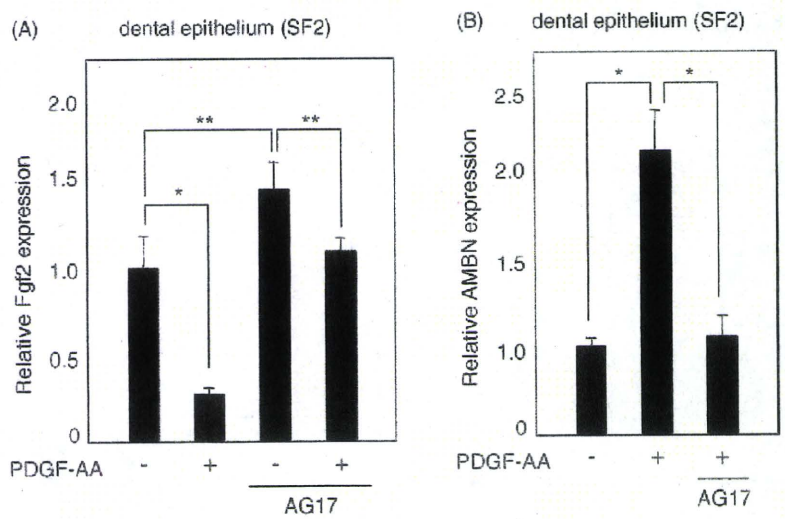

Fig. 6 – PDGF-AA regulates Fgf2 and ameloblastin expressions in SF2 cells. SF2 cells were cultured with or without 10 ng/ml of PDGF-AA and/or AG17 for 48 h, and the mRNA expressions of Fgf2 and ameloblastin (AMBN) were analysed by real-time RT-PCR. (A) Fgf2 expression was inhibited by PDGF-AA and induced by AG17. (B) Ameloblastin expression was induced by PDGF-AA and inhibited by AG17. There were no differences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, used as a control, among the various culture conditions. Similar results were obtained in at least three separate experiments. Statistical analysis was performed using analysis of variance (*, $p < 0.01$, **, $p < 0.05$).

of Bmp2, Bmp7, Fgf4, Fgf7 and Shh, while it unexpectedly increased that of Fgf2 (data not shown). To confirm that Fgf2 expression is regulated by AG17 and PDGF, we examined its expression in SF2 cells. Interestingly, AG17 induced Fgf2 expression, whereas PDGF-AA inhibited that expression in SF2 cells (Fig. 6A). Furthermore, PDGF-AA induced ameloblastin expression, whereas AG17 inhibited it (Fig. 6B). These results show that PDGF signalling functions as a modulator of other potent factors, including Fgf2 and ameloblastin, to control dental epithelium differentiation.

4. Discussion

In the present study, we found that PDGF-BB induces dental mesenchyme proliferation and PDGF-AA accelerates cusp formation. In addition, endogenous PDGFs were shown to be important for tooth germ growth and cusp formation, based on our experiments with AG17 treatment. Furthermore, our results showed that PDGF-AA regulates the expressions of Fgf2 and ameloblastin in dental epithelial cells, indicating its critical role in dental epithelium differentiation.

PDGFα, a gene coding PDGF-A, is expressed in embryos by several different types of progenitor cells that proliferate and migrate in response to PDGF. For example, PDGFα is expressed in precursor cells that become cranial neural crest cells and is thought to be required for migration of those cells into the brachial arches. PDGFα is also expressed by smooth muscle progenitors in developing lungs and widely throughout the embryonic mesenchyme, while PDGFα-deficient mice have a variety of defects in crest-derived tissues, including gross craniofacial, skeletal and cardiac abnormalities. During tooth development, PDGF-A is highly expressed in dental epithelium and weakly in dental mesenchyme (Fig. 1). PDGF-A and PDGF-B form homodimers, PDGF-AA and PDGF–BB, and a heterodimer, PDGF-AB. The only known receptor for PDGF-AA is PDGFRα, which is expressed in dental mesenchyme and inner enamel epithelium, indicating that PDGF-AA may have effects on inner enamel epithelium and neural crest-derived dental mesenchyme.

Dental cusp malformation in PDGFRα null mutants leads to a critical growth defect and shows the requirement for PDGF signalling in the determination of tooth morphology. Loss of the *Pdgfrα* gene does not have effects on proper odontoblast proliferation and differentiation in the cranial neural crest-derived odontogenic mesenchyme. However, such a lack perturbs formation of the extracellular matrix and organisation of odontoblast cells in the cusp forming area, resulting in a dental cusp growth defect.[13] PDGF-AA and PDGF-BB are able to bind to PDGFRα. In the present experiments, PDGF-BB, but not PDGF-AA, was shown to accelerate dental mesenchymal proliferation. Together, our results suggest that PDGF-BB and PDGFRβ signalling, but not PDGFRα, may be important for dental mesenchymal proliferation.

A previous study used embryonic day 10 mandibular explants cultured in serum-free media and reported that exogenous PDGF-AA enhances tooth development to reach the cap stage with increased tooth size.[17] However, in our experiments with embryonic day 13 tooth germ cultures with a low concentration of serum, we found that treatment with exogenous PDGFs did not have an effect on tooth germ growth (data not shown), in contrast to treatment with AG17. Based on these results together with those showing a high expression of PDGF-A in tooth germs, we propose that the PDGF-AA isoform and its tyrosine kinase receptor, PDGFRα, regulate tooth size and tooth development during odontogenesis via an autocrine mechanism.

We found that PDGF-A was expressed in submandibular gland epithelium, whereas PDGF-B, PDGFRα and PDGFRβ were expressed in mesenchyme. Exogenous PDGF-AA and -BB in submandibular gland organ cultures demonstrated increased levels of branching and epithelial proliferation, though their receptors were found to be expressed in mesenchyme. PDGF-AA and PDGF-BB induced the expression of Fgf7 and Fgf10, indicating that PDGFs regulate Fgf gene expression in submandibular gland mesenchyme. Also, the PDGF receptor inhibitor AG17 inhibited PDGF-induced branching morphogenesis, whereas exogenous Fgf7 and Fgf10 expressions were fully recovered. Together, these results indicate that fibroblast growth factors function downstream of PDGF signalling, and regulate Fgf expression in neural crest-derived mesenchymal cells and submandibular gland branching morphogenesis.[12] Thus, PDGF signalling is a possible mechanism involved in the interaction between epithelial and neural crest-derived mesenchyme. In tooth germ development, we speculate that an important mechanism of tooth morphogenesis via epithelial and mesenchymal interactions functions in submandibular gland morphogenesis, because of similar patterns of expression of PDGFs and their receptors in both submandibular gland and tooth germ cultures. In fact, AG17 partially decreased the expressions of Bmp2, Bmp7, Fgf4, Fgf7 and Shh, and increased that of Fgf2 in tooth germ organ cultures (data not shown). Previous studies have provided critical information regarding the functional significance of the epithelially derived enamel knot in dental cusp formation. The primary enamel knot in the cap stage tooth germ is a transient structure and serves as a signal centre for regulating cusp formation. Multiple growth factors (such as Shh, Bmps, Fgfs and Wnts), transcription factors (such as Msx2 and Lef1) and cell cycle regulators (such as p21) have important functions in regulating dental cusp formation and the fate of epithelial cells in the enamel knot.[1] A decrease in the expressions of Bmps and Shh may be associated with inhibition of cusp formation in the presence of AG17.

Organisation and remodelling of the basement membrane are also important for determination of tooth size and shape. Laminin α5, a basement membrane component, is highly expressed during tooth germ development. Laminin α5 null-mice have small teeth and inhibited cusp formation, because of decreased expressions of Fgf4 and Shh in the enamel knot.[14] Previous studies have shown a dramatic reduction in MMP-2 in neural crest-derived dental mesenchyme and inhibition of cusp formation in PDGFRα null mutants, which suggest a critical role for that extracellular proteinase in normal tooth development.[13,18] The biological function of MMP-2 is critical for breakdown of the basement membrane prior to the formation of dentin and enamel matrix, as well as for extracellular matrix remodelling, as odontoblast cells retreat towards the central part of dental mesenchyme during dental cusp development.[13] These results indicate that a proper

basement membrane is necessary for cusp formation and PDGF signalling may regulate these related processes.

In our experiments, exogenous PDGF-AA reduced Fgf2 expression in the dental epithelial cell line SF2. Previously, Fgf2 was found to potently induce both proliferation and expression of DSPP in immature pulp cells.[19] Furthermore, exogenous Fgf2 decreased the gene expressions of differentiation markers, such as amelogenin, DSPP and alkaline phosphatase, in molars at the bell stage, while abrogation of endogenous Fgf2 by antisense oligonucleotide increased the gene expressions of those differentiation makers, and also significantly enhanced enamel and dentin formation.[20] These findings suggest that Fgf2 at the bell stage regulates cell differentiation and matrix secretion. In addition, the effects of Fgf2 on tooth cells may be regulated by PDGF-AA, while exogenous PDGF-AA induces ameloblastin expression in dental epithelium. Ameloblastin plays an important role in maintaining the differentiation state of ameloblasts, and also serves as a cell adhesion molecule and regulates ameloblast differentiation.[21] That study also showed that a deficiency of ameloblastin causes severe enamel hypoplasia, accelerates the proliferation of dental epithelium and decreases the expression of amelogenin. Administration of exogenous PDGF-AA to dental epithelial cells may be useful for induction of ameloblastin and differentiation of dental epithelium to ameloblasts.

In conclusion, PDGF-A, PDGF-B, PDGFRα and PDGFRβ were found to be expressed during tooth development. AG17 inhibited tooth germ growth and cusp formation, while exogenous PDGF-BB accelerated the proliferation of dental mesenchymal cells and PDGF-AA induced cusp formation. Furthermore, PDGF-AA reduced the expression of Fgf2 and induced ameloblastin expression. Together, our results indicate that PDGFs and their receptors are necessary for tooth development.

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Conflicts of interest

None declared.

Ethical approval

Not required.

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REVIEW

Hox Genes, a Molecular Constraint for the Development and Evolution of the Vertebrate Body Plan

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Abstract : A set of *Hox* genes expressed during the embryonic process presents a crucial system to set up the body plan in animal phyla. Current systematic approaches, such as bio-imaging, epigenetic regulation and phylogenetic genome comparison, illuminated new comprehensive insights into *Hox* genes as an evolutionary constraint of the vertebrate body plan, which provides a novel counter-view to give further insights into the evolution and development of the craniofacial and dental systems as non-Hox systems.

Introduction

One of the characteristic features of the vertebral body is the anatomical repetitions along its axis, as seen in the axial skeleton (vertebrae), and their associated muscles and nervous systems[1)]. This repetitive pattern is imposed by an embryonic segmental structure : the somite (Fig. 1, A). Vertebrate somites are mesoderm-derived epithelial blocks that contain the precursors of vertebrae, skeletal muscles, and other connective tissues. Even though each somite shows a very similar epithelial cubic structure when formed, it eventually differentiates into the distinct morphology of its derivatives depending on its position along the anterior-posterior body axis, which is represented by the distinct morphology of each vertebral element. The morphological identities of somite derivatives are largely governed by *Hox* genes[2—5)] (Fig. 1). This short review will briefly overview current topics in *Hox* genes that constitute an evolutional constraint of global body patterning, which appear to make a striking contrast to craniofacial and dental systems that have been proposed to be facilitated by functional diversity and plasticity among the participating tissues during vertebrate evolution.

Hox Genes ; Temporal Collinearity and Spatial Collinearity

Vertebrate embryos develop in a similar progressive fashion, in which anterior structures are formed first, and then more posterior structures are progressively produced by gastrulation following the primitive streak and tail bud regression (Fig. 1). Therefore, the position of these structures along the anterior-posterior (AP) axis in an embryo directly reflects the timing of their production. This mode of body axis formation is highly invariable among vertebrates, sug-

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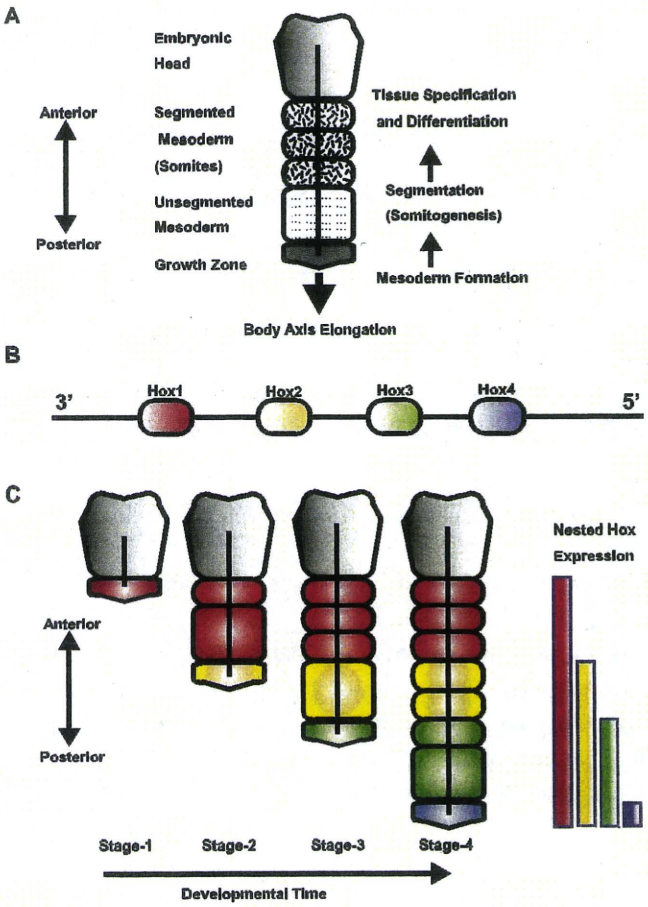

Fig. 1 A) Schematized view of vertebrate body formation. The progressive mode of mesodermal segmentation (somitogenesis) follows body axis elongation. Molecular controls of morphogenesis by *Hox* genes and the segmentation clock are initiated in the growth zone known as the primitive streak or tail bud that is located at the tip of an embryo. Tissue differentiation into skeletal elements, muscle and other connective tissues is specified in the formed somites by neighboring embryonic tissues.

B) Schematized structure of a Hox cluster. In this scheme, four *Hox* genes are arranged in a tandem manner from the 3′ to 5′ direction in a chromosomal region. The order of *Hox* genes along genome DNA correlates with their temporal and spatial expression (spatial collinearity and temporal collinearity).

C) Establishment of *Hox* expression domains in an ideal scheme. In the youngest embryo (Stage-1), the first *Hox* gene (named Hox1 and marked in red) is initiated in the growth zone. In the next stage (Stage-2), *Hox*-1-positive cells are colonized into the anterior most mesoderm and some are already incorporated into somites, whereas the second *Hox* gene (named *Hox2* and marked in yellow) begins to be expressed in the growth zone. In the following stages (Stage-3 and -4), *Hox* genes located in more 5′ regions (*Hox3* and *Hox4* and marked in green and blue, respectively) are progressively activated one by one in the growth zone. Therefore, progressive modes of *Hox* activation and body axis elongation cooperatively endow spatial nested expression patterns of *Hox* genes, which is often described as "Translation of the spatial collinearity into the temporal collinearity". Note that the colors in a body column indicate a functional *Hox* gene at a given body axis level, since the largest numbered *Hox* gene is functionally dominant over the other *Hox* genes expressed in that region, which is known as "Posterior prevalence of *Hox* genes". For instance, in the green region, *Hox3* is functionally dominant, although *Hox1* and *Hox2* are concomitantly expressed in this region (see the nested expression pattern in the right panel).

gesting an evolutionary constraint in which the temporal control of tissue formation and spatial regionalization along the AP axis must be highly coordinated during embryonic development. Although morphologically very similar, somites will differentiate into morphologically distinct vertebrae depending on their axial level which ultimately defines the axial formula (*e. g.* the occipital, cervical, thoracic, lumbar, sacral and caudal spine). This positional identity of somites is specified largely by their unique combinatorial expression of *Hox* genes in the somitic mesoderm[6] (Fig. 1, C). Mammals have 39 *Hox* genes that are organized into four clusters. Birds are believed to have the same set of Hox clusters, though the chicken genome has not yet been perfectly annotated. The genes in each cluster are arranged on the chromosome in a sequence that reflects both the chronological and spatial orders of their expression during embryogenesis (Fig. 1, B and C). The former correlation has been described as 'temporal collinearity' while the latter has been described as 'spatial collinearity'[7–9]. As described above, the vertebrate body axis progressively extends in a posterior fashion when new tissues are added from the gastrulation site at its posterior end. *Hox* clusters initiate their activity in gastrulating cells in a collinear manner in which 3′ *Hox* genes are expressed first, and then later, more 5′ *Hox* genes become progressively activated during axis elongation. The collinear activation of *Hox* genes in gastrulating cells coupled with the mode of body axis elongation results in cells being produced earlier to express more 3′ *Hox* genes and being located at a more anterior structure. This correlation led to the idea that the spatial expression of *Hox* genes along the AP axis is merely the readout of the progressive mode of axis extension in vertebrates (Fig. 1, B and C). A significant amount of evidence supports the functional relevance of the initial expression of *Hox* genes in early embryogenesis to establish the positional identities of somitic derivatives.

Relevance of Early *Hox* Gene Expression for Morphological Identity of Somite Derivatives

Chick transplantations and mouse genetic experiments have led to the idea that *Hox* gene expression is correlated to the acquisition of future somite identity as early as the unsegmented PSM stage. After heterochronic transplantation of embryonic spine precursors : the paraxial mesoderm (PSM), grafted cells still maintain their original *Hox* gene expression and tend to differentiate according to their original identity even when placed at a distinct axial level in the host[10–12]. The relevance of the precise temporal activation of *Hox* genes is also supported by mutations of *Hox* early enhancers in the mouse. These mutations, which cause an initial delay of a given *Hox* gene expression, affect skeletal patterning by largely phenocopying the null mutant of the same *Hox* even if spatial somitic expression appears to be recovered at later stages[13–15]. Therefore, the activation of *Hox* genes at an early stage of paraxial mesoderm development plays a crucial in establishing regional identity. Furthermore, expression of *Hox* genes driven by a PSM-specific promoter demonstrates a severe effect on skeletal patterning, whereas expression by a somite-specific promotor does not elicit such phenotypes[16].

Mode of Mesoderm Formation and Initial Function of *Hox* Genes

Hox genes regulate the timing of mesoderm formation through controlling the flux of epiblast cells to the primitive streak, which could provide the initial regulation of functional *Hox* expression for morphological identity[17]. This is an unique mechanism to achieve regionalization during embryonic body formation. Detailed fate mapping of the epiblast and careful observation of *Hoxb* gene initiation in the chick embryo demonstrate that *Hoxb* gene transcription is sequentially initiated in the epiblast at the level of the middle-to-posterior streak, which gives rise to mainly the lateral plate and the extraembryonic mesoderm[17]. Subsequently, the expression domain spreads anteriorly through the paraxial mesoderm territory to reach the level of Hensen's node and the posterior neural plate. A similar initiation pattern is observed in the Xenopus marginal zone and the mouse epiblast[2,8,18,19].

Current views of the mode of mesoderm formation suggest topological conservation in the mode of mesoderm formation among vertebrates in which mesodermal precursors sequentially ingress (or involute) into the gastrulation point[20—22].

Therefore, the dynamic expression of *Hox* genes follows the gastrulation movements of cells from the epiblast to the mesoderm, suggesting that *Hox* genes could play a role in mesoderm formation through gastrulation. This hypothesis was tested by electroporation of *Hox*-expression constructs containing a fluorescent reporter. The overexpression of *Hoxb* genes in chick embryos in the presumptive territory of the somites in the epiblast at the gastrula stage resulted in collinear phenotypes in terms of AP distribution of *Hox*-expressing cells. Cells overexpressing more anterior *Hox* genes contributed to more anterior portions of the somitic column, whereas cells overexpressing more posterior *Hox* genes contributed to more caudal domains. Overexpression of posterior *Hox* genes (*e. g. Hoxb7* or *Hoxb9*) causes the *Hox*-expressing cells to stay in the epiblast longer than cells expressing either control GFP or anterior *Hox* genes (*e. g. Hoxb1* and *Hoxb4*). A deletion mutant of the third helix of the *Hoxb9* homeodomain does not show this delayed effect on epiblast ingression, suggesting that DNA binding is essential for this function. Therefore, the sequential activation of *Hox* genes controls the timing of cell ingression to the primitive streak, therefore regulating the AP distribution of mesodermal cells. This function of *Hox* genes in mesodermal cell distribution along the AP axis conjugated with their temporal collinear activation most likely provides initial information to establish spatial collinearity, and could therefore provide a framework to elucidate the diversification of the axis formula among vertebrate species[5].

Genomic Structures and Collinear Transcriptional Regulation

A functional link between the tight collinear arrangement of *Hox* genes in the genome and temporal collinear activation during embryogenesis has been postulated for vertebrates[3—5,23]. Genomic relocations of *Hoxd/LacZ* transgenes at distinct positions in the *Hoxd* cluster identified a global regulatory element at the 5′ end of the *Hoxd* complex[14,15,24]. This element is essential for the early collinear activation of the cluster. Together, these observations suggest a silencing mechanism originating at the 5′ end of the cluster, which sequentially makes genes available for transcription in a 3′ to 5′ order (Fig. 2, B and C).

Hox Function on the Axial Formula

A conserved feature of the vertebrate axis is the order of morphologically distinct regions along the AP axis level (*e. g.* cervical, thoracic, lumbar, sacral and caudal vertebrae) ; however, different numbers of segments contribute to various regions, resulting in the variation of axial formulae between vertebrate species. These morphological transpositions are paralleled by corresponding transpositions of the *Hox* gene anterior expression boundaries, suggesting an evolutionally conserved role of *Hox* genes in the positioning of vertebral domains rather than in the specification of each segment[25]. This observation suggests a conserved qualitative function of *Hox* paralogues defining an embryonic area.

Posterior Prevalence, Rather than "*Hox* Code"

The idea that the most posterior genes expressed in a particular embryonic domain impose their dominant function on more anterior genes is described as the posterior prevalence or phenotypic suppression of *Hox* genes[26,27] (Fig. 2, C). In many cases, the posterior prevalence concept accounts for phenotypes of *Hox* mutants in a better way than the *Hox* code concept, in which combinatorial distribution of *Hox* products leads to the morphological diversity of individual vertebrae[6]. The *Hox* code hypothesis proposes that changes in the combinatorial outputs would impose phenotypes on the whole expression domain of these mutated genes ; however, the actual phenotypes in null mutants for all paralogues in a group are restricted to the narrow window of the anterior expression domain of these paralogues[28—30], whereas the posterior domain overlapped by more posterior

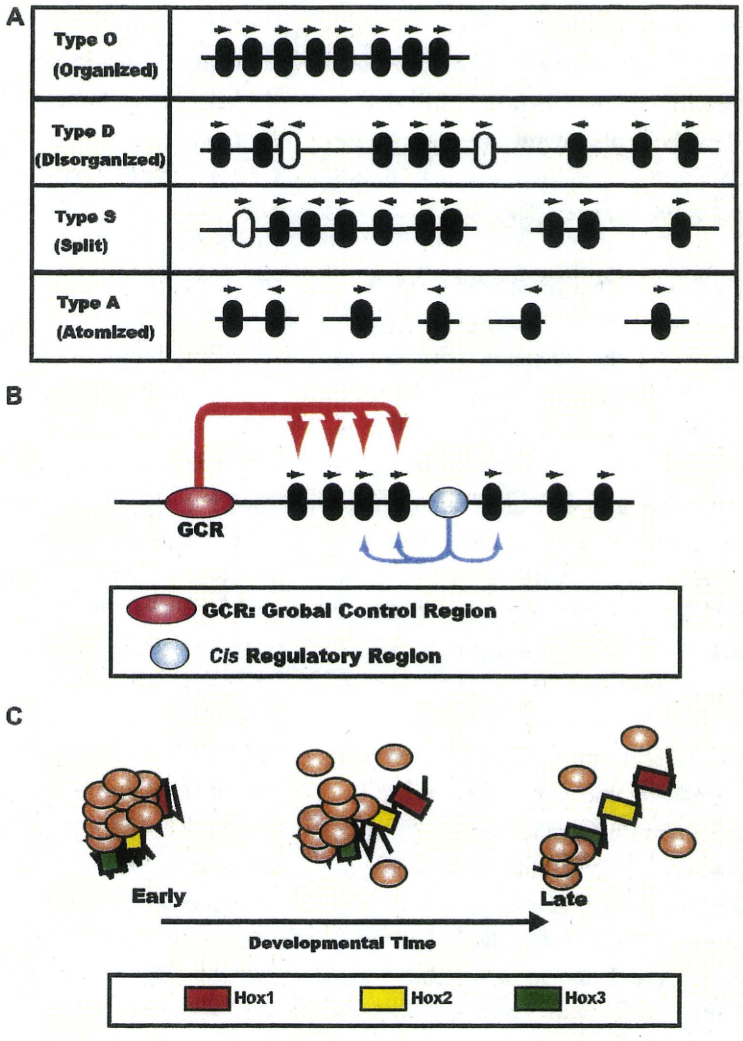

Fig. 2 A) Structural classification of *Hox* clusters[23]. The type O (organized) cluster is well organized with no insertion of foreign genes ~100 kb in genome size and all *Hox* genes (black boxes) are transcribed in the same direction, which represents vertebrate clusters. Type D (disorganized) cluster is much larger (~10 times as large as type O), and contains *Hox* genes in opposite orientations and several non-*Hox* genes (white boxes). Type D examples are found in amphioxus and in sea urchins. Split (type S) cluster appears to be divided into a couple of sub-clusters, each of which has type O or type D features. The drosophila *HOM* complex represents this type S cluster. Type A (atomized) 'cluster' represents the 'no-cluster' situation, in which *Hox* genes are found to be scattered in various genomic loci. The type A cluster is found in Oikopleura. Schemes are depicted from Duboule[23] with some modification.

B) Global control region (GCR) and local *cis*-regulatory region (s) control *Hox* transcription. Type O *Hox* cluster, mainly evolved in vertebrates, may have facilitated GCR-derived transcriptional control of a *Hox* cluster, which probably governs the sequential activation of *Hox* genes in the cluster through long-range *cis*-regulation. The GCR control could involve 'regulatory priming', which intrinsically releases a *Hox* locus and makes it progressively accessible to transcriptional mechanisms, as shown in (C). Local *cis*-regulatory region between *Hox* genes controls adjacent genes, which could have been operated in type D and S clusters.

C) Time-based activation of a *Hox* cluster through progressive chromatin remodeling. During the course of embryonic development, changes in chromatin configuration allow *Hox* genes that are initially silenced to become progressively accessible to achieve transcriptional regulation.

Hox genes shows intact morphology, which must therefore be imposed by the posterior genes. Molecular mechanisms underlying the posterior prevalence remain unclear. The possible involvement of post-translational regulation and miRNA rather than direct transcriptional repression has been discussed[4].

Collinear *Hox* System from Phylogenetic Views

A recent comparison of the genomic structures of the *Hox* gene in various animal species has challenged the conventional view of the *Hox* gene and its evolution[23] (Fig. 2, A). This intensive insight indicates that *Hox* genes are encoded in a same strand and arranged in a tandem manner without insertion by another gene, but only in vertebrates that have the best organized *Hox* clusters (type O) spanning ~100 kb. In marked contrast, many other animal groups have more disorganized (type D : disorganized), split (type S : split) or even scattered (type A : atomized) *Hox* clusters into distinct genome loci. Type D has a longer genome and mixed-up genes with the opposite direction being interspaced by non-*Hox* genes, whereas type S has split clusters of type O or D features, and type A has lost its "cluster" structure, with genes located separately in distinct genome loci. Type D examples are found in sea urchin and a cephalochordate, amphioxus. Type S and A are exemplified by *Drosophila* and *Olikopleura*, respectively. This phylogenetic comparison predicts that the ancestral cluster would be type D, suggesting that type O evolved only along the vertebrate lineage. On the other hand, type D evolved into type S and A along other lineages, implicating that evolution of the *Hox* gene cluster may not have proceeded simply by progressive expansion and diversification of the genes. Therefore, only vertebrates evolved the functional link between the tight collinear arrangement of *Hox* genes in the genome and temporal collinear activation during embryogenesis (Fig. 2) as is discussed in this review. In other words, global regulatory enhancers and associated spatio-temporal regulations of a *Hox* cluster, by which a single compacted cluster is activated as a single transcriptional unit rather than a cluster of multiple genes with distinct transcriptional regulations, has been selected only for the vertebrate body plan. Consistently, human genome sequencing demonstrates the extremely low density of interspersed repeats (as *Alu* sequence) in the four *HOX* clusters, suggesting that large- and short-scale *cis*-regulatory elements in each cluster are a strong selective constraint that cannot tolerate being interrupted by insertions[31].

Visualization of Transcriptional Control by Nuclear Organization and Chromatin Regulation

Sequential activation of the genes along the *Hoxb* cluster was observed in mouse embryonic stem (ES) cells[32] and in early mouse embryos[33] by fluorescent *in situ* hybridization (Fig. 2, C). The short exposure of ES cells to RA at a time when only *Hoxb1* is activated is sufficient to induce general decondensation of the *Hoxb* locus and selectively induce *Hoxb1* looping out of its chromosome territory (CT), while *Hoxb9* is neither expressed nor looped out. Longer RA incubation, however, induces both *Hoxb1* and *Hoxb9* looping out of the CT. In other words, the *Hoxb* locus is sequentially released from the CT in a direction from *Hoxb1* to *Hoxb9*, which appear following the temporal collinear activation of *Hoxb* genes. In gastrulating embryos, looping out of *Hoxb1* from the CT was observed where *Hoxb1* was first expressed. In the tail bud of an E9.5 embryo where both genes are expressed, both *Hoxb1* and *Hoxb9* looped out of the CT. These observations demonstrate that chromatin decondensation and successive looping out of the CT are tightly concomitant with gene transcription in both *in vitro* and *in vivo* contexts. Although a functional link between the looping out of genes and transcription remains to be established, the extrusion of a locus from the CT probably represents a poised state for transcription. Recently, systematic analysis of the chromatin status using developing mouse embryos more directly demonstrated that successive *Hoxd* gene transcription tightly correlated with directional transition in the chromatin status[34]. A clustered *Hox* locus appears to be essential to achieve this correlation, since a split *Hoxd* cluster does not exhibit this temporal transition.

Conclusion Remarks

Vertebrates have invented a highly elaborate body patterning system with genomic organization of *Hox* clusters, which does not appear to be explained by mere genomic expansion and diversification. Seeing that the craniofacial tissues, including the jaw and teeth are developed from the anterior most embryonic region that is in "*Hox* default" (*Hox*-free) status, it is intriguing to consider how discontinuation or renunciation of the *Hox* system has contributed to the evolution and development of the maxillofacial system[35,36]. Recent functional analysis of *Hox* genes using transgenic systems demonstrated that the relay of *Hox* gene activation successively regulated the formation and termination of the body axis in the mouse embryo, indicating a novel role of *Hox* genes in the body axis formation than the morphological patterning of somites[37]. More integrative models or conceptual frameworks are therefore necessary to assess the total patterning of the body plan.

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総　説

Illumination of vertebrate development by fluorescence live imaging

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脊椎動物の発生におけるライブイメージング

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抄録

個体発生は細胞の増殖や移動，機能分化が動的に協調して変遷していく過程である。分子生物学やゲノム科学の進展によって，脊椎動物の発生に関わる様々な共通ルールが明らかとなってきた。蛍光ライブイメージングは，4次元での定量的な解析を可能にし，分子から細胞，組織，器官，個体までのより包括的な理解を助ける。これによって，発生・発達生物医学は，分子の階層的記述から，より動的に理解することが可能になってきた。本稿では，脊椎動物の発生・発達生物医学における最近のトピックをいくつか挙げ，その動向と今後の展望について議論したい。

Abstract

Development of organism is a dynamic but coordinated process that involves cell proliferation, migration and changes in cell function. Molecular biology and genome science promoted this realm of biomedical science by elucidating various common rules. Fluorescence live imaging has made it possible to quantitatively analyze multicellular process in 4 dimensions, thus providing coherent understandings of distinct levels of description form molecular levels to tissue, organ and organism. Application of this approach has given further insight and comprehension of dynamic process, not mere description of molecular hierarchy, into developmental biology and medicine. In this review paper, we, through introducing current topics in the body patterning, overview how the live imaging of fluorescent proteins has shed new lights on developmental biology.

Key words : morphogenesis, development, microscopy, fluorescence, imaging, fluorescent protein

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Introduction

Embryonic development is a sequential and dynamic process in which cellular responses to patterning signals progressively restrict cell fate and localization into defined embryonic regions, which drives the growth and the shape changes of embryonic tissues. Distinct local signals induce cells to adopt specific behavior and functional potential that endow spatio-temporal organization of tissue development. Therefore, the morphological process in early embryonic development provides the fundamental framework of organogenesis.

Decades of study have established widely accepted conceptual frameworks for embryonic patterning and morphogenesis such as Spemann's organizer, epithelial-mesenchymal interactions, and morphogen gradients. Advances in molecular biology and genetics have helped decipher the molecular cascades involved in these conceptual frameworks. However, providing new answers to old questions by modern technology is concomitant with raising new questions asked to be answered by more current methods. Mere uncovering of molecular cascades and genetic hierarchy appears to fall short of providing more satisfying understanding of the spatio-temporal dynamics of biological phenomena, including embryonic development. Recent advances in fluorescence microscopy have widened this view by providing coherent insights of different scales of descriptions from the molecular level to organogenesis and ontogeny.

The purpose of this review is to illustrate current views on how the bio-imaging of fluorescent proteins and probes has contributed to the field of developmental biology through introducing current topics in the body patterning. This technology has enabled the examination of molecular and cellular behavior to study their inherent dynamics in the three-dimensional environment of living multi-layered cell organism. The application of this technology has been extended to visualize the developmental processes of vertebrate embryo in four-dimensions.

Fluorescence protein imaging in the study of vertebrate development

The properties of a fluorescent molecule arise from a chemical moiety, the fluorophore, which absorbs light at a particular range of wavelength and subsequently emits light (fluorescence) at a specific range of longer wavelength. [illegible] small organic dyes, such as fluorescein, rhodamine and AlexaFluors, and inorganic nanocrystals, also known as quantum dots. They need to be conjugated to targeting molecules, such as antibodies, because of their limited cell permeability that restricts their applications especially in targeting intracellular molecules, even though they have advantages over fluorescent proteins such as the small size of organic dyes and the exceptional photostability of quantum dots. In contrast to these fluorescent molecules, fluorescent proteins are genetically encoded and can be fused to any protein of interest. This makes their use protein-specific, minimally invasive and thus suitable for in vivo "live" studies.

Needless to say, a revolution in live fluorescent imaging has begun with the discovery of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Shimomura, et al., 1962), molecular cloning of GFP (Prasher, et al., 1992) and the expression of GFP in heterologous systems (Chalfie, et al., 1994) (Miyawaki, 2008).

GFP encodes a tripeptide within its own structure that is buried at the heart of a 2.4-nm by 4-nm beta-barrel and that undergoes an autocatalytic reaction to form a functional fluorophore without any specific exogenous factors other than molecular oxygen. The discovery and development of fluorescent proteins have fostered various biomedical fields. Developmental biology is one of the fields that have benefited greatly from fluorescent protein technology.

From cellular dynamics to embryonic morphogenesis

Cell migration plays a major role during embryonic morphogenesis. Visualization of labeled cells using fluorescent proteins has enabled the real time analysis of cell migration in vivo.

Cell migration during vertebrate gastrulation

Gastrulation is a complex 3-demensional process in which cells change position in the plane of the tissue, thereby producing the different germ layers and also providing the fundamental architecture of the embryonic body. Epiblast cells move, converge towards and ingress through a structure called the primitive streak in chick embryos, and ultimately migrate away from that streak.

The electroporation of GFP into epiblast cells and into a subset of primitive streak cells led to the characterization of the migration trajectories of the epiblast, with endodermal and mesodermal cells emerging from the primitive streak at different locations (Yang, et al., 2002). Live imaging showed that FGF4 and FGF8 behave like attractant and repellent growth factors, respectively. Combined imaging with fluorescently immuno labeled fibronectin: an extracellular matrix (ECM) and epiblast cells during primitive streak formation showed that, epiblast cells move little with respect to the ECM, which supports the notion that the majority of epiblast cell movement is associated with ECM migration (Zamir, et al., 2008).

Applying this chick live imaging with two color fluorescence cell labeling revealed a dual mode of mesoderm formation (Iimura and Pourquie, 2008, Iimura, et al., 2007). This observation indicated that the medial mesodermal structure is derived from a small portion of stem cells located in the epiblast adjacent to the Hensen's node: the chick organizer; whereas the lateral mesodermal cells are derived from the continuous ingression of epiblast cells, thus suggesting that the gastrulation mechanisms are better conserved between lower vertebrates and amniotes than previously thought.

Intrinsic molecular control on the timing of mesoderm formation by Hox genes

Collinear activation of *Hox* genes regulates the timing of mesoderm formation by controlling the flux of epiblast cells to the primitive streak during gastrulation (Iimura and Pourquie, 2006). Labeling groups of mesoderm precursor cells in the epiblast with different colored fluorescent proteins made it possible to monitor effects of different *Hox* genes expressed in these cells with unprecedented spatiotemporal resolution. This observation demonstrated unique roles of Hox genes in mesodermal cell distribution along the anterior-posterior embryonic body axis. This mechanism could thus provide a framework to understand the diversification of the axis formula among vertebrate species because the functional relevance of *Hox* genes on embryonic body plan and genomic organization has been demonstrated, especially in vertebrate evolution, (Iimura, et al., 2009, Iimura and Pourquie, 2007).

Real-time imaging of oscillatory signaling for embryonic body patterning

Body patterning of the developing vertebrate embryo is controlled by an ultradian oscillator, known as the segmentation clock. This molecular clock outputs periodic Notch, Fgf and Wnt signaling during embryonic mesoderm segmentation, known as somitogenesis, which endows the basic segmental patterning during organogenesis in the vertebrate body, including the vertebrae, and their associated muscle, tendon and nervous system. The analysis of transcriptional oscillations of the clock genes has been demonstrated by classical *in situ* hybridization methods on distinct stages of embryos because of the short oscillation periods, such as at, 30, 90 and 120 minutes for zebrafish, chick and mouse, respectively. It is, however, virtually impossible to analyze the phenotypic effect on this clock machinery, such as periodicity and amplitude. The recent development of fluorescent tools to study the dynamics of this oscillator represents a key advance in this field.

The promoter of the cyclic gene *lunatic-fringe* (Lfng) was fused to an destabilized version of the fluorescent protein Venus, and this reporter construct was used to generate transgenic reporter mice (Aulehla, et al., 2008). Despite the brevity of the oscillation period in comparison to the folding time and half-life of the Venus protein, this reporter successfully detects the oscillatory waves in cultured mouse embryos. The expression of the reporter reflects the periodic Notch response, since Lfng is a Notch target. This reporter mouse could be a powerful tool to test cross-talk among clock signals. Two-photon microscopy employing this strategy ruled out the possibility that constitutively active Wnt signaling altered the oscillation amplitude, but not the period, thus suggesting that periodic Wnt signaling controls Notch oscillations.

Probing cellular dynamics and embryonic development

Among the most important advances in laser microscopy is the capability for optical sectioning, which allows observation deeper inside tissues without interference from out-of-focus light and scatter, thus enabling unprecedented spatial and temporal resolution. This advance is achieved by the development of highly sensitive detectors and the engineering of fluorescent labeling.

enabled biologists to visualize the cell-cycle behavior of individual cells within multicellular structures.

Fuuci: fluorescent ubiquitination-based cell cycle indicator

Sakaue-Sawano et al. recently developed Fucci (fluorescent ubiquitination-based cell cycle indicator), a genetically encoded indicator for cell-cycle progression (Sakaue-Sawano, et al., 2008). This indicator system applies the regulation of cell-cycle-dependent ubiquitination of two cell cycle proteins, Cdt1 and geminin. Cdt1 and geminin have opposite effects on DNA replication during the S phase, and their protein levels oscillate accordingly throughout the cell cycle. Cdt1 protein levels are highest in the G1 phase just before DNA replication and decrease as cells transition into the S phase, whereas geminin protein levels rise during the S phase and fall during the G1 phase. Therefore, each of these two proteins appears once in distinct phase(s) of a single cell cycle in a reciprocal manner.

The original Fucci probe was generated by fusing mKO2 (monomeric Kusabira Orange2) and mAG (monomericAzami Green) to the ubiquitination domains of human Cdt1(hCdt1) and human geminin (hGem), respectively (Figure 1a). These two chimeric proteins cell cycle, reciprocally labeling nuclei of G1 phase cells in orange and those in S/G2/M phase in green. Therefore, these proteins function as effective G1 and S/G2/M markers. Furthermore, generation of transgenic mice that express Fucci in every cell enabled the characterization of the cell-cycle behavior of embryonic neural progenitor cells and the cell-cycle properties of cancer cells both *in vitro* and *vivo*.

Live imaging of cell cycle progression in a developing whole embryo

Sugiyama et al. used the Fucci system to successfully acquire a 4-dimesional view of cell cycle progression in developing zebrafish embryos (Figure 2 and 3) (Sugiyama, et al., 2009). The Zebrafish embryo is the most suitable target for live imaging using laser microscopy because of their external development at room temperature enables proper embryonic development under a microscope without any specific incubator, and its transparency allows feasible microscopic access to almost every stage of embryogenesis.

Transgenic zebrafish lines containing the ubiquitous hspa8 promoter were initially used to determine whether mammalian Fucci constructs properly indicate cell cycle in living zebrafish. This assay indicated that Cdt1 is not

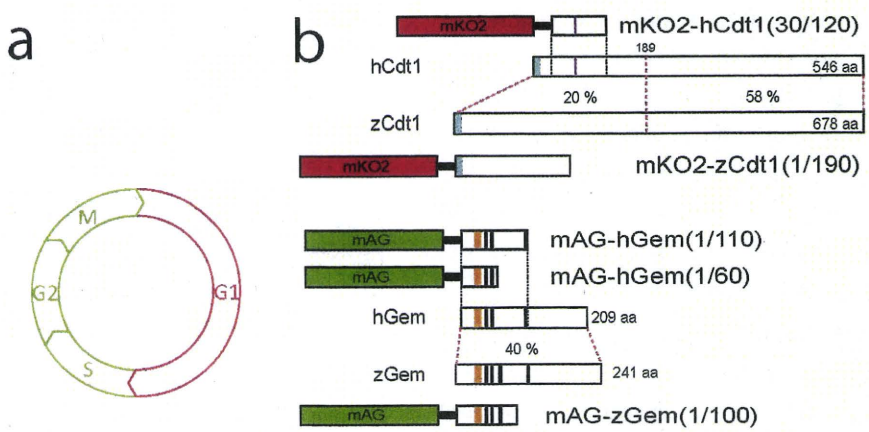

Figure 1 Fuuci: fluorescent ubiquitination-based cell cycle indicator

(a) Fucci probe was generated by fusing mKO2 (monomeric Kusabira Orange2) and mAG (monomeric Azami Green) to the ubiquitination domains of Cdt1 and geminin, respectively. These two chimeric proteins demarcate cell cycle phases of G1 and S/G2/M in orange and green, respectively.

(b) Structural comparison of mammalian (human) Fucci and zebrafish Fucci. Vertical lines indicate relative location of ubiquitination domains in Cdt1 and geminin. Note that different ubiquitination sites between human and zebrafish Cdt1 (see Sugiyama et al. 2009 for details) are used.

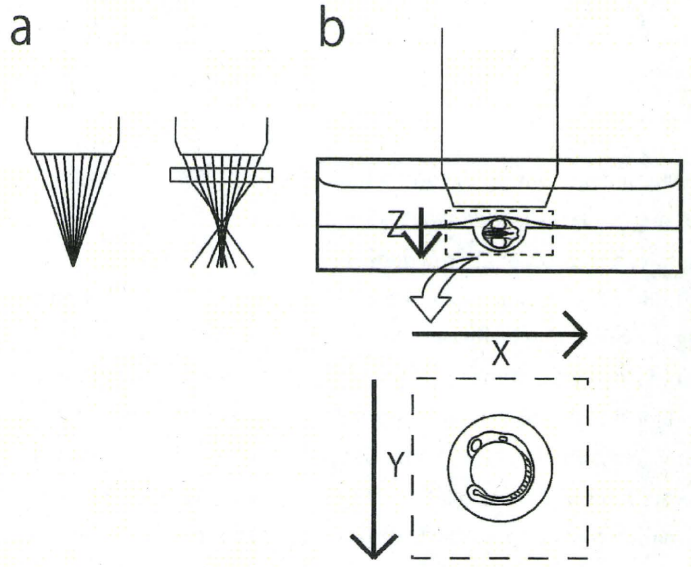

Figure 2 Use of water immersion objective to access living zebrafish embryo

(a) Standard microscopic investigations of thinly cut fixed tissue sections and living cells adhered to glass substrates can produce high-resolution images. However, attempts to image cellular dynamics inside living tissue such as an embryo where important events can occur deep within the specimen, far away from the cover glass often suffer from artifacts, including severe spherical aberration in which emission rays coming through distinct portion of objective lens do not focus at a single point (right picture). The use of a corrected water immersion objective dipped into water and direct access to the specimen is an effective approach to overcoming the aberration problems (left picture).

(b) A zebrafish embryo is supported in a small whole in a 1% agarose gel and gently oriented to determine the xy-plane of interest. Then the embryo is anesthetized with Tricane and covered with 0.3% agarose gel.

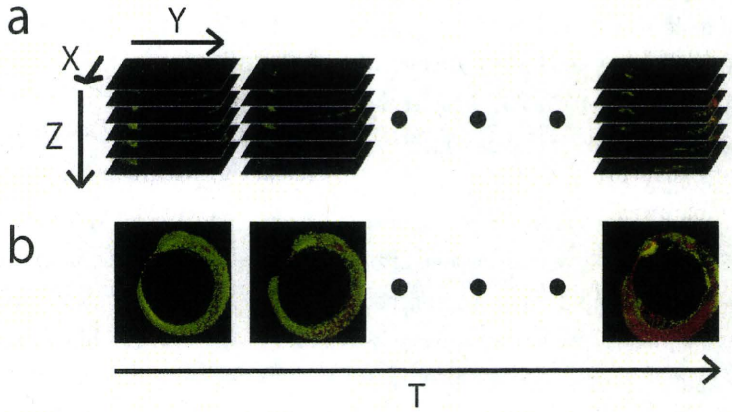

Figure 3 Four-dimensional imaging of a living whole embryo

(a) Time-lapse 3D imaging is performed in the xyz-t mode using a confocal upright microscope system equipped with a water-immersion objective. Images are acquired sequentially at 473 nm and 559 nm to avoid cross-detection of green and orange signals. Forty to fifty confocal images along the z axis are acquired every 5 minutes.

(b) Image processing including 3D reconstruction and data analysis is performed using computer software.

compatible between mammals and fish in terms of ubiquitin-mediated degradation (Figure 1b). Therefore, DNA constructs using the zebrafish homologs of Cdt1 (zCdt1) and geminin (zGem) were constructed, and then characterized using cultured fish cells, and constructed transgenic zebrafish lines. The fish-version of Fucci (named Cecyil: cell cycle illuminated) allowed observation of the dynamic patterns of cell-cycle progression in developing whole embryos. This observation illustrated intrigue correlations between cellular behavior and cell cycle progression, such as in the developing retina and notochord.

What is the behavior and contribution of individual cells within a cohort of migratory cells, and what is the morphogenetic driving force for directional growth of an embryo? The combination of fluorescence imaging and mathematical modeling provided unique answers to this question (Benazeraf, et al., 2010). Combined imaging of fluorescently immuno-labeled fibronectin in posterior mesoderm cells during embryonic body elongation demonstrated that the gradient of random cell motility downstream of FGF signaling controls posteriorly directed tissue elongation in the amniote embryo. This work uncovered the unique biological phenomenon that tissue elongation is driven by the collective regulation of graded, random cell motion rather than by the regulation of directionality of individual cellular movements or migration.

Conclusions and perspectives

The application of molecular biology and genetics to embryology led to the discovery of conserved rules of construction in the animal body plan, such as molecular hierarchy, signaling and cross talk. It is thus considered to be important to recognize that current models of how cells respond to signaling pathways *in vivo* are almost completely devoid of any quantitative information. Little is known about the sensitivity, amplification, persistence, flow and fluctuation characteristics of signaling pathways and transcriptional responses. Detectable fluctuations and changes in oscillation at the molecular and cellular levels could be associated with reproducible cellular patterns for development and even with pathological states. Such variability or diversity has often been ignored to deduce simple rules, models and even understanding or consensus. The use of fluorescent proteins, for biological imaging promotes the quantitative analysis of developmental processes because their intensity and turnover can be measured. Adopting these quantitative studies in three- or four-dimension analyses is not merely an aesthetic improvement in imaging. This type of imaging has revealed the complexity and broadened our comprehensive understanding within a coherent framework of distinct levels of description. These techniques have benefited from the insights of mathematics, physics, engineering and computer science to build and test predictive collaboration among these different fields of scientific research.

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