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Coordination between body growth and tissue growth: Wolffian duct elongation and somitogenesis proceed in harmony with axial growth

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ABSTRACT During embryogenesis, different tissues develop coordinately, and this coordination is often in harmony with body growth. Recent studies allow us to understand how this harmonious regulation is achieved at the levels of inter-cellular, inter-tissue, and tissue-body relationships. Here, we present an overview of recently revealed mechanisms by which axial growth (tail growth) drives a variety of morphogenetic events, with a focus on the coordinated progression between Wolffian (nephric) duct elongation and somitogenesis. We also discuss how we can relate this coordination to the events occurring during limb bud outgrowth, since the limb buds and tail bud are appendage anlagen acquired during vertebrate evolution, both of which undergo massive elongation/outgrowth.

KEY WORDS: *Wolffian duct, somitogenesis, body axis, chemoattraction, FGF8, limb bud, Hox gene*

Introduction

During early development, the embryo grows in size. Massive growth is most obvious in the posteriorly extending axis and in the laterally protruding limb buds. These body growths are intimately associated with internally ongoing morphogenesis. For example, the growth of the axis from anterior to posterior coincides with somitogenesis (Dubrulle & Pourquie, 2002, Hubaud & Pourquie, 2014) and the elongation of Wolffian duct (WD; also called nephric duct) (Saxen & Sariola, 1987) (Fig. 1), and limb outgrowth progressively produces cells that are ready for differentiating into skeletal cartilage along the proximal-distal (PD) axis (Tabin & Wolpert, 2007). In these contexts, body growth and internal morphogenesis need to be coordinated, and a failure of such coordination may lead to malformation of respective and /or both sides.

Recent studies have advanced our knowledge regarding how cells interact with each other at the microscopic levels of molecules and cells. However, how such coordination is achieved at the macroscopic level has largely been overlooked. We presently overview recent studies that address such old and new questions focusing on body elongation and the concomitant progression of somitogenesis and WD elongation within the elongating axis. For these types of studies, chicken molecular embryology offers a remarkable model system. We also discuss the possible commonalities between other tissues that develop in harmony with somitogenesis, and between axis elongation and limb bud outgrowth.

Coordination between axis elongation and somitogenesis

Growth of the embryonic body along the anterior-posterior (AP) axis is attributed mostly to the extension of the posterior tip, where neural and mesodermal progenitors are progressively supplied by the epiblast and/or tail bud (Shimokita & Takahashi, 2011, Catala *et al.*, 1995, Cambray & Wilson, 2002, Tzouanacou *et al.*, 2009, Olivera-Martinez *et al.*, 2012, Cambray & Wilson, 2007, Wilson *et al.*, 2009). Mesodermal progenitors participate in the formation of the presomitic mesoderm (PSM), a pair of longitudinal strips lying on either side of the neural tube. The PSM undergoes somitogenesis at its anterior end, which proceeds rhythmically at regular intervals of time and space. For this unique event, axis elongation plays a critical role. The tail bud produces a high level of FGF8,

Abbreviations used in this paper: EGFP, enhanced green fluorescent protein; FGF, fibroblast growth factor; PSM, presomitic mesoderm; WD, Wolffian duct.

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thereby creating a gradient of this protein along the AP axis, with the highest concentration posteriorly. The FGF gradient together with the segmentation clock determines the site and rhythm of somitogenesis (Dubrulle & Pourquie, 2002, Delfini *et al.*, 2005, Dubrulle & Pourquie, 2004, Dubrulle *et al.*, 2001).

Importantly, the rate of somitogenesis matches the rate of axis elongation, leaving the length of PSM constant, except for the somitogenesis-termination stage when the PSM is diminished. For the mechanisms of somitogenesis, many excellent reviews articles are available (Pourquie, 2011, Aulehla & Pourquie, 2008, Aulehla & Pourquie, 2010, Ozbudak & Pourquie, 2008, Pourquie, 2007, Baker *et al.*, 2006, Dequeant & Pourquie, 2008, Hubaud & Pourquie, 2014).

Coordination between somitogenesis and WD extension

Another tissue forming at the same rate as axis elongation is the Wolffian duct (WD), which therefore proceeds in register with somitogenesis (Saxen & Sariola, 1987, Atsuta *et al.*, 2013, Obara-Ishihara *et al.*, 1999). WD is an early primordium for the developing kidney, extending as a pair of straight tubular cords in the anterior-to-posterior direction along the lateral edges of somites/PSM (Fig. 1A). The front of extending WD, which is located posteriorly in embryo, coincides with approximately the level -3 of somitogenesis, located posteriorly to the newly forming gap with a distance of three presumptive somites (Fig. 1 B,C).

What governs this precise coordination between somitogenesis and WD elongation? Two possibilities are conceivable. One is that as the somites/PSM develop, they provide signals that cause WD to be coordinated. The other is a direct regulation by the tail bud, which would determine the rate of *both* somitogenesis and WD elongation. To distinguish between these two possibilities, technologies of tissue-specific gene manipulation and tissue- and cell-transplantation are useful, for which chickens are ideal model animals. In particular, the *in ovo* electroporation technique enables a local manipulation of cells and tissue with genes of interest (Momose *et al.*, 1999, Funahashi *et al.*, 1999, Tadokoro *et al.*, 2016, Saito *et al.*, 2012). Indeed, PSM-specific gene manipulations in chicken embryos have made critically important contributions to understanding somitogenesis (Sato *et al.*, 2002, Nakaya *et al.*, 2004, Dubrulle *et al.*, 2001, Dale *et al.*, 2003, Sato *et al.*, 2008, Watanabe *et al.*, 2009, Sato *et al.*, 2007, Takahashi *et al.*, 2008, Watanabe *et al.*, 2007).

WD-specific gene manipulation by *in ovo* electroporation

A procedure for the transgenesis of the WD has been recently developed (Atsuta *et al.*, 2013), where the unique morphogenesis of this tissue was exploited. In 10-somite embryos, the WD emerges in the presumptive pronephric region corresponding to the 6th-12th somites along the AP axis (Hiruma & Nakamura, 2003). The elongating WD receives no contribution from surrounding mesoderm (Martin, 1976). Therefore, if the somite level 6th-12th is targeted by *in ovo* electroporation, exogenous genes such as *EGFP* gene can be specifically and efficiently introduced into WD (Fig. 2A) (Atsuta *et al.*, 2013).

EGFP-electroporated cells are widely distributed in the elongating WD in a mosaic pattern, where shapes of individual cells can be visualized. Time-lapse imaging analyses have further revealed that leader cells at the elongating front are mesenchymal in shape and actively motile, with multiple cellular processes including filopodia, whereas the rear cells are more static and epithelial (Fig. 2 B-D) (Atsuta & Takahashi, 2015). These differences are consistent with the observation obtained by scanning electron microscopy showing tubular and non-tubular structures in the rear and front regions, respectively (Fig. 1D, E; photos by the authors) (Hiruma & Nakamura, 2003). Time-lapse analyses also highlight a sequence of cell division along the AP axis in the front of WD co-electroporated with H2B-EGFP (nucleus) and Lifeact-mCherry (actin filament) labels (Fig. 2F).

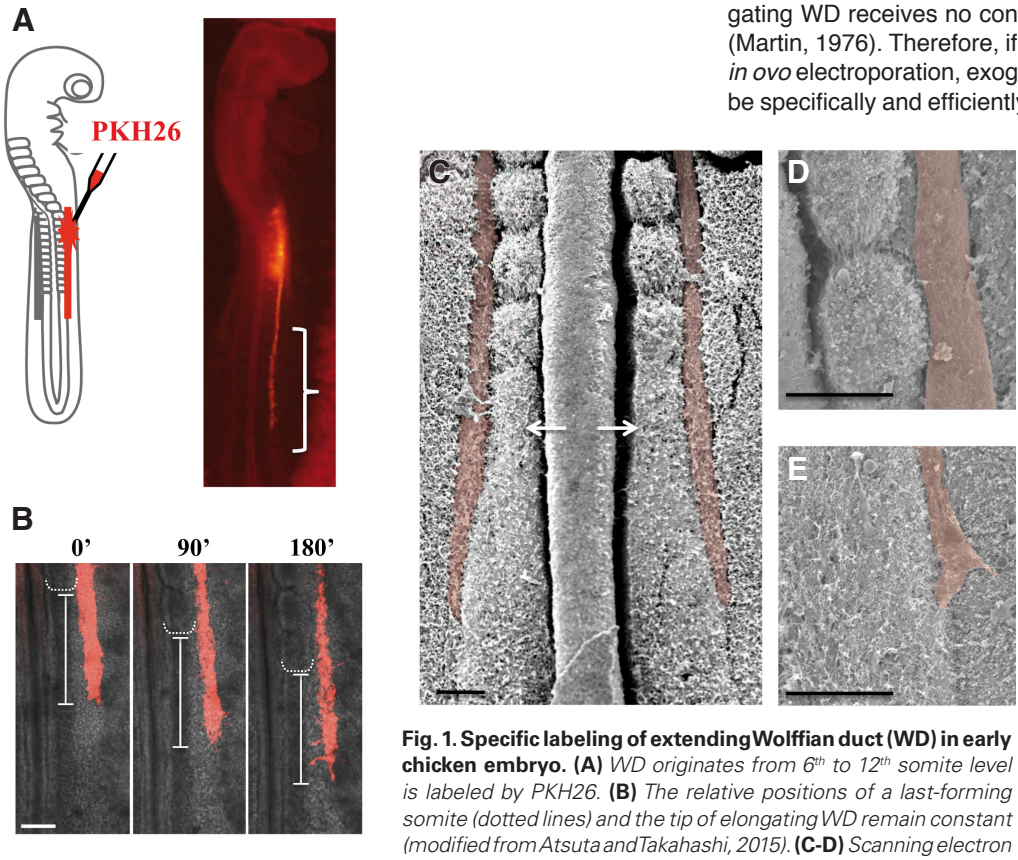

Fig. 1. Specific labeling of extending Wolffian duct (WD) in early chicken embryo. (A) *WD originates from 6th to 12th somite level is labeled by PKH26.* **(B)** *The relative positions of a last-forming somite (dotted lines) and the tip of elongating WD remain constant (modified from Atsuta and Takahashi, 2015).* **(C-D)** *Scanning electron microscopy of somites, presomitic mesoderm, and WD of 21-somite embryo. WD is digitally colored. WD located in a rear region (anteriorly in embryos)* **(D)**, *but not in the tip* **(E)**, *appears to be a tubular structure. Developing gaps between newly forming somites and PSM are indicated by white arrows. Scale bar: 50 μm. (B) Modified from (Atsuta & Takahashi, 2015).*

Migration of leader cells is regulated by FGF8

When leader cells are dissected from a donor embryo and transplanted ectopically into a stage-matched host, the transplanted cells migrate back to the original extension path. Conversely, when a piece of the WD migration path is laterally placed in a host embryo, a host WD is attracted to the transplant. A series of such embryonic manipulations, together with gene expression screening by *in situ* hybridization, have narrowed down candidate signaling and receiving molecules to FGF8 and FGFR3. The *FGF8* mRNA-positive area regresses at the same rate as the axial elongation, and this area overlaps with the position of leader cells, which express *FGFR3* mRNA. Thus, the overlapping domain remains constant as the embryo grows (Fig. 3A). The leader cells indeed transmit FGF signal intracellularly since these cells exhibit FGF-related intracellular signals including Pea3, Sef, and phosphorylated ERK (Atsuta & Takahashi, 2015).

The WD elongation can be directly regulated by FGF8, as shown by a series of experiments. First, when an FGF8-producing cell aggregate or FGF8-soaked bead is transplanted ectopically in a host embryo, WD is attracted to this implant (Fig. 3B, C). Second, when beads soaked in the FGF signaling inhibitor SU5402 are implanted near the front of WD, they prevent the extension of this tissue (Fig. 3D). Pharmacological assays have further revealed that the FGF signal within the leader cells is mediated by Ras/MAPK, and not by PI3K or PLCγ. Finally, WD leader cells cultured *in vitro* are attracted to an FGF8-bead, indicating a direct chemoattractive action by FGF8 (Atsuta & Takahashi, 2015). As mentioned above, the anteriorly decreasing FGF8 gradient is established by the tail bud during axis elongation (Dubrulle & Pourquie, 2002, Delfini *et al.*, 2005, Dubrulle & Pourquie, 2004, Dubrulle *et al.*, 2001). Thus, WD elongation and somitogenesis are driven by the axis elongation independently.

Intra-tissue coordination within the Wolffian duct

Tail bud-derived FGF signals are important not only for the coordination between somitogenesis and WD elongation, but also for progressive morphological specification *within* the WD. As mentioned earlier, the leader cells of WD are motile, whereas rear cells are static and participate in epithelial tubular construction. During WD elongation, these two types of cells are separated from each other with a constant distance between them. This raised the possibility that the rear cells undergo epithelialization because they are liberated from the influence of the tail-derived FGF.

To test this possibility, the following experiments were conducted. First, when a rear part of WD was translocated into the WD's front region, this piece underwent massive elongation like normal leader cells. Second, when the WD extension was inhibited by SU5402, leader cells not only stopped elongation but also became epithelialized. Furthermore, these ectopically epithelized cells resumed elongation when transplanted back into the normal front migration pathway. Thus, during the normal WD elongation, the rear cells are out of the FGF influence that regresses coordinately with the axial elongation (Atsuta & Takahashi, 2015).

These findings provide novel insights into how the body growth coordinately regulates the formation of internal tissues. The axial growth determines both inter-tissue (between somitogenesis and WD extension) and intra-tissue (leader cells versus rear cells in WD) coordination (Fig. 4).

Other tissues that coordinately form with axis elongation

It is known that specification and morphogenesis of the nervous system propagate antero-posteriorly. In the central nervous system, neurons in the spinal cord differentiate earlier in the anterior region than in the posterior. Neural crest cells (NCCs), a basis of peripheral nervous system, emigrate from the dorsal neural tube, and this process also occurs progressively along the AP axis. More precisely, NCCs start their emigration at the level of somite stage III, the third somite anterior to a newly forming somite. For both

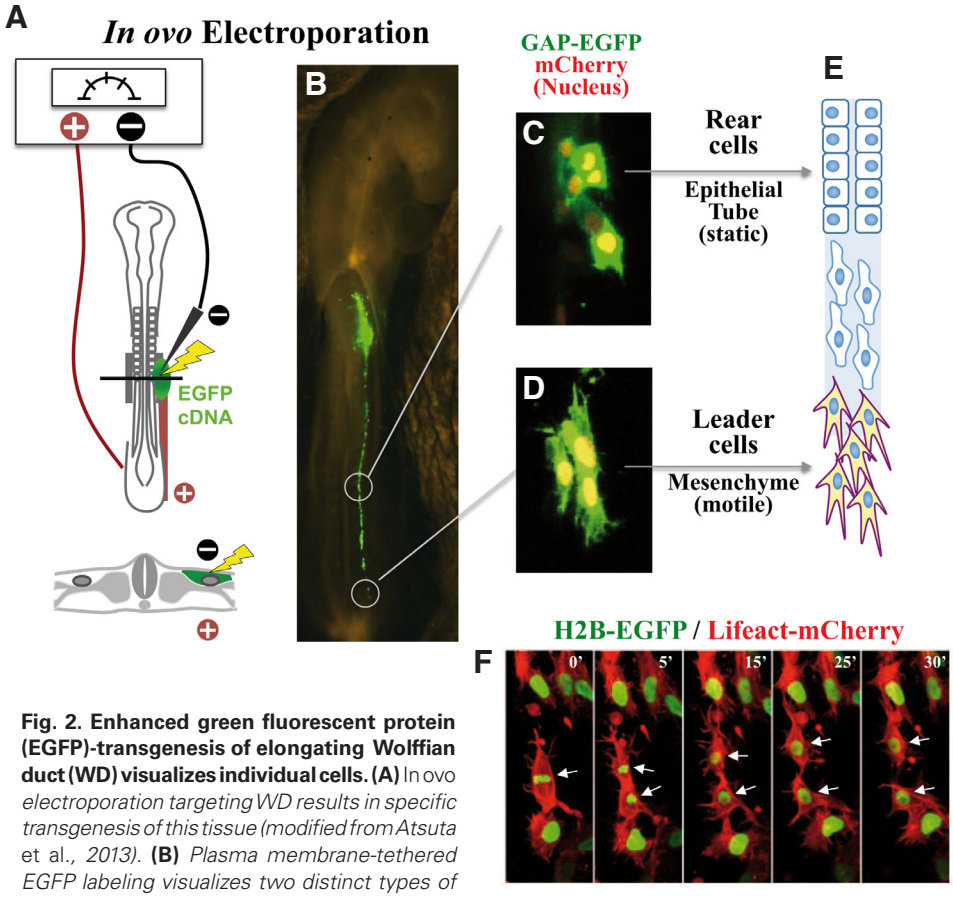

Fig. 2. Enhanced green fluorescent protein (EGFP)-transgenesis of elongating Wolffian duct (WD) visualizes individual cells. (A) In ovo *electroporation targeting WD results in specific transgenesis of this tissue (modified from Atsuta* et al., *2013).* **(B)** *Plasma membrane-tethered EGFP labeling visualizes two distinct types of cells within WD. One is static and epithelialized cells in the rear region of WD* **(C)**, *and the other is actively motile cells with multiple cell protrusion at the tip of elongating WD* **(D)**. *These differences are illustrated in* **(E)**. **(F)** *Time-laps analyses using WD co-electroporated with H2B-EGFP (nucleus) and Lifeact-mCheery (actin filament) reveal a sequence of cell division of leader cells occurring along the AP axis. (C, D) Modified from (Atsuta & Takahashi, 2015).*

events, the anteriorly decreasing gradient of FGF generated by the tail bud plays a critical role (Diez del Corral *et al.*, 2003, Diez del Corral *et al.*, 2002, Martinez-Morales *et al.*, 2011). These studies additionally reported that the posteriorly decreasing gradient of retinoic acid (RA) makes a balance with the anteriorly decreasing FGF gradient. Such opposing gradients of FGF and RA are also known to be critical for the somitogenesis (Aulehla & Pourquie, 2010, Goldbeter *et al.*, 2007), and possibly for the WD elongation as well (Fig. 3E).

Another example is the antero-posterior fusion of the major paired blood vessels, the dorsal aortae, into a mature single dorsal aorta along the midline. For this fusion, notochordal signals including chordin, a BMP antagonist, act as inhibitory signals (Garriock *et al.*, 2010). Intriguingly, the *chordin* mRNA-positive area in the notochord posteriorly regresses concomitantly with the progressive fusion of dorsal aortae such that the blood vessels that are liberated from the chordin influence start to fuse. Although not explicitly indicated in the report, the regression rate of the *chordin* mRNA-positive zone in the notochord appears to match the rate of axis elongation. We here propose the idea that the tail bud-derived FGF gradient may regulate the regression of *chordin* mRNA, in a way similar to the neural crest emigration, leading to the progressive zippering of the dorsal aortae.

The FGF gradient-regulated coordination between neural differentiation, NCC emigration, and possibly the fusion of dorsal aortae is indirect and mediated by BMP antagonists: Noggin and Chordin expressed highly in an FGF-high area prevent precocious emigration of NCCs and fusion of naïve dorsal aortae, respectively. These multi-step regulations under the FGF gradient contrast with the direct remote control of WD elongation by FGF as explained above.

Not all the tissues obey the "rule" of the axis elongation-generated FGF gradient. For example, splitting of the lateral plate mesoderm into the somatopleure and splanchnopleure, taking place in the antero-posterior direction, proceeds much faster than the axis elongation (Saito *et al.*, 2017, Funayama *et al.*, 1999), suggesting different mechanisms operating in more lateral areas of early embryos than axial and paraxial regions.

Possible commonalities in coordination between axial elongation and limb bud outgrowth?

The tail is one of the appendages acquired during vertebrate evolution. The other appendages are the fore- and hind-limbs. A commonality between the tail and limb buds is that both undergo massive elongation/outgrowth. Furthermore, the tail and limb buds, but not in the trunk, use the "caudal" clusters of Hox genes (Hox11 to Hox13) (Beccari *et al.*, 2016, Tschopp & Duboule, 2011, Andrey *et al.*, 2013). Are there any mechanisms shared between the axial elongation and limb bud outgrowth in the context of growth coordination between the body and inner tissues? Like the aforementioned axial elongation-dependent morphogenesis, opposing signals of FGF and RA are important along the proximal distal (PD) axis of the limb buds: FGFs coming from the apical ectodermal ridge (AER) located at the tip of a growing limb bud sustains the proliferation and undifferentiated state of the underlying cells, whereas RA provided from the flank antagonizes the FGF influence. This FGF-RA opposing gradients serve as the basis for the subsequent morphogenesis in the limb buds, and therefore can be regarded as a common mechanism between the axial elongation and limb bud outgrowth. Precisely speaking, however, the AER, the source of FGF protein is an epidermal component, and therefore does not give rise to the underlying mesodermal mesenchymal cells, which increase in number during the limb bud outgrowth. This contrasts with the FGF-producing tail bud, from which neural, mesodermal, and some neuromesodermal progenitors progressively supply cells to respective lineages (Wymeersch *et al.*, 2016, Shimokita & Takahashi, 2011) (Y.T. unpublished data).

During the limb bud outgrowth, except for the conspicuous cartilage specification and patterning along the PD axis into stylopod (upper arm), zygopod (fore arm), and autopod (hand plate), not much morphogenesis can be recognized to proceed in a PD dependent manner. For example, although muscle precursors known to be derived from somites colonize in the defined dorsal and ventral regions of growing limb buds and differentiate at later stages into mature skeletal muscles that are coordinated with growing cartilage and bones to construct anatomical and functional modules, this coordination is not what is discussed here.

Specification and patterning of the cartilage segments along the PD axis are intricately regulated by Hox genes, and do not undergo a simple coordination with the outgrowth of the limb buds in the context discussed above for the axial elongation. Nevertheless, it is worth discussing the possible roles of Hox genes in the patterning along the PD axis of growing

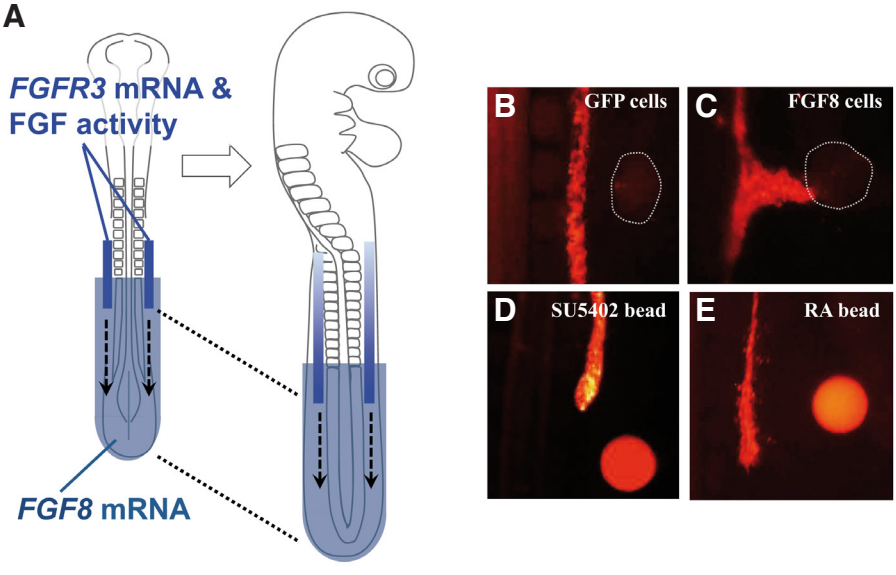

Fig. 3. The gradient of FGF8 drives Wolffian duct (WD) elongation. (A) FGF *mRNA-positive area shifts posteriorly as the body axis elongates. The tip of the WD expresses FGFR3 and transmit FGF signals intercellularly. WD is attracted to ectopically placed FGF-producing cells* **(B,C)**. *Conversely, SU5402- and RA-soaked beads inhibit WD elongation* **(D,E)**. *(B-D) Modified from (Atsuta & Takahashi, 2015).*

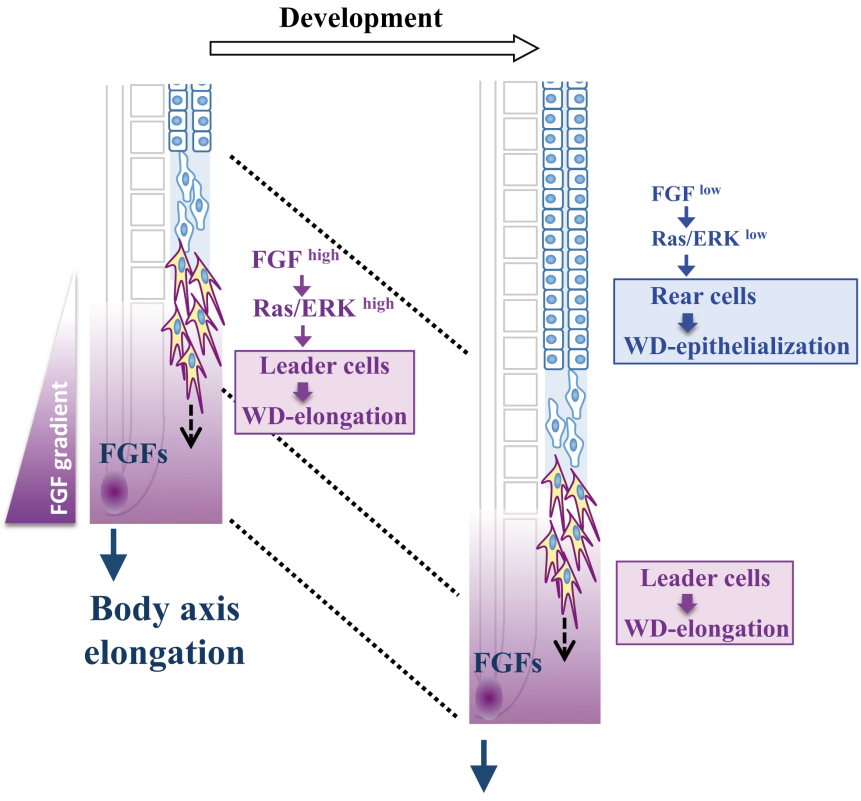

Fig. 4. Dual coordination between the body axis and Wolffian duct (WD) elongation (macroscopic level) and between motile and static cells within WD (microscopic level). *The FGF gradient created by the growing tail bud drives the WD elongation. This gradient also determines intra-tissue cell specification: high FGF for motile leader cells whereas low FGF leads to cell epithelialization and tubular formation. Modified from (Atsuta & Takahashi, 2015).*

limb buds, and relating these roles to the morphogenesis during tail bud elongation, because as mentioned earlier, only these appendages express the caudal Hox genes, Hox 11 to Hox 13. Following early proximal patterning of the stylopod, the caudal Hox11 to Hox13 genes undergo intricate regulations to define the future zeugopod and autopod. In particular, the role of Hox13, expressed latest among the Hox genes by distally derived signaling including FGFs and Shh, is critical in demarcation of the future autopod by inhibiting zeugopod-related Hox genes as elegantly revealed by Duboule's group and others (Beccari *et al.*, 2016, Tschopp & Duboule, 2011, Andrey *et al.*, 2013). In this context, it should be noted that Hoxb13 is also the latest expressed gene among the Hox genes during tail bud elongation (de Santa Barbara & Roberts, 2002). And more intriguingly, mice lacking the function of Hoxb13 show a longer tail than the wild type (Economides *et al.*, 2003). Thus, uncovering the regulation and roles of Hox13 is expected to provide information of how the terminus of appendages is determined after outgrowth/elongation.

Conclusion and perspectives

The coordination between somitogenesis and WD elongation is directly and separately controlled by the FGF gradient established by the tail bud. Other morphogenetic events occurring in axial and paraxial regions, including neurogenesis, neural crest emigration from the neural tube, and possibly the fusion of the naïve dorsal aortae are under the indirect regulation of the FGF gradient, which represses BMP action by upregulating Noggin or Chordin. In these regulations, the FGF gradient is coupled with the opposite gradient of RA. In the case of limb bud outgrowth, the opposing gradients of FGF and RA operate in the way similar to those during the axial elongation. However, the coordination between limb bud outgrowth and internal morphogenesis is not as prominent as the case of axial elongation. Nevertheless, it is worth noting that in both cases Hox13 play particular roles in determining the terminus of outgrowth. Further studies are awaited to know whether the regulations of Hox11 to Hox13 are shared among the appendages.

Growth coordination between the body and internal tissues must be important in many other contexts. For example, when the lung forms, massively branching tissues increase in size and volume, which progressively demand a growing space in the ribcage. Likewise, the skull expands in harmony with the brain growth. To understand such coordination, studies ranging from microscopic levels of gene regulation to macroscopic levels of organ/body formation must be useful, for which chicken molecular embryology offers an excellent model system.

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