Determinants of the Epithelial-Muscular Axis on Embryonic Stem Cell-Derived Gut-Like Structures

Yi Luo^a Miyako Takaki^b Hiromi Misawa^b Hiroko Matsuyoshi^b
Tomonori Sasahira^a Yoshitomo Chihara^a Kiyomu Fujii^a Hitoshi Ohmori^a
Hiroki Kuniyasu^a

Departments of ^aMolecular Pathology and ^bPhysiology II, Nara Medical University, Kashihara, Japan

Key Words

Embryonic stem cells · Hedgehog · Epithelial-muscular axis

Abstract

Dome-like structures with epithelial-muscular layers resembling the gut have been derived from mouse embryonic stem (ES) cells. These domes have been reported to show spontaneous contractions and are called ES gut. In the present study, we examined the epithelial-muscular axis of these domes by detecting differentiation markers. A normal epithelial-muscular axis was exhibited in the domes with spontaneous motility, whereas the domes without spontaneous motility showed either an inverted or obscure axis. To investigate the factors affecting the epithelial-muscular axis, we examined the expression of hedgehog signaling factors in the domes. Expression of hedgehog family factors was detected in the epithelial components of the domes with motility, whereas this expression was inverted or obscure in the domes without motility. Out of the 25 domes, 10 of the 10 motility (+) domes showed a normal epithelial-muscular axis, whereas 14 of the 15 motility (–) domes lacked a normal epithelial-muscular axis. This implies that activin A upregulated the expression of sonic hedgehog and intestinal alkaline

phosphatase in the embryoid bodies. These findings suggest that the motility of the ES gut depends on the domes' epithelial-muscular axis.

Copyright © 2010 S. Karger AG, Basel

Introduction

Embryonic stem (ES) cells are pluripotent cells which retain the potential to differentiate diverse tissues and organs [1]. ES cells are capable of generating spontaneous constructions, including dome-like structures (domes), consisting of epithelial cells, smooth muscle cells, interstitial cells of Cajal (ICC), and a few immature neurons, which bear a histological resemblance to the gut [2, 3]. This gut-like structure has been called 'gut' from mouse ES cells (i.e. ES gut). In this study, we grew ES gut using a hanging drop culture followed by an outgrowth culture [2, 3]. After 4 days in hanging drop cultures, the ES cells formed small spheroid structures called embryoid bodies (EBs). After 7 days, the EBs were transferred to outgrowth cultures. Two weeks later, the EBs had spontaneously given rise to domes with hollow inner cavities.

The gut is a highly integrated structure comprising many tissues. In gut embryogenesis, precursor cells form a primitive gut through processes involving mesoderm formation, endoderm formation, and gastrulation [4, 5]. Unlike normal gut embryogenesis, ES gut formation lacks 3-dimensional processes. Therefore, ES gut cell differentiation may be limited due to a lack of topological cell-to-cell interaction. Indeed, the yield rate of ES gut formation is not 100%. Some domes exhibit highly coordinated periodic contractions and relaxations, whereas some fail to exhibit spontaneous motility.

To explore which structural components are required for the formation of functional ES gut, we examined the relationships between epithelium, muscle, nerve, and ICC and the rates of cell proliferation and cell apoptosis in the domes. Furthermore, we examined the mechanisms governing the domes' histological structure. Finally, we examined the expressions of hedgehog (HH) homolog factors in HH signaling pathways. Sonic HH (SHH) function is essential for cell fate specification as well as for growth and patterning direction during embryonic development [6]. In this study, we examined the functional structure of the motility (+) ES gut and elucidated the factors affecting epithelial-muscular axis formation.

Materials and Methods

Culture of ES Cells and Formation of ES Gut

Undifferentiated ES cells (EB3) were maintained on gelatin-coated dishes without feeder cells in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, Mo., USA) supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL, Grand Island, N.Y., USA), 0.1 mM 2-mercaptoethanol (Wako, Tokyo, Japan), 0.1 mM nonessential amino acids (GIBCO/BRL), 1 mM sodium pyruvate (Bio-Whittaker), and 1,000 U/ml LIF (Chemicon, Temecula, Calif., USA) [2]. The EB3 cells (a kind gift from Dr. Hitoshi Niwa of the Riken Center for Developmental Biology, Kobe, Japan) carried the blasticidin S-resistant selection marker gene driven by the Oct-3/4 promoter (active in undifferentiated cells) and were maintained in medium containing 10 µg/ml blasticidin S to eliminate differentiated cells [7]. To induce EB formation, dissociated ES cells were cultured in hanging drops as previously described, with minor modifications. Two weeks later, they were shown to spontaneously give rise to a dome, i.e. the ES gut. To induce EB formation, we cultured dissociated ES cells in hanging drops [1, 8], with minor modifications. The cell density of 1 drop was 500 cells per 15 µl of ES cell medium in the absence of LIF. After 6 days in a hanging drop culture, the resulting EBs were plated onto plastic 100-mm gelatin-coated dishes and allowed to attach for the outgrowth culture [7, 9, 10]. Culturing in hanging drops was the most important process for differentiating ES gut. Each ES cell-cluster underwent a dramatic transformation into a dome with a solid and cavity-like structure, and some of them showed ambiguous beating. On approximately day 14, these

clusters proliferated to form more prominent 3-dimensional constructions with a cystic structure and began rhythmic contractions that were not necessarily frequent or regular. On approximately day 21, the clusters (ES gut) showed coordinated contraction patterns with relatively regular rhythms, although there were some incomplete domes even on day 21.

Immunohistochemistry

For the immunohistochemistry of thin sections, ES gut was fixed with 4% paraformaldehyde at 4°C and then scraped from the culture dish and embedded into 0.6% agarose-PBS, which was subsequently embedded in a paraffin block in the usual manner. Consecutive 4-µm sections were cut from each block, subjected to antigen retrieval with pepsin (DAKO Corp., Carpinteria, Calif., USA) for 20 min at room temperature, and immunostained using the immunoperoxidase technique. After the blockade of endogenous peroxidase activity by incubation for 15 min in a 3% H₂O₂-methanol solution, the sections were rinsed with PBS and incubated with a primary antibody diluted with washing solution (BioGenex, San Ramon, Calif., USA) for 2 h at room temperature. Thereafter, they were rinsed again with PBS and incubated for 1 h at room temperature with a peroxidase-conjugated secondary antibody diluted to 0.5 µg/ml (Medical & Biotechnological Laboratories Co., Ltd., Nagoya, Japan). All sections were then rinsed with PBS, color-developed using diaminobenzidine solution (DAKO), washed in water, and counterstained with Meyer's hematoxylin (Sigma). Care was taken to ensure that the antibody reaction and DAB exposure were the same for all specimens. The antibodies used in the primary reaction are listed in table 1.

Enzyme-Linked Immunosorbent Assay

For the enzyme-linked immunosorbent assay (ELISA), 1 × 10⁵ cells were cultured using a hanging drop method in 0.5 ml of stem cell medium (Sigma) with or without recombinant human/mouse/rat activin A (100 ng/ml; Cedarlane Laboratories Ltd., Burlington, N.C., USA) for 24 h. The cultured medium was filtered through a 0.2-µm filter (Becton-Dickinson, Bedford, Mass., USA). The filtered medium was used for ELISA. Concentrations of SHH and intestinal alkaline phosphatase (IAP) were detected using a Sonic Hedgehog DuoSet ELISA development kit (R&D Systems, Inc.) and an alkaline phosphatase, intestine, ELISA kit (Uscn Life Science, Inc., Wuhan, China), respectively, according to the provider's instruction.

Statistical Analysis

All statistics were carried out with StatView version 4.5 (SAS Institute, Inc., Cary, N.C., USA), and $p < 0.05$ was considered statistically significant.

Results

Dome Motility

As shown in figure 1a, domes were found after the EBs had been in the outgrowth culture for 2 weeks. On fluorescent calcium ion probe imaging, motility (+) domes showed spontaneous intracellular Ca²⁺ oscillations (fig. 1b), whereas motility (−) domes did not.

Fig. 1. Calcium oscillation in ES gut. **a** Fluorescent image of ES gut. Bar = 100 μ m. Mouse ES cells formed a dome-like structure with a hollow inner cavity at 2 weeks after outgrowth culture. **b** The intracellular calcium concentration was detected by a fluorescent probe. Calcium oscillation was also detected. The upper panel displays a spatial view and the lower panel a temporal view.

Table 1. Antibodies used for immunohistochemical analysis

Antibody	Abbreviation	Company	Clone	Marker
Cytokeratin	CK	Zymed ¹		epithelium
α -Smooth muscle actin	SMA	Zymed		smooth muscle
c-Kit		Santa Cruz ²	C-19	ICC
TFF3	TFF3	NanoTools ³		intestine
Ki67		DAKO		proliferation
ss-DNA	ssDNA	DAKO		apoptosis
Sonic hedgehog	SHH	Santa Cruz	H-160	HH ligand
Patched	PTC	Santa Cruz	G-19	HH inhibitory receptor
Smoothed	SMO	Santa Cruz	N-19	HH excitatory receptor
Glioma-associated Oncogene 1	Gli1	Rockland ⁴		HH nuclear messenger

¹ Zymed Laboratories, Inc., San Francisco, Calif., USA. ² Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA. ³ NanoTools, Teningen, Denmark. ⁴ Rockland Immunochemicals, Inc., Gilbertsville, Pa., USA.

Expression of Differentiation Markers in Domes

We examined the distribution of tissue components cyto keratin (CK), α -smooth muscle actin (SMA), c-Kit, trefoil factor 3 (TFF3), Ki67, and ssDNA in motility (+) domes as compared to motility (–) domes (fig. 2; table 2). Motility (+) domes showed a CK-positive epithelial component on their inner surface and an SMA-positive muscle component in their outer layer, whereas motility (–) domes showed an obscure distribution of CK-positive cells on their outer surface and SMA-positive cells throughout the whole wall. Motility (+) domes showed scattered c-Kit-positive ICC-suspected cells in their outer layers. However, motility (–) domes demonstrated a distribution of only a few c-Kit-positive cells. In terms of cell growth, motility (+) domes showed Ki67-positive proliferating cells and ssDNA-positive apoptotic cells mainly in their inner layer, which coincided with a CK-positive cell distribution. In contrast, motility (–) domes showed numerous Ki67-positive cells in both their inner and outer layers, and ssDNA-positive apoptotic cell debris was found within their cavities. TFF3 immunoreactivity, an intestinal epithelial marker, was found on the inner surfaces of motility (+) domes, whereas motility (–) domes showed TFF3 immunoreactivity on their outer surfaces.

In 25 domes, CK, SMA, TFF3, Ki67, and ssDNA showed opposite distributions of immunoreactive cells between motility (+) and motility (–) domes (table 2). c-Kit-positive cells were more frequently detected in motility (+) domes than in motility (–) domes.

Fig. 2. Expression of differentiation markers in motility (+) and motility (-) dome-like structures. Differentiation of epithelium, smooth muscle, nerve, ICC, and intestinal mucosa, as well as cell proliferation, and apoptosis were assessed by the expressions of CK, SMA, c-Kit, TFF3, Ki67, and ssDNA, respectively. Bar = 50 μ m.

Table 2. Expression of differentiation markers in motility (+) and motility (-) dome-like structures

	Layer	Motility (+) (n = 10)	Motility (-) (n = 15)	p value
CK ¹	Inner	10	1	<0.0001
	Outer	0	14	
SMA ¹	Inner	0	14	0.0066
	Outer	10	15	
c-Kit ²		10 (12 $\pm$ 4 cells)	3 (4 $\pm$ 3 cells)	0.0102
TFF3 ¹	Inner	10	1	<0.0001
	Outer	0	14	
Ki67 ³	Inner	76 $\pm$ 12%	86 $\pm$ 14%	NS
	Outer	12 $\pm$ 3%	92 $\pm$ 16%	
ssDNA ³	Inner	5 $\pm$ 2%	16 $\pm$ 4%	<0.0001
	Outer	1 $\pm$ 1%	22 $\pm$ 5%	

¹ When the positivity of immunoreactive cells was 30% or more, it was judged as positive. ² Positive cell number in a dome. ³ Labeling index. NS = Not significant.

Relationship between Motility Status and the Epithelial-Muscular Axis

To confirm the significance of the epithelial-muscular axis in the motility of ES gut, we examined 25 domes (table 3). In these 25 domes, a normal epithelial-muscular

Table 3. Relation of the histological axis and motor activity of ES gut

	Motility	
	(+) (n = 10)	(-) (n = 15)
Epithelial-muscle axis		
Positive	10	1
Negative	0	14
Motility status vs. epithelial-muscular axis: p < 0.0001.		

axis was found in all 10 motility (+) domes, whereas a normal axis was found in only 1 of the 15 motility (-) domes (p < 0.0001). The single motility (-) dome with a normal epithelial-muscular axis contained very few cells expressing c-Kit. These results suggest that the motility (+) domes exhibited an inner epithelial-outer muscular cell distribution (epithelial-muscular axis) with a differentiation of ICCs. In contrast, motility (-) domes exhibited an obscure inverted muscular-epithelial axis. Motility (-) domes also had high levels of proliferation and apoptosis which suggested an immature property.

Fig. 3. Expression of HH factors and Wnt pathways in motility (+) and motility (-) dome-like structures. Expression of HH pathway factors (SHH, PTC, SMO, and Gli1) was detected in motility (+) and motility (-) dome-like structures. Bar = 50 μ m.

Expression of HH Signaling Factors in the ES Gut

In order to examine the underlying mechanism generating the epithelial-muscular axis, we examined the expression of HH signaling factors (SHH, PTC, SMO, and Gli1) in the domes (fig. 3; table 4). In motility (+) domes, SHH, PTC, SMO, and Gli1 were expressed in the inner epithelial layer, whereas motility (-) domes showed SHH, PTC, and SMO in the outer layer and Gli1 in the whole wall. The single case of a motility (-) dome without the axis showed a normal distribution of SHH but not of PTH, SMO, or Gli1.

Effects of Activin A on the Activation of SHH

Activin A activates SHH in mouse embryos [11] and EBs [12]. In order to assess the effects of activin A on the domes' histological axis formation (fig. 4), we added activin A to the medium of the EB culture. Activin A increased the number of SHH-expressing cells in day-4 EBs

Table 4. Expression of hedgehog family factors in motility (+) and motility (-) dome-like structures

	Layer	Motility (+) (n = 10)	Motility (-) (n = 15)	p value
SHH ¹	Inner	10 ¹	1	<0.0001
	Outer	0	14	
PTH	Inner	10	0	<0.0001
	Outer	0	15	
SMO	Inner	10	0	<0.0001
	Outer	0	15	
Gli1	Inner	10	14	0.0274
	Outer	1	15	

¹ When the positivity of immunoreactive cells was 30% or more, it was judged as positive.

as compared to untreated day-4 EBs (fig. 4a). Subsequently, the concentrations of SHH were examined in EBs (fig. 4b, c). Activin A treatment increased the SHH in EBs 11.8-fold. Finally, we examined the effect of activin A on intestinal differentiation. The concentration of IAP, which is an intestinal differentiation marker [13], was increased 4.6-fold in activin A-treated EBs.

Discussion

In this study, we examined the motility and structure of the ES gut. Our results demonstrate that epithelial-muscular axis formation is essential for the motility of domes. We examined the distribution of the tissue components in the motility (+) and motility (-) domes using immunohistochemistry, and our results showed that a normal epithelial-muscular axis was found in the motility (+) domes but not in the motility (-) domes. Motility (-) domes showed an inverse axis and immature differentiation. Differentiation of ICC was found concurrently with normal epithelial-muscular axis formation in the dome walls. Furthermore, the HH signal, induced by activin A, played a pivotal role in epithelial-muscular axis formation. Recently, the gut-like structure was also generated from induced pluripotent stem (iPS) cells using the same methodology as with our ES gut [14]. Our findings might be applicable to iPS-derived gut-like structures.

The embryogenesis of the gut is a complicated process. The precursor cells of the gut are derived from epiblasts which form a primitive streak with mesendoder-

Fig. 4. Effects of activin A on SHH expression and intestinal differentiation. EBs were formed from ES cells in hanging drop cultures with or without activin A treatment (100 ng/ml). **a** Immunohistochemistry of SHH in day-4 EBs. Bar = 200 µm. Concentrations of SHH (**b**) and IAP (**c**) in day-4 EBs were detected by ELISA. Bars represent SD.

mal features [4]. The embryonic endoderm is generated at the ventral surface of the embryo through gastrulation from the primitive streak [5]. The embryonic endoderm forms a tube from the single cell layer sheet [5]. This multistep process of gut embryogenesis contains cell differentiation, cell-cell interaction, and spatiotemporal cognition. In contrast, ES gut genesis from ES cells retains cell differentiation and cell-cell interaction but fails to include spatiotemporal cognition. Our study showed that the concurrent expression of HH factors and Wnt pathways in the inner layer of the motility (+) domes were associated with epithelial differentiation. SHH is a known inducer of intestinal differentiation in the endoderm and induces intestinal fatty acid-binding protein [11].

Both SHH and bone morphogenetic protein-4 are involved in the development of the cell-renewable epithelium [15]. SHH is an important endodermal signal in the endodermal-mesodermal cross-talk that patterns the developing gut tube along different axes [16]. Through epi-

thelial-mesenchymal interactions, HH signaling ensures the appropriate axial patterning of the embryonic gut [6]. Thus, activation of HH pathways is associated with normal epithelial-muscular axis formation in the ES gut. Mutations in SHH and downstream signaling molecules are known to cause a variety of the congenital malformations of the gastrointestinal tract, including esophageal atresia, tracheoesophageal fistula, annular pancreas, midgut malrotation, and duodenal and anal atresia [16]. TGF-β and FGF9 also play roles in intestinal development, which should be the subject of our further studies [17, 18].

Unlike tube formation in the embryonic endoderm, internal space formation in the ES gut is poorly understood. However, apoptotic cell debris was found in the internal spaces of the motility (-) domes, suggesting that these spaces are pseudolumens. Since the motility (-) domes showed higher proliferative activity than the motility (+) domes, rapid cell cycling might induce a pseudolumen in domes without a mature functional histological structure.

Activin is a specific determinant of endodermal and mesodermal formation. Previously, by *Xenopus* animal cap assay, it was demonstrated that high concentrations of activin induce endomesoderm formation, whereas low concentrations of activin induce mesoderm formation [19]. Moreover, high concentrations of activin induce hepatocyte differentiation from the mesendoderm. In contrast, low concentrations of activin induce skeletal muscle differentiation [20]. Activin A can promote intestinal differentiation via an SHH-dependent mechanism [11]. Our data also showed an increase in SHH production and an acceleration of intestinal differentiation by activin A treatment. Activin also activates the c-Kit pathway [21] which is expressed in the ICC.

In mature gut, ICCs are essential for spontaneous intestinal motility [22]. In ES gut, spontaneous rhythmic contractions depend on the ICC network in the smooth muscle layer [3]. Moreover, inhibition of c-Kit by imatinib abrogates ICC formation and spontaneous motility in ES gut [23]. In the present study, ICCs were well developed in motility (+) domes. What is the factor affecting ICCs development? ICCs are derived from a common mesenchymal origin with smooth muscle cells [24]. Maturation of both ICCs and gut smooth muscle cells is observed at weeks 8–11, followed by the organization of muscle layers and ICC network in human embryos [25]. The expressions of c-Kit, platelet-derived growth factor, and activin are associated with the differentiation of ICCs or smooth muscle cells [26–29]. From these reports, a mechanism is

suggested to the effect that activin enhances ICC/smooth muscle cell precursor cells. After that, a distinctive differentiation of ICC from smooth muscle cells is provided by c-Kit signals; this differentiation is also enhanced by activin [21]. In future studies, a mechanism of activin activation in ES gut should be required for the effective formation of ES gut.

Acknowledgment

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

References

- Keller GM: In vitro differentiation of embryonic stem cells. *Curr Opin Cell Biol* 1995;7: 862–869.
- Takaki M, Nakayama S, Misawa H, Nakagawa T, Kuniyasu H: In vitro formation of enteric neural network structure in a gut-like organ differentiated from mouse embryonic stem cells. *Stem Cells* 2006;24:1414–1422.
- Ishikawa T, Nakayama S, Nakagawa T, Horiguchi K, Misawa H, Kadowaki M, Nakao A, Inoue S, Komuro T, Takaki M: Characterization of in vitro gutlike organ formed from mouse embryonic stem cells. *Am J Physiol Cell Physiol* 2004;286:C1344–C1352.
- Rodaway A, Patient R: Mesendoderm: an ancient germ layer? *Cell* 2001;105:169–172.
- Wells JM, Melton DA: Vertebrate endoderm development. *Annu Rev Cell Dev Biol* 1999; 15:393–410.
- Lees C, Howie S, Sartor RB, Satsangi J: The hedgehog signalling pathway in the gastrointestinal tract: implications for development, homeostasis, and disease. *Gastroenterology* 2005;129:1696–1710.
- Niwa H, Miyazaki J, Smith AG: Quantitative expression of OCT-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 2000;24:372–376.
- Robertson EJ: Embryo-Derived Stem Cell Lines. Washington, IRL, 1987.
- Hooper M, Hardy K, Handyside A, Hunter S, Monk M: HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature* 1987;326: 292–295.
- Yamada T, Yoshikawa M, Takaki M, Torihashi S, Kato Y, Nakajima Y, Ishizaka S, Tsunoda Y: In vitro function gut-like organ formation from mouse embryonic stem cells. *Stem Cells* 2002;20:41–49.
- van Eyll JM, Pierreux CE, Lemaigre FP, Rousseau GG: SHH-dependent differentiation of intestinal tissue from embryonic pancreas by activin A. *J Cell Sci* 2004;117:2077–2086.
- Mfopou JK, De Groote V, Xu X, Heimberg H, Bouwens L: Sonic hedgehog and other soluble factors from differentiating embryoid bodies inhibit pancreas development. *Stem Cells* 2007;25:1156–1165.
- Hinnebusch BF, Siddique A, Henderson JW, Malo MS, Zhang W, Athaide CP, Abedrapo MA, Chen X, Yang VW, Hodin RA: Enterocyte differentiation marker intestinal alkaline phosphatase is a target gene of the gut-enriched Kruppel-like factor. *Am J Physiol Gastrointest Liver Physiol* 2004;286:G23–G30.
- Ueda T, Yamada T, Hokuto D, Koyama F, Kasuda S, Kanehiro H, Nakajima Y: Generation of functional gut-like organ from mouse induced pluripotent stem cells. *Biochem Biophys Res Commun* 2010;391:38–42.
- Ishizuya-Oka A, Hasebe T: Sonic hedgehog and bone morphogenetic protein-4 signaling pathway involved in epithelial cell renewal along the radial axis of the intestine. *Digestion* 2008;77:42–47.
- van den Brink GR: Hedgehog signaling in development and homeostasis of the gastrointestinal tract. *Physiol Rev* 2007;87:1343–1375.
- Henry GL, Brivanlou IH, Kessler DS, Hemmati-Brivanlou A, Melton DA: TGF- β signals and a pattern in *Xenopus laevis* endodermal development. *Development* 1996; 122:1007–1015.
- Geske MJ, Zhang X, Patel KK, Ornitz DM, Stappenbeck TS: Fgf9 signaling regulates small intestinal elongation and mesenchymal development. *Development* 2008;135: 2959–2968.
- Smith JC, Price BM, Van Nimmen K, Huylebroeck D: Identification of a potent *Xenopus* mesoderm-inducing factor as a homologue of activin A. *Nature* 1990;345:729–731.
- Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ, Keller G: Development of definitive endoderm from embryonic stem cells in culture. *Development* 2004;131:1651–1662.
- Coutts SM, Childs AJ, Fulton N, Collins C, Bayne RA, McNeilly AS, Anderson RA: Activin signals via SMAD2/3 between germ and somatic cells in the human fetal ovary and regulates kit ligand expression. *Dev Biol* 2008;314:189–199.
- Takaki M: Gut pacemaker cells: the interstitial cells of Cajal (ICC). *J Smooth Muscle Res* 2003;39:137–161.
- Takaki M, Misawa H, Shimizu J, Kuniyasu H, Horiguchi K: Inhibition of gut pacemaker cell formation from mouse ES cells by the c-kit inhibitor. *Biochem Biophys Res Commun* 2007;359:354–359.
- Young HM: Embryological origin of interstitial cells of Cajal. *Microsc Res Tech* 1999;47: 303–308.
- Wallace KN, Akhter S, Smith EM, Lorent K, Pack M: Intestinal growth and differentiation in zebrafish. *Mech Dev* 2005;122:157–173.
- Beckett EA, Ro S, Bayguinov Y, Sanders KM, Ward SM: Kit signaling is essential for development and maintenance of interstitial cells of Cajal and electrical rhythmicity in the embryonic gastrointestinal tract. *Dev Dyn* 2007;236:60–72.
- Kurahashi M, Niwa Y, Cheng J, Ohsaki Y, Fujita A, Goto H, Fujimoto T, Torihashi S: Platelet-derived growth factor signals play critical roles in differentiation of longitudinal smooth muscle cells in mouse embryonic gut. *Neurogastroenterol Motil* 2008;20:521–531.
- Topouzis S, Majesky MW: Smooth muscle lineage diversity in the chick embryo: two types of aortic smooth muscle cell differ in growth and receptor-mediated transcriptional responses to transforming growth factor- β . *Dev Biol* 1996;178:430–445.
- Kanzaki M, Nobusawa R, Mogami H, Yasuda H, Kawamura N, Kojima I: Production of activin A and follistatin in cultured rat vascular smooth muscle cells. *Mol Cell Endocrinol* 1995;108:11–16.