RESEARCH ARTICLE

Brg1 plays an essential role in development and homeostasis of the duodenum through regulation of Notch signaling

Yutaka Takada, Akihisa Fukuda*, Tsutomu Chiba and Hiroshi Seno

ABSTRACT

Brg1, a core subunit of the SWI/SNF chromatin remodeling complex, is essential for development and homeostasis of various organs. However, the functional role of Brg1 in intestinal development and homeostasis, and the underlying molecular mechanisms, remain unknown. We found that deletion of Brg1 in the mouse intestine resulted in growth impairment and early death associated with abnormal crypt-villous formation, skewed differentiation into secretory lineage cells, markedly increased apoptosis, and stem cell loss in the duodenum. Furthermore, we found that the Notch signaling pathway was dramatically downregulated in Brg1-deficient duodenum. Remarkably, overexpression of the Notch1 intercellular domain (ICD) partially reversed the prognosis of intestinal Brg1 mutant mice. Notch1 ICD overexpression rescued morphogenesis, prevented over-differentiation into secretory lineage cells, and restored apoptosis to normal levels in Brg1-deficient duodenum, although stem cell loss was not rescued. Our data demonstrate that Brg1 plays an essential role in development and homeostasis, including morphogenesis, stem cell differentiation and cell survival in the duodenum. Mechanistically, the rescue of the intestinal Brg1 mutant phenotype by overexpression of the Notch1 ICD indicates that Notch signaling is a key downstream target that mediates the effects of Brg1.

KEY WORDS: Brg1, Notch signaling, Intestine, Homeostasis, Stem cell, Differentiation

INTRODUCTION

Tissue development and homeostasis are governed by activation or inactivation of multiple genes. Because chromatin is highly compacted, it must undergo structural changes to allow transcription factors to interact with nucleosomes and access binding sites on their target genes. ATP-dependent chromatin remodeling complexes alter local chromatin structure using energy from ATP hydrolysis. These structural alterations increase or decrease the accessibility of transcriptional factors and thereby activate or repress the expression of specific genes (Hargreaves and Crabtree, 2011; Helming et al., 2014; Martens and Winston, 2003; Wilson and Roberts, 2011). Brg1-null mice die during the peri-implantation stage, whereas Brm-null mice develop normally (Bultman et al., 2000; Reyes et al., 1998). Recent studies showed that Brg1 is essential for development or homeostasis of various organs and cell types, including heart (Hang et al., 2010; Takeuchi et al., 2011), vasculature (Davis et al., 2013; Griffin et al., 2008, 2011), ureter (Weiss et al., 2013), smooth muscle (Zhang et al., 2011) and neurons (Li et al., 2013; Matsumoto et al., 2006; See et al., 2005). In the intestine, Brg1 is essential for maintenance of intestinal stem cells (Holik et al., 2013). However, the functional role of Brg1 in intestinal development and homeostasis and its molecular mechanism remain to be elucidated.

The intestinal epithelium is highly organized with multiple functions, including digestion, nutrient absorption and immunological defense. Structurally, it is organized into villi, which are finger-like projections and crypts of Lieberkühn, which are invagination structures adjacent to villi. The villous epithelium consists of differentiated epithelial cells, including absorptive-type enterocytes and secretory-type goblet cells, enteroendocrine cells and tuft cells (Gerbe et al., 2011). In contrast, crypts consist chiefly of undifferentiated proliferating progenitor cells. In the bottom of crypts, intestinal stem cells, termed crypt base columnar (CBC) cells, reside between secretory-type differentiated Paneth cells (Barker et al., 2007). CBC cells can self-renew and provide progenitors, called transit-amplifying (TA) cells, which can rapidly divide and differentiate into all types of mature cells in the intestinal epithelium; subsequently, they ascend from the crypt to the villi (Barker et al., 2007).

Diverse signaling pathways are involved in intestinal morphogenesis and maintenance of intestinal homeostasis (Medema and Vermeulen, 2011). In particular, Notch signaling plays a crucial role in the regulation of progenitor cell proliferation and differentiation (Sancho et al., 2015). Notch activity promotes differentiation into the absorptive-type cell lineage rather than the secretory cell lineage (Fre et al., 2005; Jensen et al., 2000; Milano et al., 2004; Ueo et al., 2012; van Es et al., 2005; VanDussen et al., 2012; Wong et al., 2004; Yang et al., 2001). Complete inhibition of Notch signaling in the murine intestinal epithelium using γ-secretase inhibitors results in significantly elevated differentiation into secretory lineages (Milano et al., 2004; van Es et al., 2005; Wong et al., 2004). On the other hand, persistent expression of the Notch intercellular domain (ICD) expands the proliferative zone and represses secretory cell differentiation (Fre et al., 2005; Stanger et al., 2005). Notch signaling is also required for maintenance of CBC cells (Pellegrinetti et al., 2011; VanDussen et al., 2012). Notch inhibition inhibits cellular proliferation and induces apoptosis of CBC cells, thereby decreasing the overall number of these cells (VanDussen et al., 2012).
Here, we show that Brg1 plays a pivotal role in crypt-villous remodeling, differentiation and cell survival in the duodenum. Furthermore, mechanistically, our data show that the effects of Brg1 are mainly mediated by the Notch signaling pathway.

RESULTS

Intestinal Brg1 deletion results in growth impairment, early death and abnormal villous formation in the duodenum

First, we ascertained the expression pattern of Brg1 in the murine intestinal epithelium. Brg1 was expressed in all intestinal epithelial cells from embryonic development to post-natal and adult stages (Fig. 1A-C). To investigate whether Brg1 plays a functional role in intestinal development and maintenance of intestinal homeostasis, we crossed transgenic mice carrying a loxp-flanked allele of Brg1 (Brg1f/fl) (Sumi-Ichinose et al., 1997) with Villin-Cre mice (Madison et al., 2002) to generate Villin-Cre; Brg1f/f mice. Villin-Cre; Brg1f/f mice were born at Mendelian ratios, but they exhibited early post-natal death and growth impairment relative to control littermates. Fifty percent of the Brg1 mutant mice died within 4 days of birth (Fig. 1D-F). Histological analyses revealed that Villin-Cre; Brg1f/f mice had abnormal duodenal villous structure, shorter villi and disorganized intervillous pockets compared with control mice at post-natal day (P)4 (Fig. 1G,H,K). This abnormal villous structure was less striking toward the distal intestine and was much less prominent in the ileum (Fig. 1J,L). In accordance with Cre activity, almost all of the intestinal epithelial cells had lost Brg1 expression in Villin-Cre; Brg1f/f mice (Fig. 1M,N). At embryonic day (E)16.5, the duodenal villous structures of Brg1 mutant and control mice were indistinguishable, but shorter villi could be observed in Brg1 mutant mice at E18.5 (Fig. 1A-F).

Brg1 depletion results in skewed intestinal differentiation into secretory lineage cells

We next investigated the effect of loss of Brg1 on differentiation of the duodenal epithelial cells at P4. Alcian Blue staining and quantitation revealed that the number of goblet cells was markedly higher in Brg1-deficient duodenum than in control duodenum (Fig. 2A,B,M). Chromogranin A immunostaining and quantitation revealed that the number of enteroendocrine cells was also markedly higher in Brg1-deficient duodenum (Fig. 2C,D,N). Among enteroendocrine cells, GIP (glucose-dependent insulino-metropic peptide)-positive K cells were more numerous in Villin-Cre; Brg1f/f duodenum (Fig. 2E,F), whereas there were no significant differences between Brg1 mutant and control duodenum in the numbers of other types of enteroendocrine cells, including serotonin-positive EC cells, gastrin-positive G cells, secretin-positive S cells, cholecystokinin-positive I cells and Glp1 (glucagon-like peptide-1)-positive L cells (data not shown). In addition, immunostaining of Dclk1 revealed that the number of tuft cells was also significantly higher in Brg1-deficient duodenum (Fig. 2G,H). Quantitative RT-PCR (q-PCR) analysis showed that Mathl (Atoh1), a transcription factor that plays an essential role in the differentiation of intestinal secretory cell types (Yang et al., 2001), was upregulated in Brg1-deficient duodenum (Fig. 2O). Furthermore, immunostaining of the Paneth cell marker lysozyme (Sato et al., 2011) and Mmp7 (Wilson et al., 1999) revealed precociously differentiated Paneth cells in Villin-Cre; Brg1f/f duodenum, but not in control duodenum (Fig. 2I-L). In addition, Gfi1 (Bjerknes and Cheng, 2010), a transcriptional factor that is crucial for the differentiation of Paneth cells, was more highly expressed in Brg1-deficient duodenum than in controls (Fig. 2P). These data indicate that loss of Brg1 results in skewed differentiation into secretory cell types in the duodenum.

Loss of Brg1 increases the numbers of apoptotic and proliferative cells in the duodenum

Brg1 is involved in apoptosis and cell proliferation during organ development (Griffin et al., 2008; Hang et al., 2010; Li et al., 2013; Seo et al., 2005; Zhang et al., 2011). Therefore, we next investigated whether the abnormal villous structure in Brg1-deficient duodenum was associated with changes in apoptosis or cell proliferation. Remarkably, immunostaining and quantitation of cleaved caspase 3 revealed a dramatic increase in apoptotic cells in Villin-Cre; Brg1f/f duodenum relative to control duodenum at P4 (Fig. 3A-D). In addition, genes involved in apoptosis, including Noxa (Pmaip1) and Bax (Igney and Krammer, 2002), were significantly upregulated and the anti-apoptotic factor Bcl2 (Igney and Krammer, 2002) was significantly downregulated, in Brg1-deficient duodenum relative to controls (Fig. 3E).
We next investigated whether cell proliferation was affected in Brg1-deficient duodenum. Ki67 staining revealed monostratal proliferative cells in the intervillous pocket in control duodenum at P4 (Fig. 3F). By contrast, in Brg1-deficient duodenum, stratified proliferative cells were observed not only in the intervillous pocket and crypt bottoms, but also in the middle of villi (Fig. 3G). Quantitation revealed that the number of Ki67-positive cells was significantly higher in Brg1-deficient duodenum than in control duodenum (Fig. 3H). Thus, loss of Brg1 increases the numbers of both apoptotic and proliferative cells in the duodenum.

Loss of Brg1 results in depletion of intestinal stem cells

Brg1 is essential for maintenance of intestinal stem cells (Holik et al., 2013). To investigate the effect of loss of Brg1 on the development of intestinal stem cells, we used Lgr5-GFP mice, in which Lgr5+ CBC cells are GFP positive (Barker et al., 2007). Immunostaining of GFP revealed Lgr5 + CBC cells in the intervillous pocket in duodenum of Lgr5-GFP; Brg1wt/wt mice at P4. By contrast, such cells were rarely seen in the duodenum of Lgr5-GFP; Villin-Cre; Brg1f/f mice (Fig. 4A,B). Moreover, immunostaining of Msi1 (Musashi RNA-binding protein 1), a CBC cell marker (Kayahara et al., 2003; Potten et al., 2003) and a positive regulator of Notch signaling (Imai et al., 2001), revealed that Msi1-positive cells were very rare in Brg1-deficient duodenum, but they were present in the intervillous area in control duodenum at P4 (Fig. 4C,D). In addition, intestinal stem cell marker genes, including Lgr5, Olfm4, Msi1, Ascl2, Bmi1 and Tert (Barker, 2014), were markedly downregulated in Brg1-deficient duodenum relative to control duodenum (Fig. 4E). These data indicate that Brg1 is indispensable for the development of intestinal stem cells in the duodenum.

Loss of Brg1 results in downregulation of Notch signaling

Notch signaling plays an important role in maintenance of intestinal stem cells and cellular differentiation of intestinal epithelial cells. In particular, Notch inhibition results in loss of CBC cells and over-differentiation into secretory cell types (Pellegrinet et al., 2011; VanDussen et al., 2012). Therefore, we next sought to determine whether loss of Brg1 would affect Notch signaling activity in the duodenum. Immunostaining of cleaved Notch1 revealed that it was
expressed in duodenal epithelial cells in intervillous pockets in control duodenum, whereas only a few cleaved Notch1-positive cells were observed in Brg1-deficient duodenum at P4 (Fig. 5A,B). Remarkably, Hes1, a target gene of the Notch signaling pathway, was barely expressed in Brg1-deficient duodenum, whereas it was expressed in intervillous pockets and the bottoms of villi in control duodenum at P4 (Fig. 5C,D). Furthermore, Numb, which acts as an inhibitor of Notch signaling by degrading the Notch1 ICD, was markedly overexpressed in intervillous pockets in Brg1-deficient duodenum relative to controls at P4 (Fig. 5E,F). Next, we performed q-PCR analyses to determine the expression levels of genes involved in the Notch signaling pathway in the duodenum of Villin-Cre; Brg1f/f and control mice. Notch ligands, including Dll1, Dll4, Jag1 and Jag2, were significantly downregulated in Villin-Cre; Brg1f/f duodenum relative to control. Expression of Numb, which is translationally regulated by Musashi1 (Imai et al., 2001), was comparable between Brg1 mutants and controls. Notch target genes, including Hes1, Hes5 and Heyl, were markedly downregulated in duodenum of Villin-Cre; Brg1f/f mice relative to controls (Fig. 5G). Downregulation of Notch target genes was also observed in Brg1-deficient duodenum at E17.5 (Fig. S2). These results indicate that the loss of Brg1 results in marked downregulation of the Notch signaling pathway in the duodenum.

The intestinal phenotype of Brg1 mutant mice was manifested primarily in the duodenum, and was much less prominent in the ileum. Therefore, we next investigated whether Notch signaling activity is downregulated in Brg1-deficient ileum. Immunostaining revealed that Hes1 expression in the epithelial cells of the ileum was comparable between Brg1 mutant and control mice at P4 (Fig. S3A,B). The relative expression level of Hes5 was upregulated in Brg1 mutant ileum (Fig. S3C). Heyl expression was comparable between Brg1 mutant and control mice, whereas Hes1 expression was slightly downregulated in Brg1-deficient ileum compared with the control (Fig. S3C). Thus, Notch signaling activity was not downregulated in Brg1-deficient ileum compared with Brg1-deficient duodenum.

**Overexpression of the Notch1 ICD reverses growth failure and early death of Brg1 mutant mice**

Because the Notch signaling pathway was markedly downregulated in Brg1-deficient duodenum, we hypothesized that Notch1 ICD overexpression might rescue the phenotype of Brg1 mutant mice. To test this hypothesis, we used RosaNotchIC mice (Murtaugh et al., 2003), in which the Notch1 ICD is constitutively overexpressed under the control of Cre recombinase. We crossed RosaNotchIC mice with Villin-Cre mice and Brg1f/f mice to generate Villin-Cre; Brg1f/f; RosaNotchIC (VBN) mice (Fig. 6A). The body weight and survival phenotypes of Villin-Cre; Brg1f/f (VB) were partially rescued in VBN mice (Fig. 6B-D). We confirmed that Brg1 was depleted and cleaved Notch1 was expressed in the intestinal epithelial cells in VBN mice (Fig. 6E,F). As expected, Hes1 was expressed at P4 in the duodenal epithelial cells of VBN mice (Fig. 6G). Moreover, expression levels of Hes1 and Hes5 were comparable, whereas Heyl was downregulated in VBN duodenum compared to wild-type (WT) control duodenum (Fig. 6I). As expected, Numb, an upstream regulator of the Notch1 ICD, was still overexpressed at P4 in intervillous pockets in VBN duodenum, as it was in VB duodenum (Fig. 6H). Thus, Notch1 ICD overexpression partially rescued the growth failure and early death phenotypes of Brg1 mutant mice.

**Notch1 ICD overexpression rescues abnormal villous formation, skews differentiation into secretory lineages and increases apoptosis**

We next investigated whether the abnormal villous structure and skewed differentiation into secretory lineages observed in Brg1
mutant mice would be reversed in VBN mice. The length of villi in VBN duodenum at P4 was comparable to that of WT controls (Fig. 7I) and the structure of intervillous pockets was indistinguishable between VBN and WT (Fig. 7A). Alcian Blue staining and quantitation revealed that the number of goblet cells was significantly lower in VBN duodenum than in VB duodenum (Fig. 7B,J). Immunostaining of chromogranin A and GIP revealed that the numbers of enteroendocrine cells, including K cells, in VBN duodenum were comparable to those of WT controls (Fig. 7C,D,K). Furthermore, VBN duodenum contained fewer tuft cells than VB duodenum (Fig. 7E). Paneth cells were very scarce in duodenum of VBN mice, as in WT controls (Fig. 7F). In addition, expression levels of Math1 and Gfi1 were lower in duodenum of VBN mice than in VB mice (Fig. 7N). Thus, Notch1 ICD overexpression rescued impaired villous formation and skewed differentiation into secretory lineages of Brg1-deficient duodenum at P4.

We next investigated whether the elevation of apoptosis was rescued in VBN mice. Notably, immunostaining and quantitation of cleaved caspase 3 revealed that the number of apoptotic cells was significantly smaller in VBN duodenum than in VB duodenum at P4 (Fig. 7G,L). In addition, Noxa was significantly downregulated and Bcl2 was significantly upregulated in VBN duodenum at P4 (Fig. 7O,P). The number of Ki67-positive cells was comparable between VBN and WT duodenum (Fig. 7M). Therefore, Notch1 ICD overexpression rescued impaired villous formation and skewed differentiation into secretory lineages of Brg1-deficient duodenum at P4.
Bcl2 was upregulated, in VBN duodenum relative to VB duodenum (Fig. 7O). Thus, Notch1 ICD overexpression completely rescued increased apoptosis in VBN mice. In terms of the distribution of proliferative cells, Ki67-staining revealed that proliferative cells were monostratal but were still present in the middle of villi in VBN mice at P4 (Fig. 7H). The number of Ki67-positive cells was smaller in VBN duodenum than in VB duodenum, although it was still greater than that of WT controls at P4 (Fig. 7M). Thus, the number and distribution of proliferative cells was partially reversed by Notch1 ICD overexpression.

Furthermore, we investigated the phenotypes of Villin-Cre; Rosa<sup>Nothkl</sup> (VN) mice. Misexpression of the Notch1 ICD reversed the phenotypes of VBN mice to the level of wild-type control but not to that of VN mice (Fig. S4A-J). The activity of Notch signaling (e.g. expression level of Hes1) was different between VBN and VN mice (Fig. S4A-J).

Taken together, these data indicate that the phenotype of Brg1 mutant mice, including impaired morphogenesis, skewed differentiation into secretory lineages and elevated apoptosis, was due to downregulation of the Notch signaling pathway in Brg1 mutant mice.

We next investigated intestinal stem cells of VBN mice. Immunostaining revealed that Ms1-positive cells were very scarce in VBN duodenum at P4 (Fig. 7P). In addition, expression levels of intestinal stem cell marker genes, including Ms1, Lgr5, Olfm4, Ascl2 and Bmi1, were comparable in the duodenum of VBN and VB mice (Fig. 7Q). Thus, Notch1 ICD overexpression did not rescue intestinal stem cell loss in Brg1-deficient duodenum.

**Stabilization of β-catenin does not reverse the phenotypes of Brg1 mutant mice**

Wnt signaling pathways also play a crucial role in development and homeostasis of the intestine (Sato and Clevers, 2013). Therefore, we investigated whether loss of Brg1 affected Wnt signaling activity in the duodenum. Q-PCR analyses revealed that the expression levels of genes involved in the Wnt signaling pathway were markedly downregulated at multiple levels in Brg1-deficient duodenum (Fig. S5A). Therefore, we next sought to determine whether activation of the Wnt signaling would reverse the phenotypes of Brg1 mutant mice including duodenal stem cell loss. To this end, we used Ctnnb1<sup>flox;cre</sup> mice (Harada et al., 1999), in which Cre recombination removes exon 3 of Ctnnb1 to stabilize β-catenin, and generated Villin-Cre; Brg1<sup>fl/fl; Cre<sup>flox;cre</sup></sub> (VBb) mice. Interestingly, β-catenin stabilization did not rescue the phenotype of Brg1 mutant mice, including impaired crypt-villous remodeling, skewed differentiation into secretory lineages, elevated apoptosis and cell proliferation (Fig. S5B-E,H-K). Moreover, of note, Lgr5<sup>+</sup> CBC cells were rarely observed in the duodenum of VBN mice at P4, similar to their distribution in VB mice (Fig. S5F). Intestinal stem cell marker genes associated with the Wnt signaling, including Lgr5 and Ascl2, were significantly downregulated in the duodenum of VBN mice (Fig. S5L). Of note, the Notch signaling pathway was still downregulated in VBN mice, although some of the Notch-related genes, including Dll4, Jag2 and Hes1, were slightly upregulated in VBN mice compared with VB mice (Fig. 7Q). Therefore, these data indicate that Brg1 plays a pivotal role for development and homeostasis of the duodenum through the regulation of Notch signaling in a manner that is independent of Wnt signaling.

**DISCUSSION**

In this study, intestinal Brg1 mutant mice presented with early postnatal death associated with impaired crypt-villous formation, skewed differentiation into secretory lineage, markedly increased apoptosis and stem cell loss in the duodenum. Furthermore, the Notch signaling pathway was dramatically downregulated in Brg1-deficient duodenum. Notably, Notch1 ICD overexpression rescued impaired morphogenesis, over-differentiated into secretory lineage and elevated apoptosis in the absence of Brg1, demonstrating that Brg1 plays a pivotal role for intestinal development and homeostasis via a Notch signaling-dependent mechanism.

In terms of the functional relationship between Brg1 and the Notch signaling, we found that Notch ligands including Dll1, Dll4, Jag1 and Jag2 were strongly downregulated in Brg1-deficient duodenum. Reduced expression of these four Notch ligands can most likely explain the downregulation of Notch signaling. Misexpression of the Notch1 ICD reversed the phenotypes of VBN mice to the level of the WT control but not to that of Villin-Cre; Rosa<sup>Nothkl</sup> (VN) mice. The other Notch cleaved components (e.g. Notch2, Notch3, or Notch4) might be involved in reversing the expression level of Notch target genes and consequently reverting the phenotypes of VBN mice to the level of VN mice. Regarding to the relationship with Wnt signaling, we found marked downregulation of the Wnt signaling in Brg1-deficient duodenum. Of note, the Notch signaling activity was still downregulated in the model of β-catenin stabilization (VBB mice), although some Notch components, including Dll4, Jag2 and Hes1, were slightly upregulated in VBB mice compared with VB mice. Thus, our data support the notion that the mechanistic link between Brg1 and Notch is not predominantly mediated by Wnt signaling. Our findings demonstrate that Brg1 plays an indispensable role in neonatal crypt-villous remodeling by regulating Notch signaling in a manner that is independent of Wnt signaling.

Brg1 is required for inhibition of apoptosis in the development of various cell types and organs, including neurons (Li et al., 2013), smooth muscle (Zhang et al., 2011) and blood cells (Griffin et al., 2008); consequently, knockout of Brg1 increases the rate of apoptosis in these tissues. Our data showing that elevated apoptosis was reversed by Notch1 ICD overexpression in VBN mice demonstrate that marked downregulation of Notch signaling is responsible for increased apoptosis in Brg1-deficient duodenum. This finding is consistent with a previous report showing that Notch inhibition by conditional knockout of Hes1, Hes3 or Hes5 results in increased apoptosis in murine intestinal epithelial cells (Ueo et al., 2012). A previous study shows that apoptotic cells are increased in young adult Brg1 mutant mice (Holik et al., 2013). Similarly, crypt-restricted apoptosis increased in the adult Notch inactivation models (Milano et al., 2004). In contrast, in the embryonic Notch inactivation models, increased apoptosis is widely observed in the intestine (Ueo et al., 2012). This is similar to what we observed in our embryonic Brg1 mutant mice. Our data, together with the previous study, underscore the specific requirement for Brg1 and Notch in preventing apoptosis during neonatal crypt-villous remodeling that has not been appreciated in previous adult studies.

Brg1 is essential for intestinal stem cell homeostasis (Holik et al., 2013), but the molecular details of the requirement for Brg1 in intestinal stem cell maintenance remain unknown. Notch signaling is essential for survival of intestinal stem cells (Pellegrinet et al., 2011; VanDussen et al., 2012) and treatment with the Notch inhibitor DBZ causes intestinal stem cell loss (VanDussen et al., 2012). However, our results show that intestinal stem cell loss was not reversed by Notch1 ICD overexpression in intestinal Brg1 mutant mice. Furthermore, although Wnt signaling was also...
markedly downregulated in Brg1 mutant mice, the activation of Wnt signaling by β-catenin stabilization did not reverse the stem cell loss of Brg1-deficient duodenum. The inability to completely rescue the intestinal phenotype by overexpression of Notch1 ICD or Wnt further suggests that the effects of Brg1 deletion are complex and not merely due to alterations of either signaling pathway. Future studies should seek to clarify the molecular mechanism by which loss of intestinal Brg1 expression results in stem cell loss in the duodenum.

Notably, we observed very different phenotypes between the duodenum and ileum in intestinal Brg1 mutant mice. Brm was barely expressed in the duodenum in both Brg1 mutant and control mice, but was widely expressed in the ileum in both Brg1 mutant and control mice at P4 (Fig. S6A-D). Also, our results showed that the Notch signaling pathway was dramatically downregulated in the duodenum, but barely affected in the ileum. These data suggest that the difference in the Brm expression pattern might contribute to the difference in phenotype between the duodenum and ileum, through regulation of the Notch signaling pathway, in Brg1 mutant mice. Future studies should seek to determine whether double loss of Brg1 and Brm in the intestine would lead to a severe phenotype in the ileum.

Brg1 acts as a tumor suppressor in the murine pancreas (von Figura et al., 2014). In addition, inactivating mutations in Brg1 and other subunits of SWI/SNF complexes have been identified in various human cancers, including the pancreas and intestine (Helming et al., 2014; Wilson and Roberts, 2011). Furthermore, Brg1 is involved in cell proliferation during embryonic development (Hang et al., 2010; Seo et al., 2005; Li et al., 2013), a period in which Brg1 depletion increases the number of proliferative cells in neural plate (Seo et al., 2005). In line with these data, we found that the number of proliferative cells was elevated in Brg1-deficient duodenum. Although the precise mechanism is unknown, proliferative cells might be more abundant in Brg1-deficient duodenum in order to compensate for stem cell loss and a dramatic increase in apoptosis. The increase in cell proliferation caused by loss of Brg1 raises the possibility that Brg1 plays a tumor-suppressive role in the duodenum. Future studies are required to determine whether Brg1 functions as a tumor suppressor in murine and human duodenum.

MATERIALS AND METHODS

Mouse lines

Experimental animals were generated by crossing Villin-Cre mice (Jackson Laboratories #004586) (Madison et al., 2002), Brg1flox mice (gift from D. Reisman, University of Florida, with permission from P. Chambon) (Sumi-Ichinose et al., 1997), ROSA26NOSLACCD mice (Jackson Laboratories #008159) (Murtaugh et al., 2003), Lgr5-EGFP-RES-CreERT2 mice (Barker et al., 2007) (Jackson Laboratories #008875) and Cinnb1lox(ies) mice (Harada et al., 1999). Villin-Cre; Brg1flox mice were normal and used as a parental line. Villin-Cre mice were also normal and used as a parental line or controls. To generate Villin-Cre; Brg1flox mice, we crossed Villin-Cre; Brg1flox mice with Villin-Cre; Brg1flox mice. Villin-Cre; Brg1flox mice. Villin-Cre; Brg1flox mice. ROSA26NOSLACCD mice were generated by crossing Villin-Cre; Brg1flox mice with Villin-Cre; Brg1flox mice. All experiments were approved by the animal research committee of Kyoto University and performed in accordance with Japanese government regulations.

Histological analysis

Mouse tissues were fixed with 4% buffered paraformaldehyde solution, paraffin-embedded and sectioned (5 µm thickness). Sections were deparaffinized, rehydrated and stained with Hematoxylin and Eosin (H&E) or Alcian Blue and counterstained with Nuclear Fast Red (KPL). For immunohistochemistry, sections were incubated with primary antibodies overnight at 4°C and with biotinylated secondary antibody for 1 h at room temperature; immunoperoxidase labeling was performed with Vectastain ABC kit (Vector Laboratories) and then sections were colored with diaminobenzidine (DAB) substrate (Dako) and counterstained with Hematoxylin. For immunofluorescence, sections were incubated with primary antibodies overnight at 4°C and with fluorophore-conjugated secondary antibody (Invitrogen) for 1 h at room temperature. Primary antibodies used in this study were obtained from the indicated suppliers: rabbit anti-Brg1 (1:100; sc-10768, Santa Cruz Biotechnology), rabbit anti-actin (1:200; ab9276, Abcam), rabbit anti-GFP (1:200; HP4021612, Sigma-Aldrich), rabbit anti-Dclk1 (1:200; ab 31704, Abcam), mouse anti-α-cadherin (1:100; 610182, BD Transduction Laboratories), rabbit anti-lysozyme (1:200; A0099, DAKO), rabbit anti-Mmp7 (1:100; 3801S, Cell Signaling Technology), rat anti-serotonin (1:100; sc-73025, Santa Cruz Biotechnology), goat anti-gastrin (1:100; sc-7783, Santa Cruz Biotechnology), goat anti-secretin (1:100; sc-22630, Santa Cruz Biotechnology), goat anti-cholecystokinin (1:100; sc-21617, Santa Cruz Biotechnology), mouse anti-GLP-1 (1:10,000; ab23468, Abcam), rabbit anti-cleaved caspase 3 (1:100; 9664, Cell Signaling Technology), rabbit anti-p53 (1:500; VP-P956, Vector Laboratories), rat anti-Ki67 (1:100; M7249, Dako), chicken anti-GFP (1:200; ab13970, Abcam), rat anti-Musashi1 (1:1000; gift from Prof. Hideyuki Okano, Keio University, School of Medicine, Department of Physiology, Tokyo, Japan), rabbit anti-Cleaved Notch1 (1:100; 2421, Cell Signaling Technology), rabbit anti-Hes1 (1:1000; sc-25392, Santa Cruz Biotechnology), rabbit anti-Numb (1:100; ab14140, Abcam), and rabbit anti-Brm (1:400; ab15597, Abcam). Antigen retrieval for all primary antibodies was achieved by boiling in 10 mM citrate buffer pH 6.0 for 15 min. For quantitative analysis, cell counting or measurement of villi length was performed in at least ten sections from three animals for each genotype.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from murine intestine using the RNeasy Mini kit (Qiagen). Single-stranded cDNA was prepared using Superscript III (Invitrogen). Quantitative RT-PCR was performed on a Light-cycler FastStart DNA Master SYBR Green 1 kit (Roche Diagnostic). Expression levels were normalized to GPDH levels using the ΔΔCt method. Primers are listed in Table S1.

Statistics

Student’s t-tests were performed to determine statistical differences, with P<0.05 considered to be statistically significant. Kaplan–Meier analysis was used to analyze percentage survival. Values are presented as means±s.d. unless otherwise noted.

Acknowledgements

We thank D. Reisman, University of Florida, with permission from P. Chambon, for sharing Brg1flox mice. We also thank T. Yoshioha, Y. Yamaga, Y. Kimura, T. Maruno, Y. Nakamishi, N. Nakatsui, K. Kanda, and T. Ueo for technical assistance and helpful suggestions.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.T., A.F. and H.S. analyzed the data. Y.T., A.F., T.C. and H.S. wrote the manuscript. Y.T. and A.F. conceived and designed the study. Y.T. performed the experiments. Author contributions Y.T., A.F. and H.S. analyzed the data. Y.T., A.F., T.C. and H.S. wrote the manuscript.

Funding

This work was supported by the Japan Society for the Promotion of Science (JSPS) [KAKENHI 25112707, 25130706, 26293173 to H.S.]: the Ministry of Education, Culture, Sports, Science, and Technology of Japan [15K15290 Research program of the Project for Development of Innovative Research on Cancer Therapeutics (P-Direct) to H.S.]: the Ministry of Health, Labour and Welfare [Health and Labour Sciences Research Grants for Research on Intractable Diseases, Hepatitis and The innovative development and the practical application of new drugs for hepatitis B to T.C.; Comprehensive Research on Life-Style Related Diseases including Cardiovascular Diseases and Diabetes Mellitus to H.S.]: The Development of Innovative Therapeutic Drug for the Intractable Inflammatory Bowel Disease to H.S.]: Kobayashi Foundation for Cancer Research (to H.S.): The Naito Foundation
Supplementary information
Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.141549.supplemental

References

http://dev.biologists.org/lookup/doi/10.1242/dev.141549.supplemental

Development (2016) 143, 3532–3539 doi:10.1242/dev.141549