Maternally acquired genotoxic *Escherichia coli* alters offspring's intestinal homeostasis

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The neonatal gut is rapidly colonized by a newly dominant group of commensal *Escherichia coli* strains among which a large proportion produces a genotoxin called colibactin. In order to analyze the short- and long-term effects resulting from such evolution, we developed a rat model mimicking the natural transmission of *E. coli* from mothers to neonates. Genotoxic and non-genotoxic *E. coli* strains were equally transmitted to the offspring and stably colonized the gut across generations. DNA damage was only detected in neonates colonized with genotoxic *E. coli* strains. Signs of genotoxic stress such as anaphase bridges, higher occurrence of crypt fission and accelerated renewal of the mature epithelium were detected at adulthood. In addition, we observed alterations of secretory cell populations and gut epithelial barrier. Our findings illustrate how critical is the genotype of *E. coli* strains acquired at birth for gut homeostasis at adulthood.

Introduction

Upon birth, the infant gut undergoes a massive microbial colonization which has been suggested to exert marked effects on basic host physiology, immunity and metabolism later in life (for a review see refs. 1 and 2). This initial extra-uterine microbial load originates from the mother and birth environment, and then dynamically evolves toward a complex consortium throughout host development and environmental exposures.[3-9] Although diverse among individuals, the composition of the infant fecal microbiota reaches a state of relative equilibrium in humans by the end of the first year of life[10] and adopts an adult-like configuration within the 3-y period following birth.[9] The host provides a niche in which bacteria ensure their transmission and retention within the gut. In turn, the microbiota, especially in the neonate, contributes to essential host functions such as nutrient supply, energy balance,[11-13] metabolic signaling,[14] resistance to pathogens colonization,[15-17] and has a key role in promoting the postnatal maturation of the intestinal mucosal barrier.[18-23]

The commensal bacteria *Escherichia coli* are one of the first colonizer of the mammalian gut at birth.[10,24,25] The mother represents the most influential environmental factor for the transmission of *E. coli* regardless of delivery mode.[26] *E. coli* dominates the gut microbiota within a few days after birth and contributes to the generation of an anaerobic environment favorable for the further establishment of *Bacteroides*, *Bifidobacterium*, and *Clostridium* species. *E. coli*, among other *Enterobacteriaceae*, becomes the predominant facultative anaerobic bacteria within the mature gut microbiota in which it establishes a stable lifelong interaction with the host.[10,27,28]

Although *E. coli* exhibits a very high genomic plasticity, its core genome representing less that 40% of the genes present in each *E. coli* strains, its genetic structure is predominantly clonal.[29] Phylogenetic analyses of *E. coli* populations revealed at least five main phylogenetic groups: A, B1, B2, D, and E.[30] Over the past 30 y, epidemiologic studies have established a shift from the phylogenetic group A to the phylogenetic group B2 strains in the fecal microbiota of populations living in industrialized countries,[28,29,31,32] including infants.[33-35] Enriched dietary habits or increased level of hygiene are presumably the main factors accounting for this major modification in the phylogenetic group distribution of fecal *E. coli* population. Moreover, up to 30% of commensal B2 strains have acquired a genomic island, called *pks*, by horizontal transfer.[36] Fecal carriage of commensal B2 *pks*+ *E. coli* strains in infants has been estimated in Western countries and revealed that up to 18% of Swedish infants are colonized three days after birth, in a study performed 10 y ago.[37] Carriage of the *pks* island was also associated with long-term persistence of such neonatal acquired commensal B2 strains.[37]

The *pks* genomic island encodes for a nonribosomal peptide synthetase (NRPS) and polyketide synthetase (PKS) machinery that allows the synthesis of a peptide-polyketide hybrid genotoxin, named colibactin.[36] A short and direct contact of cultured human cells or mouse enterocytes, in colon loop experiments, with *pks*+ *E. coli* strains induced transient DNA double strand breaks, characterized by the formation of phosphorylated histone H2AX (γH2AX) foci. This was followed by incomplete DNA repair, leading to anaphase bridges, chromosomal abnormalities and senescence in dividing cells.[38,39] In IL10⁻/⁻, azoxymethane (AOM)-treated, *pks*+ *E. coli* monocolonized mice, a significant increase of γH2AX foci was observed in colonocytes suggesting that inflammation creates an environment potentiating the genotoxicity of *E. coli* strain.[40] In addition, the presence of *pks*+ *E. coli* accelerated the progression from dysplasia to invasive carcinoma in mono-associated AOM/IL10⁻/⁻ mice whereas *pks*+ *E. coli* mono-associated AOM/WT mice did not develop any dysplasia/tumors.[40] Taken together, these studies suggest that *pks*+ *E. coli* could promote colorectal carcinogenesis in a pro inflammatory environment.

We hypothesized that the postnatal period is a critical period where direct interaction between *pks*+ *E. coli* and immature intestinal barrier is susceptible to modulate intestinal homeostasis. Persistence of such neonatal gut residents is actually not known and impact of early exposure to these genotoxic strains on the developing intestine has never been explored. Therefore, we developed a novel animal model that mimics the natural transmission of *E. coli* from mothers to newborns in order to investigate whether colibactin can alter the development and function of the intestinal epithelial barrier. We observed that early colonization with *pks*+ *E. coli* triggers genotoxicity in the gut epithelium of neonates whereas γH2AX foci were no longer detected in adults. Nevertheless, adult rats early exposed to genotoxic *E. coli* strains exhibited an increased cell turnover in both small intestine and colon. Further examination of the gut epithelium revealed that occurrences of anaphase bridges and crypt fissions were abnormally high, whereas pattern of differentiated epithelial cells was reshaped in adult rats

early exposed to *pks*+ *E. coli*. In addition, these phenomenons were transmissible across generations. The analyze of the intestinal epithelium permeability revealed that the integrity of the gut barrier was altered in animals early colonized with *pks*+ *E. coli*. In conclusion, we characterized an example of long lasting regulation of the host intestinal barrier function by genotoxic *E. coli* strains acquired at birth and demonstrated a transferability of host intestinal phenotypes across generations according to the phenotype of the pioneer gut bacteria.

Results

Prevalence of B2 *E. coli* carrying the *pks* island in neonates

We analyzed a collection of stools from 184 healthy neonates collected between October and November 2010 at the University Hospital of Limoges in France. At three days of age, 56.5% of infants were colonized with *E. coli* (104 neonates) and 47.1% of them carried at least one B2 *E. coli* strain (49 neonates), corresponding to 26.6% of all infants. Over the 52 B2 *E. coli* isolates collected and further characterized (**Table S1**), 30 carried the *pks* island. The rate of *pks*+ *E. coli* colonization at three days of life was finally 26.9% in infants colonized with *E. coli* or 15.2% (28 neonates) in the entire infant population.

These *pks*+ *E. coli* isolates were further analyzed by multilocus sequence typing (MLST)[41] and serotyping. Subgroup VII (STc14) isolates were the most prevalent, all of them except two, being from serogroup O75 (**Table S1**).

Generation of non-genotoxic isogenic mutant in a commensal B2 *E. coli* strain carrying the *pks* island

M1/5 *pks*+ *E. coli* strain (*E. coli* WT), further used in this study, was isolated from the stool of a healthy adult (**Table S2**). This *pks*+ *E. coli* strain of serogroup O75, which belongs to the subgroup VII of the phylogenetic group B2 and for which shotgun sequence data was available, induced the formation of nuclear γH2AX foci in infected rat intestinal epithelial cells (IEC-6), as previously observed with other *pks*+ *E. coli* strains.[36,42] The *clbA* gene coding for a 4'-phosphopantetheinyl transferase (Pptase), which is essential for the synthesis of colibactin, has been deleted to generate an isogenic *E. coli* Δ*clbA* mutant unable to induce the generation of γH2AX foci. Chromosomal complementation of the Δ*clbA* mutant (*E. coli* Δ*clbA::clbA*) fully restored this genotoxic activity (**Fig. S1A and B**).

Genotoxic and non-genotoxic commensal *E. coli* strains are transmitted from mother to the offspring and equally colonized the gut from birth to adulthood in an asymptomatic manner

We developed an experimental animal model mimicking the early colonization of neonates by *E. coli* from the mother microbiota (**Fig. 1A**). Pregnant Wistar rats were fed with *E. coli* WT, *E. coli* Δ*clbA*, *E. coli* Δ*clbA::clbA* or PBS. These B2 *E. coli* strains were the dominant *Enterobacteriaceae* species isolated in mother fecal microbiota during the perinatal period (**Fig. S2A and B**). The natural transmission of *E. coli* strains to the offspring was detected as soon as post-natal day 2 (PND2). All neonates were then equally colonized and gut *E. coli* population reached the highest density between PND4 and PND8 (**Fig. 1B**). Fecal
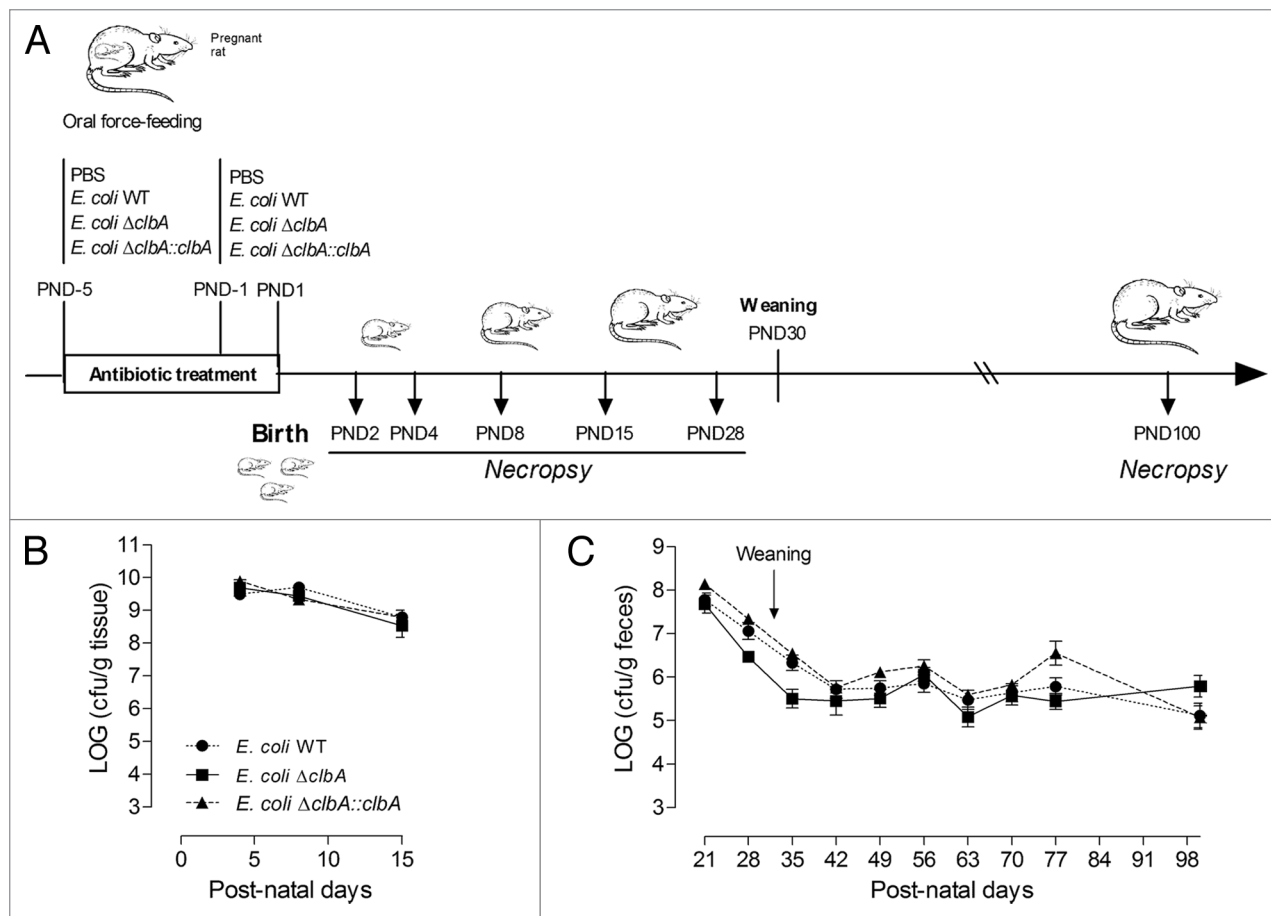
Figure 1. Commensal *E. coli* strains are transmitted from mother to the offspring and persistently colonized the gut at adulthood. (**A**) Experimental design of a rat model mimicking long-lasting colonization of the gut by commensal *E. coli* strains from birth to adulthood. Pregnant rats were treated with streptomycin (5 g/L) in drinking water and were inoculated twice with 10^9 CFU by intragastric gavage before parturition with *E. coli* WT, Δ*clbA* or Δ*clbA::clbA* strains or with PBS. Newborns were sacrificed at Post-Natal Days (PND) PND2, PND4, PND8, PND15, PND28 and PND100. (**B**) Evaluation of gut colonization in cecum/colon homogenate of neonates at early time-points (PND4, PND8 and PND15). (**C**) Evaluation of gut colonization in feces homogenate at later time-points (PND21, PND28, PND35 to PND100). Weaning is highlighted with a black arrow. Groups of 3–4 rats (PND4), 6–7 (PND8), 6–8 (PND15) and 15–20 (PND21-PND100) were used. Mean values ± SEM are shown.

bacteria counts gradually decreased from PND21 to PND35, then stabilized and the adult fecal *E. coli* strain levels remained around $10^{5,6}$ CFU per gram of feces (**Fig. 1C**). This decline of *E. coli* population was associated with the weaning transition and may result, as previously described in the literature, from the increased diversity of the gut microbiota composition and redox modifications of the gut microenvironment.[43] *E. coli* originating from the mothers was always the predominant bacterial specie among *Enterobacteriaceae* present in the offspring gut microbiota (**Fig. S2C**). During the four-month period of our experiment, the animals were healthy and we did not observe any alteration of body weight or overall health status in early colonized animals with commensal *E. coli* strains (**Fig. S3**). In conclusion, using this novel rodent model, we observed that both kinetic and dynamic of gut colonization with commensal B2 *E. coli* strains reflected a similar pattern than the one previously described in humans, in a completely asymptomatic manner.

Maternally acquired commensal genotoxic *E. coli* strains exacerbate DNA damage in the gut epithelial cells of neonates

We asked whether maternally acquired commensal B2 *E. coli* strains were genotoxic to the neonatal epithelium. From PND2 to PND8, we observed a significant increased number of γH2AX⁺ cells along the entire length of the intestinal mucosa in pups originating from *clbA*⁺ (WT or Δ*clbA::clbA*) *E. coli* treated mother as compared with pups originating from *E. coli* *clbA*⁻ (Δ*clbA* or PBS) treated mother (**Fig. 2A and B**). During this first week of life, the intensity of γH2AX signature observed in the intestinal epithelium of neonates colonized with *clbA*⁺ *E. coli* strains was similar to that observed with 0.5-Gy-whole body γ irradiation (**Fig. S4A and B**). Once pups reached PND8, γH2AX signal progressively declined, and genotoxicity was no longer detectable in rats older than PND28, when the number of genotoxic bacteria was estimated to be below 10^7 CFU per gram of feces (**Fig. 1C**).
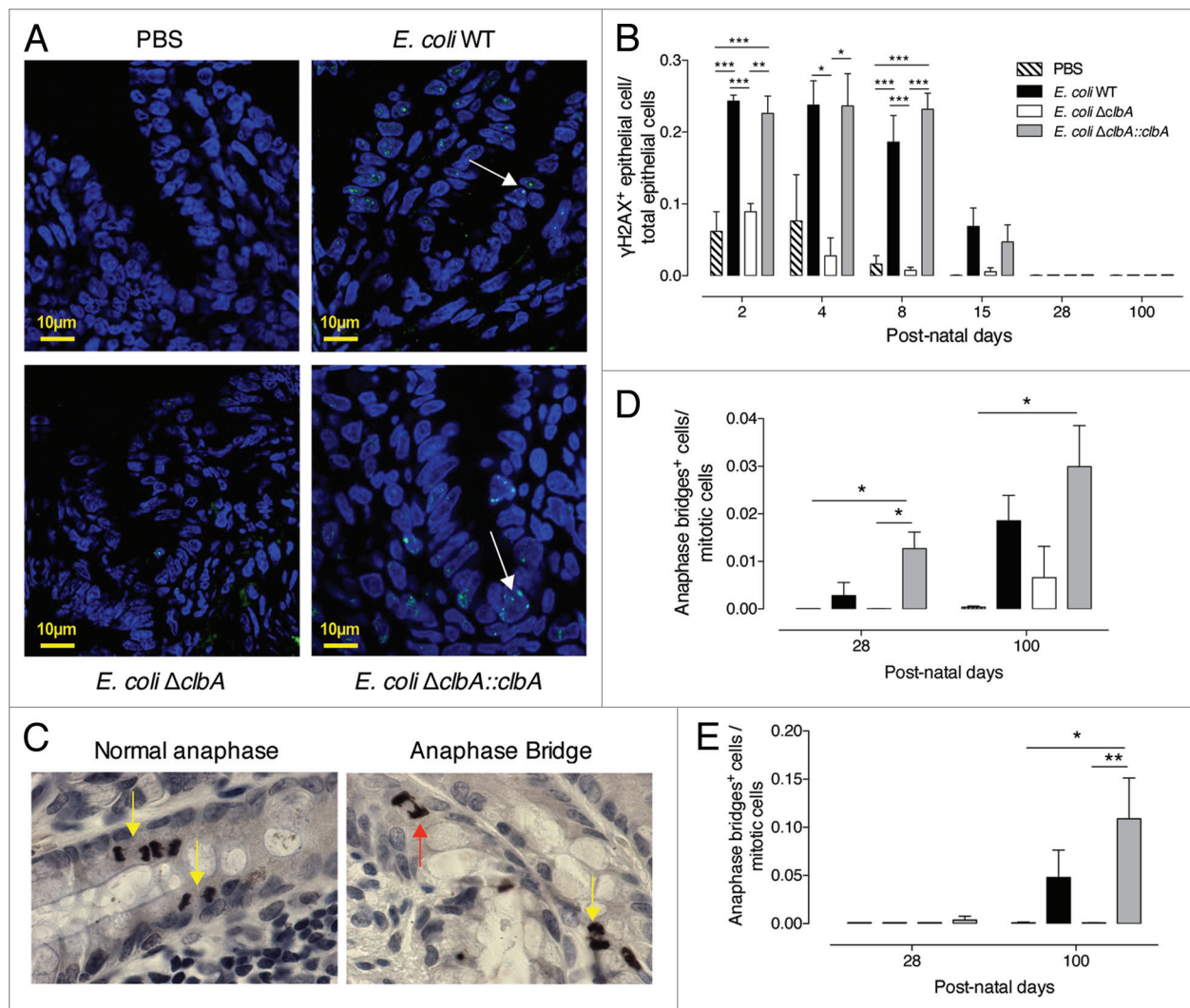
Figure 2. Gut colonization with commensal *E. coli* strains producing colibactin exacerbates DNA double-strand breaks in gut epithelial cells of neonates and induces signs of increased chromosomal instability at adulthood. Immunofluorescence analysis of intestinal and colonic epithelium of neonates and adults (PND2-PND100) colonized since birth by commensal *E. coli* WT, *E. coli* ΔclbA or *E. coli* ΔclbA::clbA strains or treated with PBS. (**A**) Representative colon frozen sections at PND4. DNA was stained in blue and γH2AX foci in green. Scaled bars = 10 μm. White arrows show γH2AX foci in nucleus. (**B**) Quantification of γH2AX-positive epithelial cells. Groups of 3–6 rats were analyzed. Mean value ± SEM are shown. Two-way ANOVA with Bonferroni Multiple Comparison test, * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. Immunohistochemistry analysis of intestinal and colonic epithelium of rats at PND28 and PND100. (**C**) Representative colon sections at PND100. Phospho-H3 was stained in black and allows detection of normal anaphases (left, yellow arrows) and abnormal mitosis with anaphase bridge (right, red arrow). Magnification used for photomicrographs = x20. Yellow arrows show normal anaphase figures and red arrow shows anormal mitotic cell with anaphase bridge. (**D-E**) Quantification of mitotic cells with anaphase bridge in small intestine (**D**) and colon (**E**). Groups of 5–10 rats were analyzed. Mean ± SEM are shown. Two-way ANOVA with Bonferroni Multiple Comparison test, * $P \leq 0.05$ and ** $P \leq 0.01$.

Adult gut stably colonized since birth with a commensal genotoxic *E. coli* displays epithelial cells with signs of increased chromosomal instability

Misrepaired double-strand breaks (DSBs) can induce chromosome fusions resulting in anaphase bridging.[44,45] We previously observed such chromosomal instability biomarker within cultured epithelial cells shortly exposed to *pks*+ *E. coli*.[38] Thus, we quantified the anaphase bridge index within different segments of the adult gut epithelium in rats early colonized with

genotoxic or non-genotoxic *E. coli* strains (**Fig. 2C**). Interestingly, a significant increased number of anaphase bridges was detected in the small intestine of animals early colonized with *clbA*+ *E. coli* strains as compared with *clbA*- *E. coli* at PND28 and PND100 (**Fig. 2D**). A similar pattern was also observed in the colon (**Fig. 2E**), suggesting that DNA repair that follows DSBs triggered by *clbA*+ *E. coli* strains during the neonatal period was likely effective but DNA exhibited scars of misrepaired DSBs that will lead to genomic instability at adulthood.

Adult gut stably colonized since birth with a commensal genotoxic _E. coli_ displays increased rate of epithelial cell apoptosis

Intestinal epithelial cells continuously undergo spontaneous apoptosis and shedding as a result of constant renewal of the epithelial monolayer throughout postnatal life. Given that colibactin-induced DNA damage, following in vitro infection with _pks+ E. coli_, leads to cell death by apoptosis[38] and that intestinal epithelial cells displayed DNA damage in rats colonized with _clbA+ E. coli_ (**Fig. 2B**), we quantified apoptosis in epithelial cells along the gut at PND100. Regardless of the gut compartment, a significant increased number of apoptotic cells was observed in animals early colonized with _clbA+ E. coli_ strains as compared with animals early colonized with the _clbA-_ strain or controls (**Fig. 3A and B**). This phenomenon may occur as soon as PND28 (**Fig. S5A and B**). However, in offspring, gross histological architecture of the crypt-villus units did not reveal apparent differences according to mother treatment (**Fig. S6A–D**).

Adult gut stably colonized since birth with a commensal genotoxic _E. coli_ displays increased rate of epithelial cell proliferation

A higher rate of apoptotic cells without any change in the gut epithelium morphology, including villus and crypt depth, prompted us to examine the proliferative cell compartment within intestinal crypts at adulthood. Immunohistochemical staining of phosphorylated histone H3, a marker of mitotic cells, revealed that increased apoptosis rates previously observed in the mature gut epithelium of rats early exposed to _clbA+ E. coli_, was associated with a marked increase of mitotic cells number (**Fig. 3A and C**; **Fig. S5C and D**). Consistently, staining of these adult gut sections with a marker of the replicative S-phase (Proliferative Cell Nuclear Antigen, PCNA) revealed that the expansion of the proliferative compartment was restricted within the crypts of adult rats early exposed to _clbA+ E. coli_ (**Fig. 3A and D**; **Fig. S5E and F**).

Early gut colonization with a commensal genotoxic _E. coli_ hastened intestinal epithelial cells migration at adulthood

In order to investigate whether the increased proliferation and apoptosis in the crypt compartment of adult rats early colonized with genotoxic _E. coli_ strains have an impact on cell turnover, we monitored epithelial cell migration along the crypt-villus axis, by performing a 12–24 h BrdU chase (**Fig. S7A**). We observed that the migration front of BrdU-positive cells was significantly higher in rats early colonized with _clbA+ E. coli_ strains as compared with rats early colonized with _clbA- E. coli_ or controls (**Fig. S7B and C**). Moreover, 48 h after BrdU injection (**Fig. S7D**), intestinal epithelial cells in rats from _clbA+ E. coli_ treated mother progressed more rapidly along the crypt-villus axis as compared with those from _clbA- E. coli_ treated mother or controls. This functional assay indicated that early acquired commensal genotoxic _E. coli_ modulates intestinal epithelium renewal in adult rats.

Adult gut stably colonized since birth with a commensal genotoxic _E. coli_ displays an abnormal crypt fission rate

Crypt proliferation is a critical process driving the postnatal growth of the gut in rodents and humans. When crypts divide, two flask-shaped based joining in a single unit are observed at the top of the crypt. This feature is characteristic of crypt fission. The number of crypt fission increases in the gut throughout the three first postnatal weeks in rats and then decreases significantly with age.[46-48] Using our model, we observed that the percentage of crypt fission segregated according to the _E. coli_ strain acquired at birth (**Fig. 3A and E**; **Fig. S5G and H**). Indeed, it remained abnormally elevated in 100 d-old rats colonized with _clbA+ E. coli_ strains as compared with control rats or rats colonized with _clbA- E. coli_. Increased number of crypt fissions is considered as a dominant mechanism of early adenoma development.[49,50]

Maternally acquired commensal genotoxic _E. coli_ strains influence secretory intestinal cell lineage at adulthood

The proliferative cells spend approximately two days in the crypt, in which they divided 4–5 times before terminally differentiated into absorptive cells, mucous-secreting goblet cells, hormone-secreting enteroendocrine cells or bactericidal defensin peptides and lyzozyme-secreting Paneth cells.[51] Based on our previous results showing a significant increase of proliferative cells in the intestine of adult rats from _clbA+ E. coli_ treated mother, we next assessed cell lineage selection using secretory cell differentiation markers. We did not detect any modification of colonocyte differentiation in the offspring at PND100 (**Fig. 4B and C**). However, staining of small intestinal sections with Periodic-Acid Shiff revealed an increase of mucous-secreting goblet cells in rats early colonized with the genotoxic _E. coli_ (**Fig. 4A and B**). In addition, we noticed a significant reduction of enteroendocrine cells, as determined by the immunohistochemistry analysis of chromogranin A, in _clbA- E. coli_-colonized rats as compared with _clbA+ E. coli_-colonized rats (**Fig. 4A and C**). Finally, we observed an increased number of Paneth cells, stained with anti-lyzozyme antibody, in adult rats colonized at birth with a genotoxic _E. coli_ strain (**Fig. 4A and D**).

Commensal genotoxic _E. coli_ strains maternally acquired since birth increase intestinal epithelial permeability at adulthood

We have established that the mature gut epithelium of rats early colonized with commensal genotoxic _E. coli_ displays altered patterns of apoptosis, proliferation, and differentiation. We next assessed whether these alterations may alter epithelial barrier function. We analyzed the transcellular permeability to intact HRP in small intestine using Ussing chambers (**Fig. 5A**). No significant changes were observed; suggesting that transcytosis of high molecular weight macromolecules in intact form is not affected by commensal B2 _E. coli_ colonization. We next examined intestinal paracellular permeability to 4kDa dextran-FITC and electrical resistance (R) (**Fig. 5B and C**). Rats early colonized with _clbA+ E. coli_ strains exhibited, at both PND28 and PND100, a significant increase in apical to serosal cumulative permeation of 4kDa dextran-FITC as compared with control rats or rats early colonized with the _clbA- E. coli_ strain. In accordance with this increased 4kDa dextran-FITC permeability, a significant decrease of the electrical resistance (R) was observed in rats early colonized with the _clbA+ E. coli_ strains, suggesting that adult rats early colonized with genotoxic _E. coli_ have persistent epithelial barrier dysfunction.
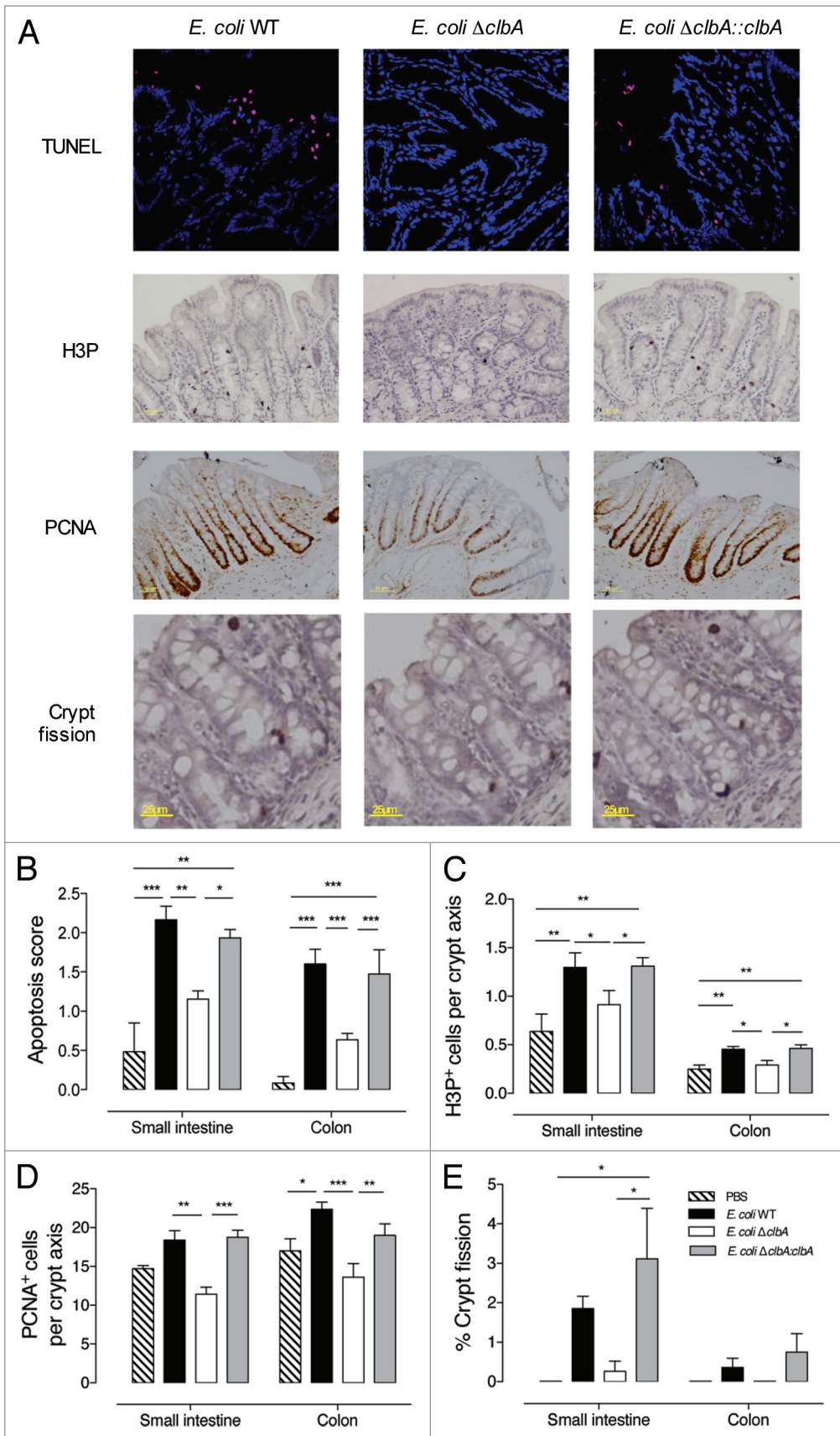
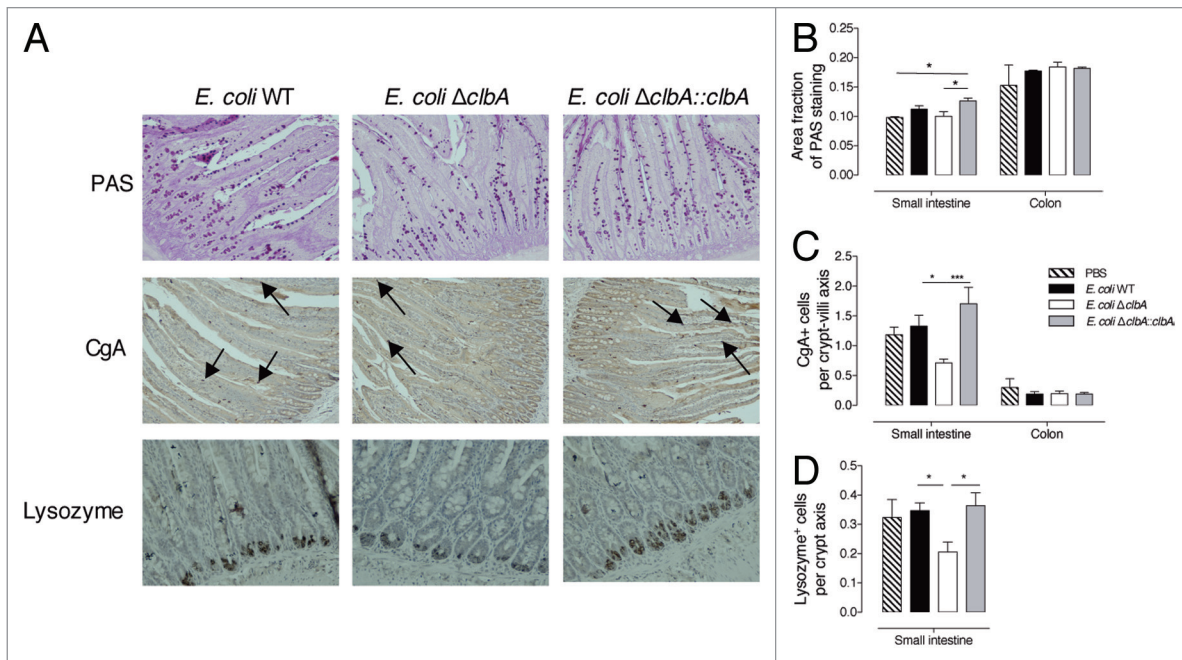**Figure 3.** For figure legend, see page 319.

Figure 4. Early gut colonization with commensal *E. coli* strains producing colibactin modulates small intestine epithelial cell differentiation at adulthood (PND100). Histological analysis of the intestinal epithelium of adult animals early colonized by commensal *E. coli* WT, Δ*clbA* or Δ*clbA::clbA* strains or treated with PBS. (**A**) Representative small intestine sections stained with Periodic-Acid Shiff (PAS) method showing goblet cells in magenta. Enteroendocrine cells (black arrows) were stained in brown with anti-chromogranin A (CgA) antibody. Paneth cells were stained in brown with anti-Lysozyme (Lyz) antibody. (**B**) Quantification of PAS staining area on total epithelial surface. (**C**) Quantification of CgA+ cells along the crypt-villus axis. (**D**) Quantification of Lyz+ cells along the crypt-villus axis in the small intestine. Magnification used for photomicrographs = x20. Groups of 5–10 rats were used. Mean values ± SEM are shown. One-way ANOVA with Bonferroni Multiple Comparison test, * $P \leq 0.05$, ** $P \leq 0.01$.

Alterations observed in the gut epithelia of rats colonized with a maternally acquired genotoxic commensal *E. coli* strain are transmissible across generations

We extended this study by examining transgenerational impact of the natural transmission of a maternally acquired genotoxic *E. coli*. To this end, adult rats colonized since birth with *E. coli* WT or *E. coli* Δ*clbA* strain as well as control rats were bred (**Fig. 6A**). Fecal bacteria analysis of the second generation revealed that both genotoxic and non-genotoxic *E. coli* strains were naturally transmitted to the offspring with comparable levels than in the first generation (**Fig. 6B**). As expected, at PND10, was significantly increased within the gut epithelium of neonates colonized with genotoxic *E. coli* (**Fig. 6C**) and associated, at PND56 with an increased occurrence of anaphase-bridges (**Fig. 6D**), mitotic cells (**Fig. 6E**), and crypt fission (**Fig. 6F**). Collectively, these findings demonstrated that a specific *E. coli* strain present at a physiological level of colonization in the gut microflora of the mother can be transmitted across generations. The genotoxic potential of these early colonizing *E. coli* strains elicits short- and long-term physiological alterations of the gut physiology.

Discussion

Bacterial colonization of the gastrointestinal tract occurs during the first year of life. After this period, the microbiota composition is rather stable throughout adulthood.[52] Adult human intestine harbored approximately 10^{14} bacterial cells, comprising more than 500 different bacterial species.[53] This dense microbial environment affects metabolic, neurologic, inflammatory and immunologic host functions.[18,54] Nonetheless, the neonatal period remains largely unexplored while likely having a tremendous impact on metabolic and cellular programming of the host.[56] Perturbations in this fragile ecosystem, during this highly vulnerable period may have consequences in the later life.

E. coli belongs to the pioneer microbiota that colonized the mammalian gut within few days after birth.[29] As reported

Figure 3 (See opposite page). Early gut colonization with commensal *E. coli* strains producing colibactin increases the intestinal epithelial cells apoptosis, proliferation and crypt fission at adulthood. Histological and immunofluorescence analysis of the intestinal and colonic epithelium of adult animals early colonized by commensal *E. coli* WT, Δ*clbA* or Δ*clbA::clbA* strains or treated with PBS. (**A**) Representative colon frozen sections at PND100. DNA was stained in blue and apoptotic cells were stained in red with anti-TdT antibody (Tunel). Scale bars = 10 μm. Representative colon sections at PND100. Phospho-H3 was stained in black. PCNA was stained in black. Representative colon sections at PND100 and stained with hematoxylin-eosin. (**B**) Quantification of intestinal apoptotic score (see Methods section). (**C**) Quantification of H3P+ cells per crypt. (**D**) Quantification of PCNA+ cells per crypt. (**E**) Quantification of crypt fission. Black arrows show the fission of crypt in the colon. Magnification used for photomicrographs = x20. Groups of 5–10 rats were used, Mean values ± SEM are shown. One-way ANOVA, Bonferroni Multiple Comparison test, * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.
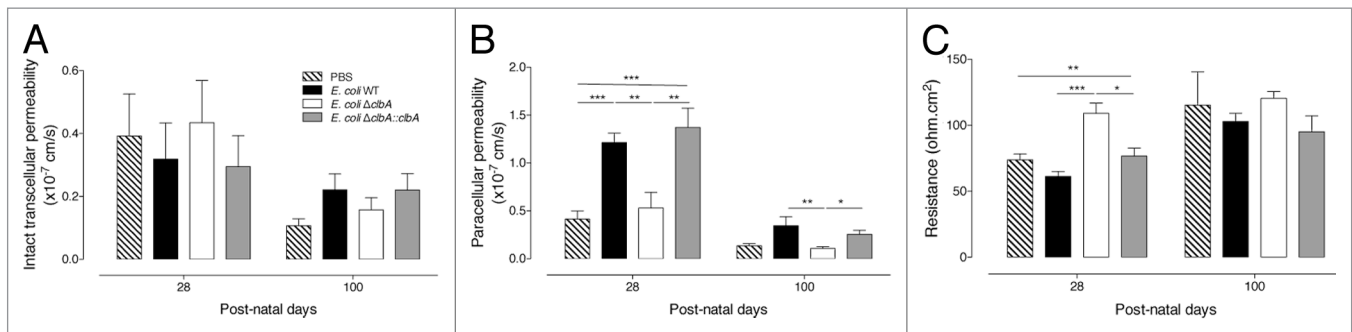
Figure 5. Early gut colonization with commensal *E. coli* strains producing colibactin alters intestinal permeability at adulthood (PND28 and PND100). Duodenal permeability was analyzed in adult animals early colonized by commensal *E. coli* WT, Δ*clbA* or Δ*clbA::clbA* strains or treated with PBS. (**A**) Transcellular permeability was assessed measuring mucosal to serosal flux of HRP. (**B**) Paracellular permeability was assessed measuring mucosal to serosal flux of FITC-dextran. (**C**) Electric transepithelial resistance was recorder during the permeability analysis. Groups of at least 10 rats were used. Mean values ± SEM are shown. One-way ANOVA with Bonferroni Multiple Comparison test, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

by our group,[36] B2 *E. coli* strains harbored a specific genomic island called *pks*, which encodes the genotoxin colibactin. These colibactin-producing *E. coli* induced DNA double strand breaks, chromosomal abnormalities and genomic instability in mammalian cells.[38] Therefore, we did a survey for the presence of *pks⁺ E. coli* in early life. In this study, we showed that *pks⁺ E. coli* strains belong to the early microbiota of around 15% of three-days-old French neonates. This is in accordance with data generated from a 10 y old Swedish cohort in which 18% of three-days-old neonates was colonized by *pks⁺ E. coli*. Thus, the investigation of the impact of an early exposure with these genotoxic strains on the developing intestine was of particular interested. In this context, we developed an innovative and realistic animal model of natural colonization by vertical transmission. This model reproduced the natural sequence of events that precedes human gut colonization, with levels reaching the same order of magnitude than those observed in neonates[7,26,57] and adults.[29,58] In addition, this model eliminated pups manipulation and thus circumvented the neonatal stress that is known to impact the gut mucosal barrier.[59-61] The use of germfree or gnotobiotic animals gave insights into the causative role of microbiota in shaping host gut responses but lacked of accuracy as the result of large intestinal developmental defects or an artificial monocolonized environment. Thus, this vertical model allowed us to investigate in a global and natural way the role of pioneer genotoxic commensal *E. coli* strain on the intestinal homeostasis.

E. coli harboring the *pks* island have been shown to be more frequently detected in colorectal tissue from inflammatory bowel disease (IBD) and colorectal colon cancer (CRC) patients as compared with non-IBD/non CRC controls,[40,62] and may directly impact carcinogenesis. However, it is not clear whether dysbiosis promoting the emergence of *E. coli* is a cause or a consequence of CRC. We hypothesized that *E. coli*, which is the major pioneer bacteria colonizing the gut of neonates, may be involved in the initiation steps of colon carcinogenesis. Using our model allowing maternal transmission of *E. coli*, we demonstrated that early acquired colibactin-producing B2 *E. coli* establishes a lifelong niche in the gut ecosystem behaving as an autochthonous microbe. As a consequence, B2 colibactin-producing *E. coli* altered intestinal

epithelial cells integrity during the neonatal period, by activating the DNA damage response, and at adulthood, by increasing signs of genotoxic damage including anaphase bridges, crypt fission, intestinal proliferation and cellular renewal. These data strongly suggested that early colonizing colibactin-producing *E. coli* strains could participate in intestinal tumorigenesis. Several studies have reported that the use of genotoxic therapies in young children exacerbates the risk to develop cancer at adulthood.[63-65] Our data revealed that the intestinal genotoxicity intensity observed in animals early colonized by colibactin-producing *E. coli* was similar to that observed with 0.5Gy whole-body γ-irradiation. Beside, epidemiological follow-up of atomic-bomb survivors indicated that low-dose (~1Gy) increased the frequency of mutation associated with colorectal cancer.[66] Elaboration of longitudinal birth cohort in order to further explore a potential link between carriage of colibacin-producing B2 *E. coli* strain at birth and predisposition to colorectal cancer is required.

Moreover, the present study revealed a role of colibactin-producing B2 *E. coli* in lineage-specific maturation of intestinal epithelial cells. While in the small intestine of animals early colonized by colibactin-producing *E. coli*, amplified differentiation of Paneth, goblet and enteroendocrine cell lineages might reflect an increase in proliferative dynamics, the crypt hyperplasia observed in the colon was not related to any modification of epithelial cell differentiation. This selective cell lineage commitment in the small intestine suggest that rather than a passive effect of deregulated cell proliferation, early intestinal colonization with colibactin-producing *E. coli* might affect intestinal cell programming. The renewal of the intestinal epithelium from stem cells to functionally mature cells is tightly regulated by multiple factors and pathways involving epithelial-epithelial and epithelial-mesenchymal interactions.[67] In this study, we showed that it may also respond to environmental conditions and especially to the composition of the neonatal gut microbiota.

A concomitant increase of Paneth and goblet cell numbers has been reported after a systemic treatment with the DNA-damaging agent doxorubicin and may be related to regeneration processes.[68] Indeed, it has been demonstrated that Paneth cells may influence
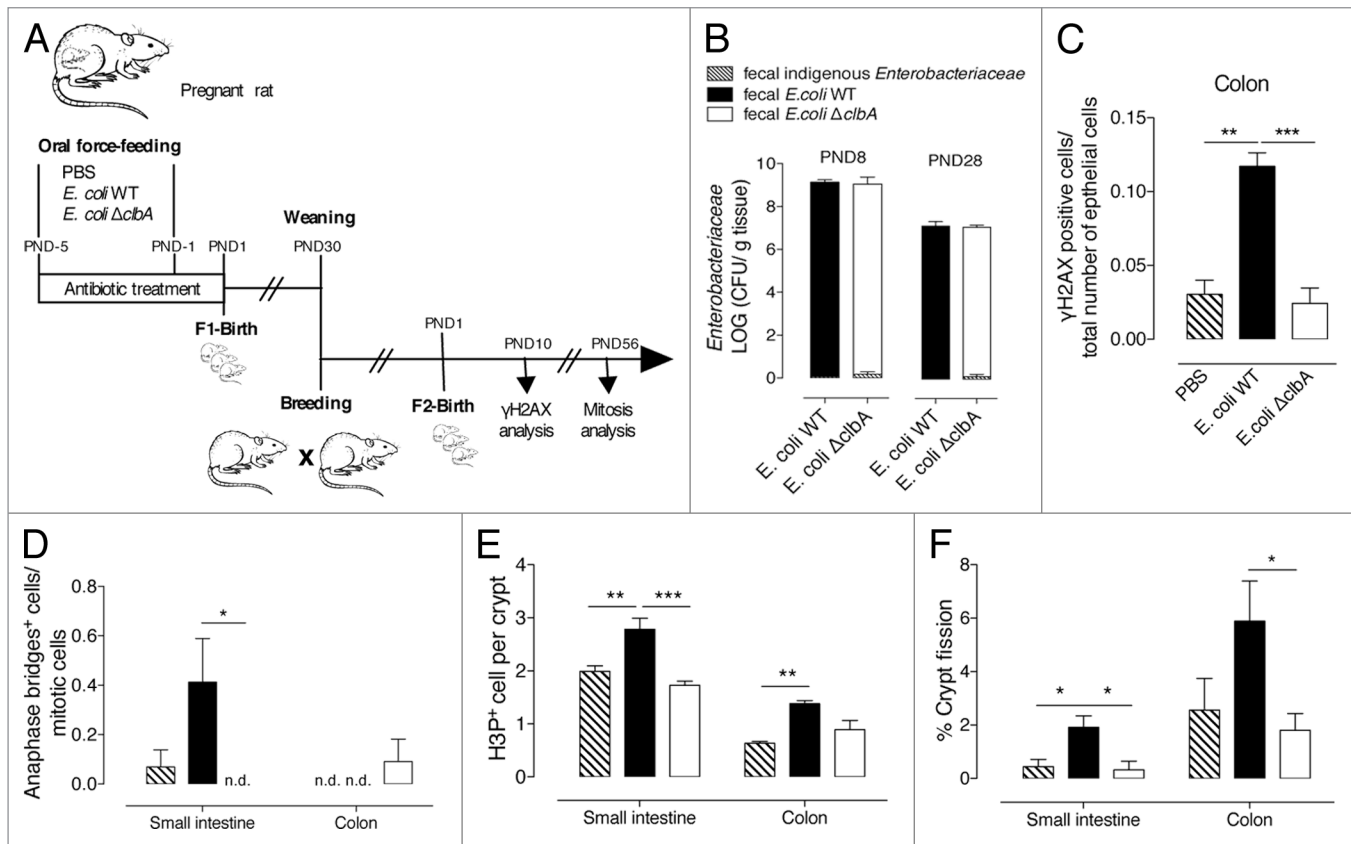
Figure 6. Alterations in intestinal physiology, induced by early gut colonization with commensal *E. coli* producing colibactin, are transmitted across generations. (**A**) Experimental design of a rat model mimicking transgenerational colonization of the gut with commensal *E. coli* strains. Adult rats, early gut colonized with commensal *E. coli* WT, Δ*clbA* strains or treated with PBS (F1), are breed and give rise to a second generation (F2) that is subsequently analyzed. (**B**) Evaluation of gut colonization in cecum/colon homogenate of neonates at PND8 and feces homogenate of adults at PND28. (**C**) Quantification of colonic γH2AX-positive epithelial cells. (**D**) Quantification of mitotic cells with anaphase bridge in small intestine and colon. (**E**) Quantification of H3P^{+} cells per crypt in small intestine and colon. (**F**) Quantification of crypt fission in small intestine and colon. Groups of 6 rats were used. Mean values ± SEM are shown. One-way ANOVA with Bonferroni Multiple Comparison test, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

the stem cells micro-environment and function.[70,71] In addition Paneth cells synthesize and secrete numerous factors that are known to modulate proliferation and migration of enterocytes.[72] It is likely that intestinal DNA damage induced during the neonatal period, where the intestinal mucosal barrier is largely immature, by colibactin-producing *E. coli* may be exemplified by an increased numbers of Paneth and goblet cells at adulthood, probably due to the recruitment of cells from a pre-existing pool of secretory intermediate progenitor cells which are known to be induced after DNA damage.[73] This specific point was not examined in the present study and will be the focus of future experiments.

Intestinal epithelial cells are key players of the intestinal barrier by forming a physical barrier allowing the transport of nutrients to the lamina propria while keeping larger molecule and bacteria in the lumen. Intestinal permeability controls interplay between host and external environment. Mechanisms regulating the integrity of the gut epithelial barrier, and thus the maintenance of tolerance between the host and the gut environment, critically involve the gut microbiota.[54] Our experiments revealed that the presence of genotoxic *E. coli* dampened paracellular

permeability. However, we did not observe any difference in transcellular permeability but we did not analyze the transcytosis of macromolecules in degraded form. Indeed, luminal antigens crossing the epithelium can reach the serosal compartment in intact (10%) or degraded form (90%).[74] Our results exclude an effect of *E. coli* colonization on intact transcellular permeability but did not exclude an effect on the lysosomal pathway involved in degraded antigens transcytosis. There are mounting evidences suggesting that disturbance of intestinal barrier function and especially enhanced gut permeability may be involved in several gut dysfunctions, including irritable bowel syndrome (IBS)[75] and IBD.[76] In addition, enhanced translocation of luminal components could alter tolerance between the host and its environment leading to the development of allergic or auto-immune manifestations. Indeed, recent epidemiological and clinical studies indicate an association between the composition of intestinal microbial communities and the increased incidence of allergic asthma, eczema or rhinitis.[77-81] Taken together with the results of our study, these data lead us to hypothesize that early gut colonization with genotoxic B2 *E. coli* might be a risk factor in the incidence of such diseases. In addition, we established

that the early life is a critical period for the host and that the composition of the intestinal microbiota and its interaction with the intestinal epithelium is likely to give important information about the development of diseases that manifest both during infancy and adult life.

The gut microbiome is approximately 150 times larger than the human genome, with an estimated 3.3 million microbial genes. In this study, we have shown that the disruption of a single gene in the genome of B2 *E. coli* strains acquired at birth is critical for gut homeostasis of the host. As suggested in an assay wrote in 2009 by Blaser and Falkow,[82] it is predictable that social and medical progress that affects the composition of the microbiota will have consequences for our physiology and health. Such evolution is observed not only at the level of the microbiota but also at the level of phylogenetic groups, bacterial species and genes. There is a concomitant increase in population of industrialized countries of immune-mediated diseases and colorectal cancer along with a shift in *E. coli* population from the declining phylogenetic group A to the newly dominant phylogenetic group B2.[29] A large proportion of these B2 *E. coli* strains are both genotoxic and pioneer bacteria colonizing the gut of neonates. Thus, our data lead us to hypothesize that early gut colonization with the newly dominant B2 *E. coli* group associated with genotoxic activity might be a risk factor in the incidence of immune-mediated diseases and colorectal cancer.

Material and Methods

Analysis of feces in neonates

Healthy neonates were defined as asymptomatic neonates, which did not receive any antibiotic after birth. Sex, gestational age, mode of delivery as well as per-partum antibiotic prophylaxis during labor were recorded anonymously with approval of local ethics committee. Characterization of *E. coli* isolates is described in Supplemental Materials and **Table S1**.

Bacterial strains, mutagenesis procedures and growth Conditions

Bacterial strains used in this study are listed in **Table S2**. The commensal *Escherichia coli* strain M1/5, kindly provided by Pr. Ulrich Dobrindt, was isolated from the stool of an asymptomatic human and maintained with minimal genetic manipulation. *Pks* chromosomal isogenic mutant and complemented strain were generated in the laboratory. Procedures, primers and templates used for gene mutagenesis and resultant mutants are presented in the Supplemental Materials and **Table S2** respectively. Before oral administrations, all *E. coli* strains were grown 6 h in LB broth supplemented with antibiotics at 37 °C with shaking. These cultures were diluted 1:100 in LB broth without antibiotics and cultured overnight at 37 °C with shaking. Bacterial pellets from these overnight cultures were suspended in sterile PBS to the concentration of 10^9 colony forming units (CFU)/ml.

Experimental animal model of long-lasting gut bacterial colonization

Primiparous timed-pregnant WISTAR female rats were obtained from Janvier on gestational day 15 and housed separately under specific-pathogen-free conditions and had access to food and water supplemented with streptomycin (5g/L) ad libitum. Pregnant females were inoculated twice with 10^9 bacteria by intragastric gavage at PND-5 and PND-1. Toxalim animal facility (INRA, UMR 1331, Toulouse) is licensed by the French Ministry of Agriculture (agreement n° B31.555.13). All animal experiments complied with the European Union regulation, reviewed by the regional ethics committee (CNREEA n°1; MP/03/62/11/11). Bacterial colonization, body weight, gut development and maturation of offspring were specifically monitored from birth to weaning (PND2 to PND28) or at adulthood (PND56 and PND100) when gut maturation was completed (**Fig. 1A**).

Colonic bacterial load

Before PND15, cecum and colon homogenates were prepared in 0.6 mL of isotonic saline solution using Precellys tissue homogenizer (Bertin Technologies). After PND15, feces homogenates were prepared. 10-fold serial dilution of homogenates was plated on MacConkey agar plates (Biovalley) supplemented or not with appropriate antibiotics. Plates were incubated at 37 °C and the numbers of CFU were enumerated after 24 h. Colonies found growing on MacConkey's agar plates without antibiotic were considered to be *enterobacteria*-like bacteria belonging to the family *Enterobacteriaceae*.

Immunofluorescence analysis

Small intestine and colon samples were placed immediately in optimum cutting temperature (OCT, Sakura) compound and snap-frozen in liquid nitrogen. Sections (5µm) were fixed in 4% formaldehyde, permeabilized with PBS-0,25% Triton X100, blocked with PBS-0.1% Tween20–5% NGS and stained with mouse anti-phospho H2AX antibody (Millipore, clone JBW301) followed by goat anti-mouse-FITC antibodies (Zymed). DNA was stained for 1 min with TO-PRO-3 (Invitrogen) and mounted in VectaShield containing DAPI (Vector Laboratories).

Cell death was quantified by terminal deoxynucleotidyl transferase-mediated tetramethylrhodamine-dUTP (TMR-dUTP) nickened labeling (TUNEL kit, Roche). Frozen sections of small intestine and colon were fixed with 4% paraformaldehyde and permeabilized with PBS-0.1% Triton X100–0.1% Sodium citrate. DNA was labeled with TO-PRO-3 (Invitrogen). Scoring (0–4) was based on the number of TUNEL-positive cells in intestinal epithelium. 0 = no TUNEL-positive cells; 1 = < 5 TUNEL-positive cells; 2 = 5 to 10 TUNEL-positive cells; 3 = 11 to 40 TUNEL-positive cells; 4 = > 41 TUNEL-positive cells. Images were acquired with an Olympus IX70 laser scanning confocal microscope equipped with the Fluoview software FV500. Confocal aperture was set to achieve a z optical thickness of ~0.5 µm for foci quantification and ~0.26 µm for γH2AX foci localization analysis.

Histology, immunohistochemistry, and cell lineage analysis

Small intestine and colon samples were collected, fixed with 10% formalin for 24 h, dehydrated and embedded in paraffin according to standard histological procedures. Sections (5 µm) were mounted on SuperFrost® Plus Slides then dewaxed in a xylene bath for 10 min and rehydrated in graded alcohol baths. Slides were stained with hematoxylin and eosin (H&E) and

analyzed for intestinal morphometry (crypt depth, villi length, and crypt fission). For immunohistochemistry, endogenous peroxidase activity was quenched using 5% H_2O_2 in PBS (or methanol). Antigen retrieval was performed by heating section in 10mM sodium citrate (pH 6.0) for 10 min. Non specific binding was blocked, incubating with 2.5% normal goat serum for 1 h at room temperature (RT). Sections were incubated with primary antibody anti-phospho-Histone3 (H3P, 1/50e, Cell Signaling Technology, overnight at 4 °C), anti-chromogranin A (CgA, 1/500e, Immunostar, 1 h at RT) or anti-lysozyme (Lyz, 1/100e, Invitrogen, 1 h at RT) antibodies. Detection was performed by incubating with biotin secondary antibody followed by ABC complex (Vectastain, Vector Laboratories).

Sections were incubated with anti-PCNA (Proliferating Cellular Nuclear Antigen, 1/1000e, Santa Cruz) 1 h at RT. Detection was performed using IMPRESS reagent (Kit anti-mouse IMPRESS reagent, Vector laboratory). Staining was developed with DAB substrate (Vector Laboratories) and sections were counterstained with Hematoxylin QS (Vector Laboratories). After dehydratation, sections were mounted in Vectamount (Vector Laboratories) and images were acquired with a Leica DMRB fluorescence microscope or Nicon Eclipse 90i and analyzed with NIS Elements AR program. For goblet cells observation, slides were stained using periodic-acid Schiff method (PAS): incubation with 0.5% of periodic acid for 7 min at RT followed by Schiff reagent for 15 min at 4 °C.

Cell proliferation was analyzed by assessing the number of mitotic cells (H3P$^+$ cells), proliferative cells (PCNA$^+$ cells) per crypt axis (25 to 100 crypts were analyzed per sections). Goblet cells number was evaluated by measuring the mean area of PAS staining on 5 photomicrographs per sample. Enteroendocrine cells (CgA$^+$ cells) and Paneth cells (Lyz$^+$ cells) were enumerated per crypt-villus axis (25 to 100 crypts were analyzed per sections). 1–3 sections were analyzed per rat. Crypt depth and villus length were obtained from at least 10 well-oriented crypt-villus axes per animal. The number of fissioning crypts was obtained by observing at least 100 crypts per animal.[48] All slides were analyzed by two investigators who were blinded for the treatment. Images were acquired with a Leica DMRB or Nikon Eclipse 90i microscope. Analyses were performed using Image J software (http://rsb.info.nih.gov/ij/).

Intestinal permeability

Two specimens of duodenum fragments were mounted in Ussing chambers (Easy Mount, Physiologic Instruments) exposing a surface area measuring 0.1 cm^2. They were bathed on each side with 1 ml of oxygenated thermostated Kreb's solution (Sigma). Electrical parameters, including potential difference, short-circuit current (Isc) and total electrical resistance (R), were recorded at regular intervals during the 2-h period of experimentation. A change in electrical resistance was considered an index of altered paracellular permeability. Horseradish peroxidase (HRP) transport was measured as an index of macromolecular permeability, and FITC-dextran 4kDa epithelial passage was measured as a marker of paracellular permeability to small molecules. After equilibration of electrical parameters, HRP was added to the mucosal compartment at a final concentration of 0.4 mg/ml, and FITC-dextran 4kDa at a final concentration of 2.2 mg/ml. The 2 markers were applied simultaneously in the mucosal compartment. Epithelial permeability to intact HRP was determined by an enzymatic assay measuring HRP activity using an automatic Infinite M200 microplate reader (Tecan). Epithelial permeability to FITC-Dextran 4kDa was determined by measuring the fluorescence intensity (FI) 485nm/525nm using an automatic Infinite M200 microplate reader (Tecan). By measuring the specific activity of the markers expressed in FI/µg or HRP activity/µg and the FI or HRP activity values found in the serosal compartment, intestinal fluxes (µg/2 h·cm^2) were calculated. Permeability was calculated as the ratio of flux/concentration, as previously described.

Statistical analysis

Statistical evaluation of differences between the experimental groups was determined by using one-way or two-way analysis of variance (ANOVA) followed by a Bonferroni post-test (which allows comparison of all pair of groups). All tests were performed with GraphPad Prism 5.03 (GraphPad Software Inc.). All data are presented as mean ± standard error of the mean (SEM). A P value < 0.05 was considered significant.

Supplemental Materials

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