# Effects of bacteria-mediated reprogramming and antibiotic pretreatment on the course of colitis in mice

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**Abstract.** Since the original study by Takahashi and Yamanaka in 2006, there have been significant advances in the field of induced pluripotent stem cells. However, to the best of our knowledge, all of the studies published to date are based on *ex vivo* gene delivery and subsequent reimplantation of the cells. By contrast, *in vivo* reprogramming allows the direct administration of DNA encoding the reprogramming factors into the target tissue. In our previous study we demonstrated the beneficial effects of Salmonella-mediated oral delivery of genes into colonic mucosa as a therapy for colitis. In the present study, the effect of the bacterial vector *Salmonella typhimurium* SL7207, carrying a plasmid encoding the reprogramming factors Sox2, Oct3/4 and Klf4, on colitis in mice was investigated. Therapeutic intervention, consisting of repeated gavaging following the induction of colitis, did not exhibit beneficial effects. However, preventive oral administration of the therapeutic bacterial strain resulted in improvements in weight loss, colon length and stool consistency. Recently it has been shown that antibiotic pretreatment may alleviate chemically induced colitis in mice. Therefore, in the present study it was investigated whether antibiotic pretreatment of mice was able to enhance colonization of the administered bacterial strain in the colon, and therefore improve therapeutic outcome. C57BL/6 mice were administered streptomycin and metronidazole for four days, prior to multiple oral administrations of therapeutic bacteria every other day. Following three gavages, mice were administered dextran sulfate sodium in their drinking water to induce colitis. Disease activity parameters, including stool consistency, weight loss and colon length, were improved in the group receiving antibiotics and bacterial vectors. These results indicate that antibiotic pretreatment may enhance bacterial gene delivery into the colon. Furthermore, the anticipated *in vivo* reprogramming of colon cells appears to have a beneficial effect on the severity of colitis. These effects, however, still require further analyses.

## Introduction

The two major forms of inflammatory bowel disease (IBD) are ulcerative colitis and Crohn's disease. The pathogenesis of IBD is complex, and the most common treatment includes the use of immunosuppressive agents, antibiotics and biological agents (1,2). However, this approach is symptom-oriented with harmful side effects; therefore, new insights for novel therapeutic strategies are required. The fact that intestinal bacteria have a role in the pathogenesis of IBD presents a potential strategy for the reduction of bowel inflammation (3). A number of previous studies manipulated the intestinal microbiota via the administration of probiotics in the treatment of IBD (4-6). Bacteria-mediated gene delivery in IBD has previously been investigated and found to be successful. Previous studies have demonstrated that bactofection of the colonic mucosa using *Salmonella typhimurium* SL7207 carrying genes encoding antioxidant, anti-inflammatory and anti-angiogenic factors has been effective in the treatment of dextran sulfate sodium (DSS)-induced colitis in rodents (7-9). Several studies have also investigated the effects of antibiotic treatment on the course of experimental colitis in the view of bacterial (probiotic) therapy. Antibiotic pretreatment may enable certain invasive bacterial strains to better colonize the gut during DSS-induced colitis (10). A number of antibiotics, including minocycline, exert intestinal anti-inflammatory effects and attenuate the activation of experimental colitis. Furthermore, these antibiotics show an additive effect on the recovery of intestinal damage when used along with probiotic bacterial strains (11).

Notably, previous studies have investigated therapy based on stem cell administration for the treatment of IBD animal models. Amelioration of experimental colitis has been shown using hematopoietic stem cells (12), mesenchymal stem cells (13) and colonic stem cells (14). However, despite the promising data on stem cell therapy of IBD, the use of induced pluripotent stem (iPS) cells for the treatment of IBD has not yet been assessed. In two recent reviews, we presented the rationale behind *in vivo* reprogramming using bacterial vectors for gene delivery into the colon tissue (15,16). We hypothesized

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that reprogramming intestinal cells into a pluripotent state could demonstrate potential for IBD therapy and prevention. This theory was based on the presumption that adult somatic cells with the capacity to return to the pluripotent state have the ability to differentiate into the desired cell phenotype, this phenotype exhibiting resistance to the IBD-inducing stimuli. Several studies have shown that a pre-emptive stem cell therapy may lead to a protective phenotype later when the damaging event occurs (17,18). However, the concept of *in vivo* reprogramming has yet to be verified experimentally.

The present study aimed to investigate the use of bacterial vectors for oral delivery of genes encoding reprogramming factors into the colon and the effects of this treatment on the course of DSS-induced colitis in mice. The therapeutic and preventive approaches were compared, and the role of antibiotic pretreatment in this process was also investigated.

## Materials and methods

*Bacteria and plasmids*. The bacterial strain *Salmonella typhimurium* SL7207, which is suitable for bactofection of eukaryotic cells, was transformed with eukaryotic expression plasmids pcDNA3-RFP (Addgene, Cambridge, MA, USA) (8), carrying the gene encoding red fluorescent protein (RFP), or pCX-OKS-2A (Addgene), carrying genes encoding the reprogramming factors Oct3/4, Klf4 and Sox2 (OKS). The bacterial strain possessed a natural resistance to streptomycin and the plasmid encoded a gene for resistance to ampicillin. SL7207 bacteria with the appropriate plasmid were grown in standard Luria-Bertani (LB) medium in the presence of ampicillin (100 $\mu$g/ml) and streptomycin (50 $\mu$g/ml). The bacterial culture was incubated without agitation until an optical density of 0.4 was reached, measured at 600 nm. The culture was then centrifuged (10 min, 5,000 x g, 4˚C) and the pellet washed three times with 15% glycerol in phosphate-buffered saline (PBS). Bacteria were subsequently resuspended in 15% glycerol in PBS to achieve $4x10^9$ colony forming units (CFU) per ml. The final solution was divided into 1-ml aliquots and stored at -80˚C until use for oral gavage. The bacterial count was confirmed by plating serial dilutions of bacterial stock onto LB plates containing the appropriate antibiotic.

*In vivo survival of SL7207 bacteria*. In the single gavage experiment, mice were fed via gastric gavage with $10^9$ CFU SL7207-OKS or SL7207-RFP in 0.25 ml. At several different time-points (4, 8, 12, 24, 36 and 48 h) a stool sample was taken, serially diluted in PBS and plated onto LB plates containing streptomycin and ampicillin. In the multiple gavage experiment, mice were fed four times via gastric gavage every other day and the stool samples were taken 4 h after the first gavage and then every 24 h. The average number of surviving bacteria was expressed as CFU per mg of stool.

*Colitis model*. Male C57BL/6 mice (aged 12-16 weeks) were obtained from Charles River Laboratories (Prague, Czech Republic). All mice were kept in a controlled environment with a 12/12-h light/dark cycle with *ad libitum* access to water and food. Mice received either water or 2% DSS (MP Biomedicals, Solon, OH, USA; molecular weight, 36,000-50,000) for seven days *ad libitum* in drinking water starting from day 0. From day 7 DSS was changed back to water for a further three days. Body weight and stool consistency (0, normal; 1, soft-formed; 2, watery; 3, watery with blood) were monitored every day until the end of the experiment. Weight loss was expressed as a percentage of the initial weight of the animal at the beginning of the experiment. Mice were sacrificed on day 10. The animal experiments were approved by the institutional review board and Ethics Committee of Comenius University Faculty of Medicine (Bratislava, Slovakia).

*Therapeutic vs. preventive bacterial treatment*. For the therapeutic experiment, 48 male C57BL/6 mice were divided into six groups (n=8 per group) as follows: (i) DSS PBS; (ii) H₂O PBS; (iii) DSS RFP; (iv) H₂O RFP; (v) DSS OKS; and (vi) H₂O OKS. Colitis was induced according to the aforementioned protocol. On days 0, 2, 4 and 6 of the colitis experiment mice in all groups were administered 0.25 ml of the respective bacterial strain ($10^9$ CFU; groups 3-6) or 15% glycerol in PBS (groups 1 and 2) using a gastric gavage. For the preventive treatment experiment, 24 male C57BL/6 mice were divided into four groups (n=6 per group): (i) DSS RFP; (ii) H₂O RFP; (iii) DSS OKS; and (iv) H₂O OKS. On days -4, -2 and 0 of the colitis experiment mice in all groups were administered 0.25 ml of the respective bacterial strain ($10^9$ CFU) using a gastric gavage.

*Antibiotic pretreatment*. Male C57BL/6 mice (n=32) were divided into four groups (n=8 per group): (i) H₂O PBS (CTRL); (ii) DSS PBS; (iii) DSS ATB; and (iv) DSS ATB OKS (ATB indicates antibiotic treatment). Mice in groups 3 and 4 were administered metronidazole (750 mg/l) and streptomycin (1 g/l) in drinking water for four days prior to the start of the experiment (days -8 to -4). On days -4, -2 and 0 of the colitis experiment mice were administered 0.25 ml OKS bacterial strain ($10^9$ CFU; group 4) or 15% glycerol in PBS (groups 1, 2 and 3) using a gastric gavage. Colitis was induced according to the aforementioned protocol. On days -4, -3, -1, 1 and 3 of the experiment, a stool sample was taken from four mice from each group, serially diluted and plated onto LB medium containing streptomycin and ampicillin. Samples were obtained immediately prior to the first gavage (day -4), 24 h after the first, second and third gavage (days -3, -1 and 1) and 48 h after the third gavage (day 3). The average bacterial count in the stool was calculated from the number of colonies on the plates.

*Statistical analysis*. Data were analyzed using one- and two-way analysis of variance, where appropriate. The Bonferroni post hoc test was used to evaluate the differences between groups. P<0.05 was considered to indicate a statistically significant difference. The analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as the mean ± standard error of the mean.

## Results

*In vivo survival of SL7207 bacteria*. In the *in vivo* survival and passage analysis, the administered bacteria were recovered from the stool after 36 h. Fig. 1 shows the time course of the bacterial recovery from the stool following a single administration of $10^9$ CFU SL7207-OKS or SL7207-RFP in 0.25 ml
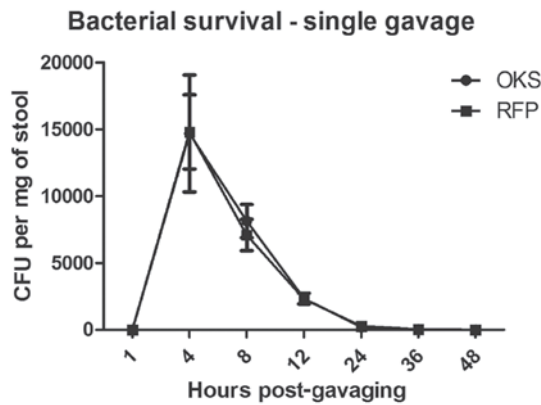
## Bacterial survival - single gavage



Figure 1. Time course of bacterial recovery from the stool following a single gavage. Peak levels of bacteria are observed after 4 h. No bacteria were present in the stool 48 h after a single gavage. CFU, colony forming units; OKS, reprogramming factors Oct3/4, Klf4 and Sox2; RFP, red fluorescent protein.

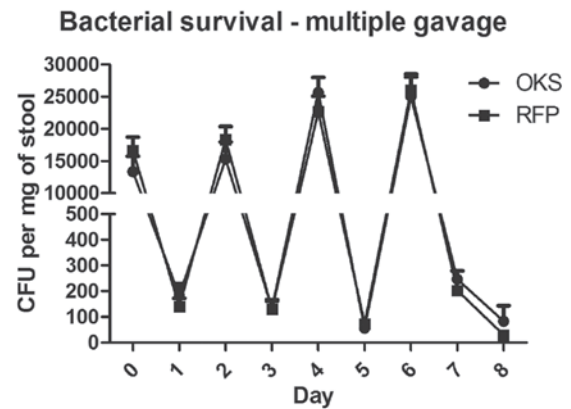## Bacterial survival - multiple gavage



Figure 2. Time course of bacterial recovery from the stool following multiple gavages. Repeated administration of bacteria slightly increased the bacterial count in the stool on the day of gavage (days 0, 2, 4 and 6). Bacteria were also detected 48 h after the last gavage (day 8). CFU, colony forming units; OKS, reprogramming factors Oct3/4, Klf4 and Sox2; RFP, red fluorescent protein.

using gastric gavage. No bacteria were present in the stool 48 h after the single gavage.

To determine whether repeated administration of the bacterial vector was likely to improve colonization of the colon, multiple gavages of the bacteria were analyzed. As shown in Fig. 2, four consecutive administrations of the bacterial vector every other day resulted in detectable bacterial counts in the stool 48 h after the final gavage (125 and 45 CFU/mg for OKS and RFP, respectively) compared with no detectable bacteria following a single gavage.

*Therapeutic vs. preventive bacterial treatment of colitis.* In the present study it was investigated whether the bacterial strain SL7207 carrying the plasmid with genes encoding three reprogramming factors (OKS) could have a positive effect on the course of DSS-induced colitis. This was analyzed using basic disease activity parameters, including weight loss, stool consistency and colon length. In the first experiment, therapeutic bacteria were administered concurrently with DSS treatment by four gastric gavages every other day, starting from day 0. The weight loss curves of all groups with colitis (DSS PBS, DSS RFP and DSS OKS) indicated a similar course of ongoing colitis in all groups, resulting in a significant weight loss at day 10 (75.8, 74.7 and 76.9%, respectively) compared with the control groups with no DSS treatment. No significant differences were found among the DSS groups throughout the course of colitis experiment (data not shown). In addition, no significant differences among the DSS groups were observed in stool consistency (mean score, 2.33, 2.11 and 2.00, respectively) and colon length (mean length, 6.7, 6.4 and 6.5 cm, respectively) on the day of sacrifice.

In the preventive bacterial treatment, bacterial vectors were administered prior to induction of colitis by three consecutive gastric gavages every other day, starting from day -4. The group treated with bacteria carrying reprogramming genes (DSS OKS) showed significantly improved weight loss compared with the group receiving control bacteria (DSS RFP) (Fig. 3). This improvement was apparent from day 6 to the end of the experiment. Similar differences between the DSS OKS and DSS RFP groups were observed for other parameters,
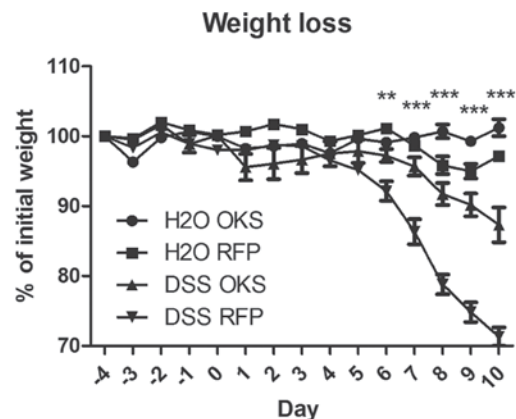
## Weight loss



Figure 3. Weight loss curve in the preventive treatment experiment. A significant improvement was observed in the DSS OKS group compared with the DSS RFP group, starting from day 6. **$P < 0.01$ and ***$P < 0.001$, DSS OKS compared with DSS RFP. OKS, reprogramming factors Oct3/4, Klf4 and Sox2; RFP, red fluorescent protein; DSS, dextran sulfate sodium.

including stool consistency (starting from day 5) (Fig. 4) and colon length (on the day of sacrifice) (Fig. 5).

*Antibiotic pretreatment.* Since the preventive approach was found to be more beneficial than the therapeutic approach, the effect of antibiotic pretreatment was then assessed. The same preventive treatment protocol was performed, consisting of three gastric gavages of bacterial vector or PBS every other day, starting from day -4, and followed by the induction of colitis by DSS. Mice in the DSS ATB and DSS ATB OKS groups received metronidazole and streptomycin in their drinking water for four days prior to the first gavage. Antibiotic pretreatment allowed the administered bacterial strain SL7207-OKS to efficiently colonize the gut of mice in the DSS ATB OKS group, as shown by plating the stool samples onto LB medium containing streptomycin and ampicillin (Fig. 6). A significant number of resistant bacteria were detected, even three days after the last gavage. No bacteria resistant to both antibiotics were observed in the stools of mice in other groups at any time-point (CTRL, DSS and DSS ATB).
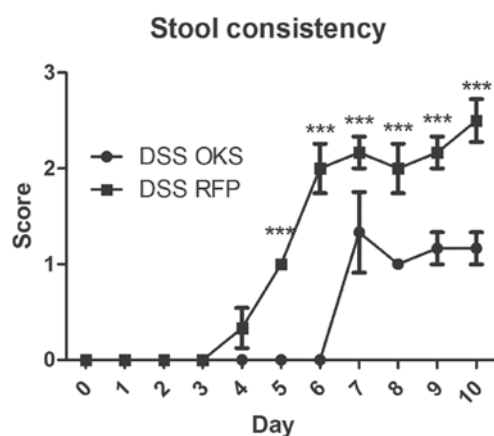
Figure 4. Stool consistency in the preventive treatment experiment. The DSS OKS group had significantly improved stool consistency compared with the DSS RFP group, starting from day 5. ***P<0.001, DSS OKS compared with DSS RFP. OKS, reprogramming factors Oct3/4, Klf4 and Sox2; RFP, red fluorescent protein; DSS, dextran sulfate sodium.



Figure 6. Time course of bacterial recovery from the stool in the antibiotic pretreatment experiment. Bacteria were administered via gavage on days -4, -2 and 0. The graph shows the data for the DSS ATB OKS group. Significant numbers of bacteria resistant to streptomycin and ampicillin were found in the stool three days after the final gavage (day 3). No bacteria were recovered from the stools of mice in other groups at any of the time-points. CFU, colony forming units; OKS, reprogramming factors Oct3/4, Klf4 and Sox2; DSS, dextran sulfate sodium; ATB, antibiotic treatment.
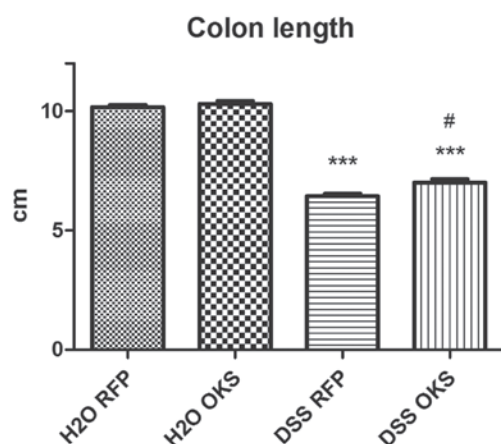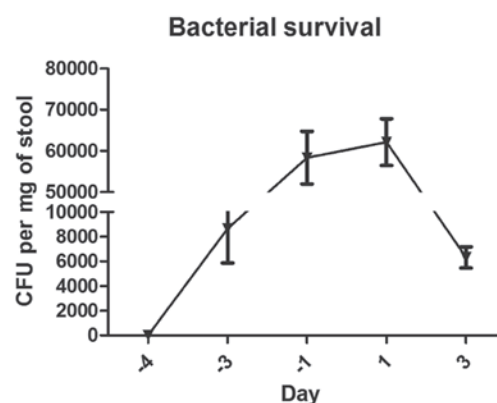


Figure 5. Colon length in the preventive treatment experiment. The groups treated with DSS showed significantly lower colon length compared with the control groups. A significant difference was found between the DSS RFP and DSS OKS groups. ***P<0.001, compared with the respective control group; #P<0.05, compared with the DSS RFP group. OKS, reprogramming factors Oct3/4, Klf4 and Sox2; RFP, red fluorescent protein; DSS, dextran sulfate sodium.
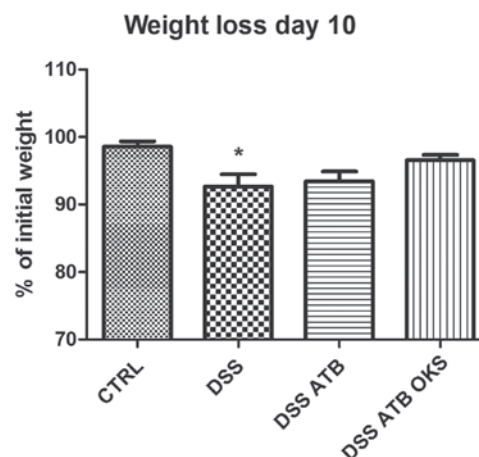


Figure 7. Weight loss in the antibiotic pretreatment experiment at day 10. The DSS group showed significant weight reduction; however, neither of the groups receiving antibiotic pretreatment (DSS ATB and DSS ATB OKS) showed significant weight reduction. *P<0.05, compared with the control group. OKS, reprogramming factors Oct3/4, Klf4 and Sox2; DSS, dextran sulfate sodium; CTRL, control; ATB, antibiotic treatment.

The DSS group with no treatment showed significant weight reduction compared with the control group at the end of the experiment (Fig. 7). Neither of the groups that received antibiotic pretreatment (DSS ATB and DSS ATB OKS) showed significant weight reduction, and the DSS ATB OKS group showed only a slight weight loss (96.5%) compared with the control group (98.6%). Furthermore, the DSS groups pretreated with antibiotics exhibited significantly improved stool consistency at the end of the experiment compared with the DSS group with no antibiotic or bacterial treatment (Fig. 8). In addition, no significant difference was observed between the DSS group receiving antibiotic and bacterial treatment (DSS ATB OKS) and the control group without colitis. The DSS and DSS ATB groups showed significantly lower colon length compared with the control group (Fig. 9). This effect, however, was not observed in the DSS group with both antibiotic and bacterial treatment. Furthermore, the DSS ATB OKS group had significantly improved colon length compared with the DSS group with no treatment.

## Discussion

The present study analyzed the effects of the Salmonella-mediated delivery of reprogramming genes into the colon and pretreatment with the antibiotics metronidazole and streptomycin on chemically induced colitis. Two gavaging protocols, therapeutic and preventive, were initially compared for the administration of the bacterial vectors. It was shown that pre-emptive bacterial treatment was superior to the therapeutic treatment in terms of the basic parameters of colitis activity. The lack of efficiency of the therapeutic approach may be due to the requirement for healthy tissue for the bacterial vectors to enter the host and release the expression plasmid into the host cells. However,
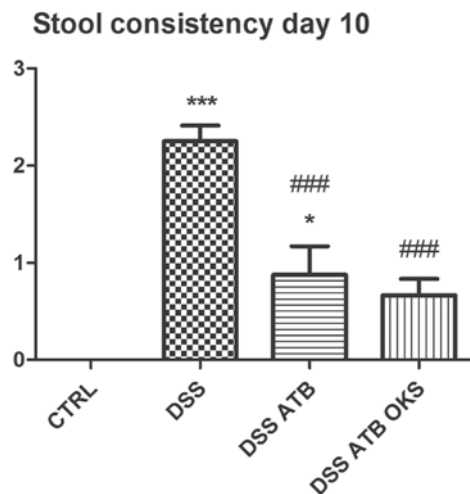
## Stool consistency day 10



Figure 8. Stool consistency in antibiotic pretreatment experiment at day 10. The DSS and DSS ATB groups exhibited significantly higher scores than the control group. The two groups with antibiotics (DSS ATB and DSS ATB OKS) had improved stool consistency compared with the DSS group. $^{*}P<0.05$ and $^{***}P<0.001$, compared with the control group; $^{###}P<0.001$, compared with the DSS group. OKS, reprogramming factors Oct3/4, Klf4 and Sox2; DSS, dextran sulfate sodium; CTRL, control; ATB, antibiotic treatment.
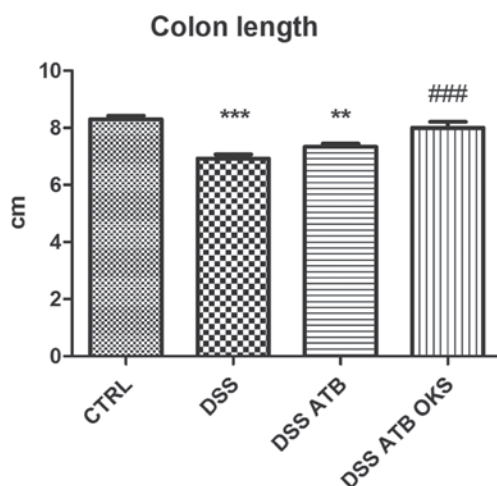
## Colon length



Figure 9. Colon length in the antibiotic pretreatment experiment. The DSS and DSS ATB groups showed significantly lower colon length than the control group. Colon length in the combination treatment group (DSS ATB OKS) was significantly higher than that in the DSS group. $^{**}P<0.01$ and $^{***}P<0.001$, compared with the control group; $^{###}P<0.001$, compared with the DSS group. OKS, reprogramming factors Oct3/4, Klf4 and Sox2; DSS, dextran sulfate sodium; CTRL, control; ATB, antibiotic treatment.

efficient delivery of the genes by SL7207 bacteria into colonic cells, even during DSS-induced colitis, has been shown previously (7,8). Assuming that reprogramming did occur in the colon, the enhanced beneficial effects of the preventive gene delivery of reprogramming factors are in accordance with the preliminary data published on stem cell therapy that indicate that a pre-emptive stem cell therapy may lead to a protective phenotype later when the damaging event occurs (17,18).

Although there has been some investigation into the differentiation of iPS cells into intestinal cells (19,20), little is known regarding the specific conditions that are required for the efficient reprogramming of intestinal cells into a pluripotent state.

To the best of our knowledge, the generation of iPS cells from intestinal somatic cells has not yet been reported. Given that the degeneration of intestinal mucosal cells has an essential role in the pathogenesis of IBD, therapy based on their reprogramming provides a rationale for the protection of these cells against the harmful effects and thus prevention of damage in IBD. It appears that the reprogramming processes could occur in the intestine using *in vivo* gene delivery, despite the fact that low transfection and reprogramming efficiencies are expected.

As a follow-on to our initial experiment, the preventive administration protocol was employed and the effect of antibiotic pretreatment was investigated. It was demonstrated that the administered bacteria were present only in the stool of mice with colitis that received both antibiotics and SL7207-OKS (the DSS ATB OKS group). Notably, significant amounts of the administered bacteria were detected in the stool even 72 h after the last gavage. This indicates that antibiotic pretreatment markedly improved the exposure time of the bacterial vector to the colonic mucosa, thus increasing the efficiency of gene transfer. The actual invasion of SL7207 bacteria into the colon tissue was shown in our previous study (8).

Although neither antibiotics nor bacterial reprogramming significantly altered the weight of the animals in comparison with colitis control mice, the trend towards reducing weight loss was clear. Colon length, as a measure of colitis activity, was significantly improved by the combination of antibiotic pretreatment and bacteria-mediated reprogramming compared with the control colitis group. Similar results were found for stool consistency, another measure of colitis activity. Antibiotics alone and in combination with reprogramming genes led to an improved stool consistency score, with the lowest score observed in the combination group. These results indicate that antibiotic pretreatment may enhance bacterial gene delivery into the colon. Furthermore, *in vivo* reprogramming of colon cells appears to have a preventive effect on the course of colitis.

It has been shown that gut inflammation stimulates horizontal gene transfer between the members of the gut microbial community, therefore spreading the determinants of fitness, virulence and antibiotic resistance (21). The disruption of microbial flora composition observed following antibiotic treatment may favor several specific bacterial species that contain the necessary factors, which are further spread across the community if the inflammation takes place. This may explain the positive effect of antibiotic pretreatment on the course of DSS colitis.

The present study does not provide direct evidence of the expression of the reprogramming factors, nor is there evidence of the effect of antibiotic pretreatment on the microbial flora in the gut. In addition, the study lacks histological and biochemical quantification of the inflammation. These limitations make this study a preliminary investigation. However, this experiment should be viewed as a first attempt to use bacterial infection-mediated gene delivery in the context of a microflora modified by antibiotics. It also shows for the first time, to the best of our knowledge, the potential for therapeutic treatments for IBD based on reprogramming the cells *in vivo*. The data in the present study may therefore be used for further experiments investigating the mechanism of *in vivo* reprogramming in IBD.

## Acknowledgements

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