IMPLICATION OF SHORT CHAIN FATTY ACIDS PRODUCED BY THE GUT MICROBIOTA IN THE ACTIVATION OF G-PROTEIN COUPLED RECEPTOR 43 AND THEIR POSSIBLE ROLE TO CONTROL PROLIFERATION OF CANCER CELLS

A) Introduction

The gut microbiota is involved in important metabolic and immune functions with a marked effect on energy balance and the nutritional and health status of the host (Neish, 2009). Recent evidences, primarily from investigations in animal models, suggest that the gut microbiota could play a crucial role in the regulation of host energy metabolism, insulin sensitivity and inflammatory response (Bäckhed et al., 2010, Delzenne & Cani, 2011; Delzenne et al., 2011). The gut microbiota digests complex dietary carbohydrates to monosaccharides and short chain fatty acids (SCFA) such as acetate, propionate and butyrate. As end products of bacterial fermentation, SCFA represent an energy source and may also act in the gut as signalling molecules. The aim of the project is to study novel biological systems prone to relate microbial and host metabolism: the G-protein coupled receptors GPCR41&43 (also named free fatty acid receptors FFAR 3& 2) which bind the SCFA produced through microbial fermentation of carbohydrates. Propionate and acetate are ligands for these GPCRs that are expressed by gut epithelial and enteroendocrine cells, but also by adipocytes (Xiong et al., 2004; Brown et al., 2003; Milligan et al., 2009). Previous works showed that acetate and propionate inhibit isoproterenol-induced lipolysis in 3T3-L1 adipocytes (Hong et al., 2005) and also that the activation of GPR43 by acetate *in vivo* results in reduced plasma levels of free fatty acids, showing the inhibition of lipolysis (Ge et al., 2008). Acetate and propionate also seemed to exhibit anti-inflammatory properties in human monocytes and mice model colitis (Cox et al., 2009; Maslowski et al., 2009)

The initial aim of the visit was to analyse if SCFA resulting from gut microbiota fermentations are able to regulate the expression of GPR43 in the adipose tissue. We decided to perform preliminary studies in BaF3 cells, a good model to study the implication of SCFA on differentiation/proliferation in order to assess *in vitro* the possible role of GPR43 in the control of proliferation in presence of propionate.

B) Material and Methods

B.1. Cell cultures and chemicals used

Murine proB BaF3 cells, containing ectopic expression of Bcr-Abl, were selected as cancer cells because of their high levels of GPR43 expression (Maslowski et al., 2009). The Philadelphia (Ph) translocation t(9;22)(q34;q11) generates the *bcr-abl* fusion gene characteristic for chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia-Ph +(Deininger et al., 2000). Bcr/Abl is a chimeric oncogene that can cause both acute and chronic human leukemias. Bcr-abl encodes a constitutively active cytoplasmic tyrosine kinase that is necessary and sufficient to induce and maintain leukemic transformation but the mechanisms of transformation are largely unknown. (Daley et al., 1990; Lugo et al., 2000; Huettner et al; 2000).

Cells were maintained in RPMI1640 medium(PAA clone) supplemented with 10% fetal calf serum, streptomycin 100µg/ml, penicillin 100 IU/ml, and 1% of non-essential amino acids solution (Gibco) at 37°C in humidified 5% CO2 (Beck *et al.*, 2011).

BaF3 cells were incubated in presence of propionate. The agonist of GPR43 phenylacetamide [4-chloro-α (1-methylethyl)-N-2-thyazolyl- benzeneacetamide] and the inhibitor of cAMP phosphodiesterase, the 3-isobutyl-1-methylxanthine (IBMX) were also employed.

Mouse Lewis lung carcinoma (LLC) cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, streptomycin 100µg/ml and penicillin 100 IU/ml (Gibco) at 37°C in humidified 5% CO_2.

B2. Cell proliferation

MTT Assay: Cells were incubated in a 96-well plate in presence of different chemicals (propionate, phenylacetamide, IBMX) at different concentrations and at different times. Cell proliferation *in vitro* was measured by the cleavage of the yellow thiazolyl blue tetrazolium bromide (MTT) to purple formazan crystals by metabolically active cells. 10µl of MTT 5mg/ml was added to each well. After centrifugation, 50µl of medium was removed and formazan crystals were dissolved by addition of 100µl

isopropanol-HCl 0.04M. The absorbance of the formazan was measured at 570nm, from which was subtracted a background value measured at 650nm. The results are presented as percentage of cell proliferation in control conditions.

All the experiments were performed at least in three different independent experiments by triplicate.

B.3. Cells GPR43 mRNA analysis

Total RNA was isolated from cells using the TriPure isolation reagent kit (Roche Diagnostics). cDNA was prepared by reverse transcription of 1 µg total RNA using the Kit Reverse transcription System (Promega). Real-time polymerase chain reactions (RT-qPCR) were performed with the StepOne Plus real time PCR system and software (Applied Biosystems) using SYBR-Green for detection. All samples were run in duplicate in a single 96-well reaction plate. The sequences of the targeted gen GPR43 and Ribosomal protein L19 (RPL19) RNA, chosen as housekeeping gen, are described elsewhere (Dewulf et al., 2011).

B.4. Western Blot analysis

Protein expression of GPR43 was examined by western blot analysis (WB) in adipose tissue, brain, spleen, muscle and in BaF3 and LLC cells. Appropriate protein amounts (25 µg) of different tissues and different cell culture lines were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (10% SDS-PAGE). After electrophoresis (100V, ~2 hours), proteins were transferred to nitrocellulose membranes. The membranes were blocked for 2.5 h in Tris-buffered saline tween 20 (TTBS) (pH 7.4) containing 5% powdered milk protein and then incubated overnight at 4ºC with a dilution of the primary antibody. β-actin was used as control. The membranes were washed six times with TTBS and incubated for 60 min with a dilution of secondary antibody. Protein bands were then detected by chemiluminiscence, using Super Signal West Fento and Super Signal West Pico kits from Thermo.

C) Results

C.1. Propionate decreases BaF3 cell proliferation *in vitro*

Preliminary results showed that incubation of BaF3 cells in presence of propionate was characterized by a time- and dose-dependent decrease in the proliferation, as evidenced by a MTT assay. (**Figure 1)**

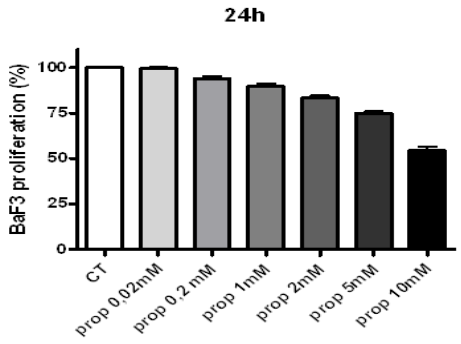

Figure 1: BaF3 proliferation *in vitro* (%) after 24h of incubation in presence/absence of propionate

C.2. GPR43 activation decreases the proliferation of BaF3 cells *in vitro*

Propionate is one of the most potent known ligand of GPR43. We hypothesized that the anti-proliferative effect of propionate on BaF3 cells could be mediated by GPR43, so we decided to incubate BaF3 cells in presence of phenylacetamide, a potent synthetic agonist of GPR43 recently designed (Lee et al., 2008; Wang et al., 2010;) to test if GPR43 was sufficient to reduce the proliferation of these cancer cells. We observed that this agonist reduced BaF3 proliferation in a time- and dose-dependent way (MTT assay), so GPR43 activation seems to be effective and the results showed a reduction in BaF3 cell growth.

It is well known that GPR43 displays a dual coupling through Gi and Gq protein families, as shown in CHO cells transfected with mouse GPR43 and in HEK293 transfected with human GPR43 (Brown et al, 2003; Lepoul et al; 2003). We focused in the study of the possible role of Gi pathway in the activation of propionate. For this purpose we used the IBMX, an inhibitor of cAMP phosphodiesterase that blunts the activation of Gi pathway. BaF3 cells were incubated 1 hour in absence or presence of 3-

isobutyl-1-methylxanthine (IBMX) 316 µM, and then incubated in absence or presence of phenylacetamide or propionate for 24h. IBMX reduced the anti-proliferative effect of phenylacetamide and decreased the anti-proliferative properties of propionate at high doses (10mM) (**Figure 2**). These results suggest that a decrease in cAMP level (inhibition of Gi pathway) might be implicated in the reduced cell proliferation mediated by propionate and phenylacetamide but further experiments should be performed to confirm these data.

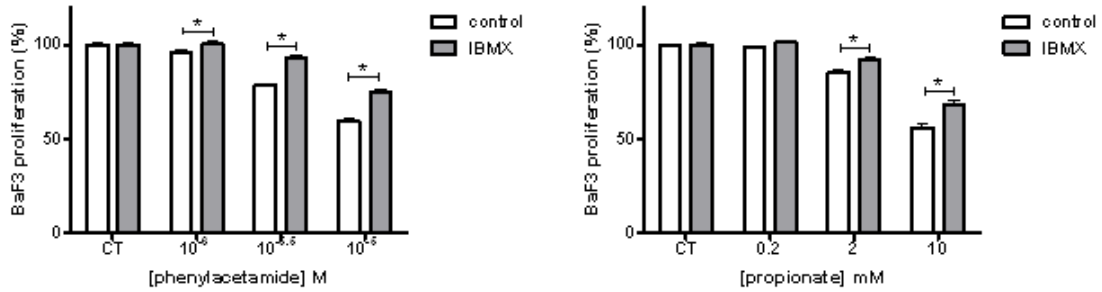

Figure 2: BaF3 proliferation *in vitro* (%) after 1h incubation of IBMX. These cells were then incubated in the absence/presence/absence of phenylacetamide or propionate for 24 h (MTT assay). * p< 0.05.vs control, two ways ANOVA with Bonferroni post-hoc test.

C.3. GPR43 mRNA levels are higher in BaF3 than in LLC cell lines

The mRNA level of GPR43 measured by RT-qPCR was thousand-fold lower in LLC cells than in BaF3 cells (**Figure 3**). These results were expected because preliminary assays performed showed that this cell line incubated in presence or absence of propionate was less sensitive towards the anti-proliferative effect of propionate (MTT assay).

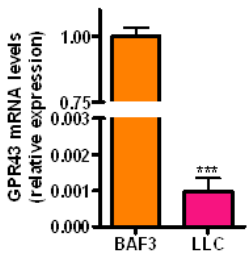

Figure 3: GPR43 mRNA levels in BaF3 and LLC cells. *** p<0.05 vs. BaF3, Student's t-test.

C.4. Western Blot analysis

According to UniProt and ensemble website the GPR43 has a calculated weight of 37 kDa. However, since this protein is also glycosylated, the actual band is detected at a higher molecular weight (~43 kDa) if we followed instructions recommended by the supplier.

The first WB experiments performed were not satisfactory to detect GPR43 because the gels showed different bands that are not complementary to GPR43. The use of β-actin as a positive control showed that the protocol performed was working (data not shown) but the primary antibody used against GPR43 didn't seem to be specific because in expected positive tissues for the expression of GPR43 protein -like adipose tissue or spleen- we did not detect neither a band at 37 kDa nor at 43kDa (**Figure 4).** Regarding cell lines, we detected neither a specific band for GPR43 in BaF3 cells that is established they have higher expression of GPR43 protein. Further experiments will be performed using different protocols or different antibodies.

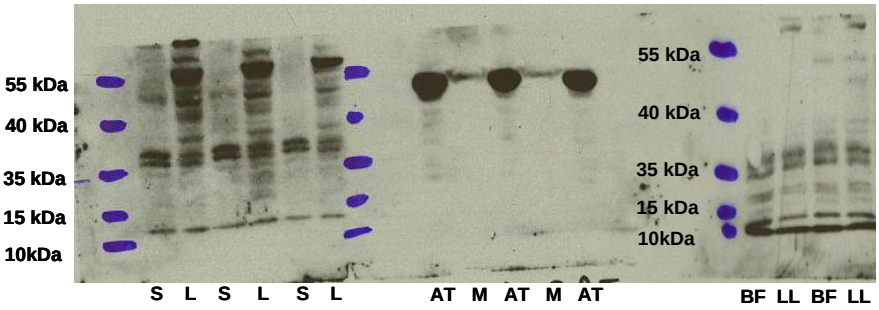

Figure 4: Western blot gels obtained in spleen, liver; adipose tissue, muscle, BaF3 and LLC cells. S= spleen, L= liver, AT= adipose tissue, M= muscle, BF=BaF3 cells; LL=Lewis lung carcinoma cells

D. Future collaboration with host institution

My participation in the Exchange Grant from the ENGHIR programme allowed me to acquire direct experience with cell cultures and the western blot techniques. Moreover the grant promoted new ideas to be exchanged and provided me the opportunity to start a new postdoctoral project next year with the host receptor.

E. References

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