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Ffar2 expression regulates leukaemic cell growth *in vivo*

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Background: Activation of free fatty acid receptor 2 (FFAR2) by microbiota-derived metabolites (e.g., propionate) reduces leukaemic cell proliferation *in vitro*. This study aims to test whether *Ffar2* expression *per se* also influences leukaemia cell growth *in vivo*.

Methods: Bcr-Abl-expressing BaF cells were used as a leukaemia model and the role of *Ffar2* was evaluated in Balb/c mice after lentiviral shRNA transduction.

Results: Our data formally establish that reduced leukaemic cell proliferation is associated with increased *Ffar2* expression *in vivo* and *in vitro*. Going beyond association, we point out that decreasing *Ffar2* expression fosters cancer cell growth *in vitro* and *in vivo*.

Conclusions: Our data demonstrate the role of *Ffar2* in the control of leukaemic cell proliferation *in vivo* and indicate that a modulation of *Ffar2* expression through nutritional tools or pharmacological agents may constitute an attractive therapeutic approach to tackle leukaemia progression in humans.

Short-chain fatty acids (SCFA), such as acetate, propionate and butyrate, are microbial byproducts of fermentation that are involved in the gut microbiota–host crosstalk through several pathways (Bindels *et al*, 2013; Canfora *et al*, 2015). Free fatty acid receptor 2 (FFAR2), also known as GPR43 or FFA2, is a G-protein coupled receptor that binds propionate. Its activation reduces leukaemic cell proliferation *in vitro* (Bindels *et al*, 2013). In a mouse model of leukaemia consisting in the transplantation of Bcr-Abl-transfected BaF cells, feeding inulin-type fructans (ITF), a non-digestible fermentable fiber changing the gut microbiota, leads to increased portal propionate levels and reduced hepatic leukaemic cell proliferation (Bindels *et al*, 2012). We and others have documented that beside the canonical histone deacetylase pathway, the FFAR2 pathway could also mediate the anti-proliferative effect of SCFA such as propionate (Tang *et al*, 2011; Bindels *et al*, 2012; Shi *et al*, 2014). While overexpression of *Ffar2* induces apoptosis (Tang *et al*, 2011; Shi *et al*, 2014), our data reveal that its knocking down increases the growth of leukaemic cells *in vivo*.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), except CMTB [4-chloro-α-(1-methylethyl)-*N*-2-thiazolyl-benzeneacetamide] (Ambinter, Paris, France) and imatinib mesylate (Cayman Chemical, Tallinn, Estonia).

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Cell culture. BaF cells transfected with Bcr-Abl were a kind gift of Dr K Bhalla (MCG Cancer Center, Medical College of Georgia, Augusta, GA, USA). Cell maintenance, MTT (thiazolyl blue tetrazolium bromide) cell growth assays and counting of intact BaF cells are described in Bindels *et al* (2012). U937 and HL-60 cells were maintained in RPMI medium 1640 or Iscove's Modified Dulbecco's Medium, respectively, each medium being supplemented with 10% foetal bovine serum (PAA clone, PAA, Pasching, Austria) and streptomycin $100\,\mu g\,ml^{-1}$ and penicillin $100\,IU\,ml^{-1}$ (Gibco, Inchinnan, Scotland). For mRNA analysis, BaF cells were incubated with chemicals or the corresponding vehicle as indicated in figure legends. After incubation, the cells were harvested, washed with PBS, and frozen in Tripure Isolation Reagent (Roche Diagnostics, Penzberg, Germany). To silence *Ffar2* expression, BaF cells were infected with lentiviral particles containing short hairpin RNA (shRNA) sequences. Briefly, pLKO 0.1 lentiviral vectors expressing control shRNA (Addgene 1864, Cambridge, UK) and FFAR2 shRNA (clone TRCN0000027562; Sigma-Aldrich) were used to generate viral particles with the lentiviral packaging mix (Sigma-Aldrich) according to the manufacturer's instructions. After 24 and 48 h, viral supernatant was harvested, titrated, and used to infect BaF cells. Puromycin selection ($10\,\mu g\,ml^{-1}$) was applied after lentiviral infection.

Animals. In a first series of experiments, BaF cells (1×10^6 cells in 0.1 ml saline) or an equal volume of saline was injected into the tail vein of anesthetised 5-week-old female BALB/c mice (Charles River, France). One day after inoculation, half of the mice that were transplanted with BaF cells received 0.2 g per day of ITF (Orafti p95, Beneo-Orafti, Oreye, Belgium) in their drinking water for 12 days. In a second series of experiments, 10-week-old male C57BL6/J mice (Charles River, France) received or not 0.2 g per day of ITF for 4 weeks in the drinking water. In a third series of experiments, BaF-shCT or BaF-shFFAR2 (1×10^6 cells in 0.1 ml saline) or an equal volume of saline was injected into the tail vein of anesthetised 5-week-old female BALB/c mice. Mice were necropsied 13 days after inoculation. White blood cell count was performed on a MS9-3 Hematology Analyzer (Melet Schloesing Laboratoires, Osny, France).

Mice were housed in a 12 h light/dark cycle. Mice were necropsied under anesthesia. Blood samples and organs were harvested and stored at $-80\,^{\circ}C$. Mouse housing and all *in vivo* experiments were approved by the Ethics Committee of the Université catholique de Louvain (Agreement LA 1230314).

mRNA and western blot analyses. mRNA analyses and western Blot of STAT3 were performed as previously described (Bindels *et al*, 2016; Cacace *et al*, 2016). Amplification product size and absence of genomic DNA contamination were checked on agarose gel for a subset of samples (Supplementary Figure S1). Primer sequences are indicated in Supplementary Table S1.

Statistical analysis. Results are expressed as mean ± s.e.m. Student *t*-test was used to compare two groups and one-way ANOVA with Dunnett's post-tests to compare three or more groups. Two-way ANOVA with Bonferroni post-tests was used to examine the influence of two categorical variables. $P<0.05$ was considered statistically significant (Graph-Pad Prism Software, San Diego, CA, USA).

RESULTS

We previously showed that increasing portal levels of propionate by the administration of ITF leads to decreased leukaemic cell accumulation in mouse liver, as evidenced by a decreased Bcr-Abl expression (Figure 1A) (Bindels *et al*, 2012). Interestingly, it was associated with an increased *Ffar2* expression in the liver of ITF-fed leukaemic mice (Figure 1B). Induction of *Ffar2* expression was likely restricted to the remaining BaF cells. Indeed, we found that *Ffar2* is 70-fold more expressed in BaF cells than in the mouse liver (BaF cells: 1.000 ± 0.059, liver: 0.015 ± 0.003, $n=3$), and the administration of ITF to healthy mice for 4 weeks did not influence hepatic *Ffar2* expression (Figure 1C).

We further analysed this phenomenon *in vitro* in BaF cells, where propionate simultaneously reduced cell proliferation and increased *Ffar2* expression in a dose-dependent manner (Figure 2A and B). Reduced cell proliferation and increased *Ffar2* expression were also observed when mimicking the action of propionate on FFAR2 and histone deacetylases using CMTB, a FFAR2 synthetic agonist (Wang *et al*, 2010), and trichostatin A, an inhibitor of histone deacetylases (Charrier *et al*, 2006) (Figure 2C–F). Thus, both pathways could be involved in the modulation of *Ffar2* expression by propionate. Imatinib, a tyrosine kinase inhibitor targeting Bcr-Abl (Druker, 2008), also reduced cell growth and increased *Ffar2* expression (Figure 2G and H), expanding the concept of an association between cell proliferation and *Ffar2* expression beyond propionate-dependent pathways. Further mechanistic studies using actinomycin D, an inhibitor of mRNA synthesis, revealed that CMTB regulates *Ffar2* expression at the transcription level independently of an activation of STAT3 (Supplementary Figure S2). Incubation of two human leukaemic cell lines (HL-60 and U937) in the presence of trichostatin A and doxorubicin revealed a similar association between cell proliferation and induction of *Ffar2* expression (Supplementary Figure S3), allowing to extend the relevance of our finding beyond one mouse cell line.

We then sought to explore the impact of a modulation of *Ffar2* expression on cancer cell proliferation *in vivo*. As overexpression of *Ffar2* was previously shown to induce apoptosis in HEK293T cells (Shi *et al*, 2014), we rather investigated the impact of its knocking down. We used shRNA in a 13-day *in vivo* experiment. Transduction of BaF cells with a shRNA targeting FFAR2

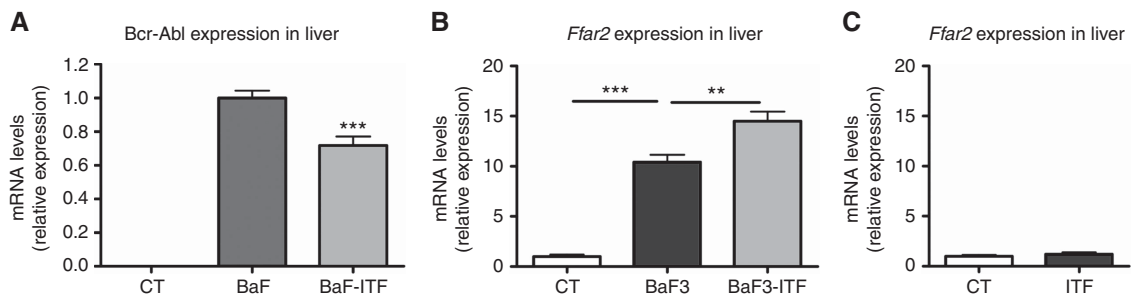

Figure 1. Reduced cell proliferation is associated with increased *Ffar2* expression *in vivo*. Bcr-Abl and *Ffar2* expression in the liver of BaF-transplanted mice with or without ITF (**A**, **B**). *Ffar2* expression in the liver of mice with or without ITF (**C**). CT: control mice. $^{**}P<0.01$ and $^{***}P<0.001$ *vs* BaF, $n=8$. Figure 1A published in Bindels *et al*, 2012.

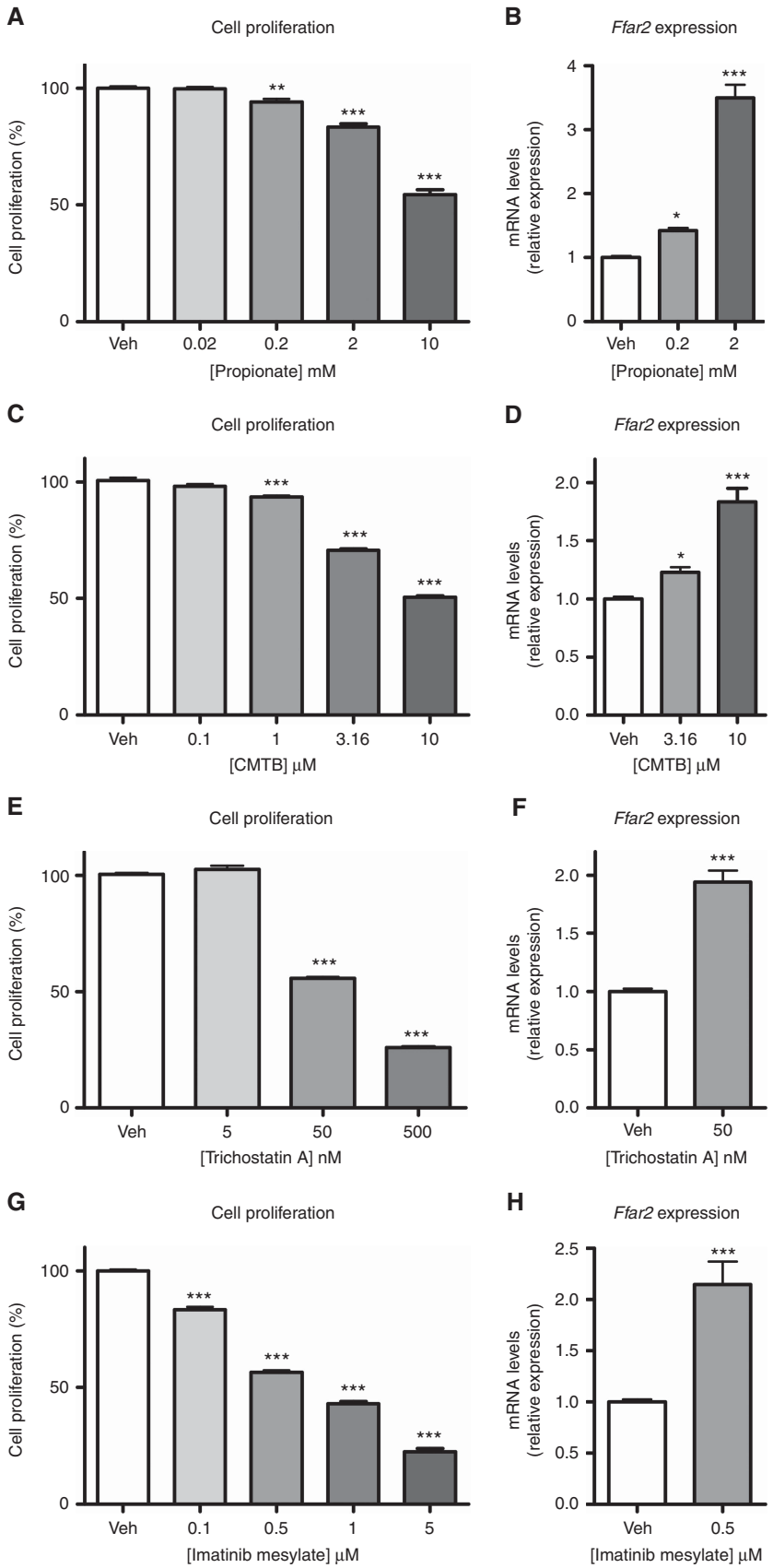

Figure 2. Reduced cell proliferation is associated with increased *Ffar2* expression *in vitro*. Cell proliferation and *Ffar2* expression after 24 h incubation in presence of propionate (**A**, **B**), CMTB (**C**, **D**), trichostatin A (**E**, **F**) and imatinib mesylate (**G**, **H**). Results of three independent experiments performed at least in triplicate are shown. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs* vehicle (Veh).

(shFFAR2) reduced target expression by 90% (Figure 3A) and increased cell proliferation (Figure 3B). After *in vivo* transplantation, compared to BaF-shCT cells, BaF-shFFAR2 cells were found at higher levels in the blood, spleen, lungs, subcutaneous adipose tissue, but not in the liver (Figure 3C–G). In the liver, *Ffar2* expression was increased in mice transplanted with BaF-shCT, but not in mice transplanted with BaF-shFFAR2 cells (Figure 3H), further indicating that the increased hepatic expression of *Ffar2* observed in our model is due to the expression of *Ffar2* in BaF cells and not to changes of the expression of *Ffar2* in hepatic cells.

DISCUSSION

Our data formally establish that reduced leukaemic cell proliferation is associated with increased *Ffar2* expression *in vivo* and *in vitro*. Furthermore, we show that knocking down *Ffar2* expression directly promotes cancer cell spreading *in vivo*, at least in the serum, lungs, spleen and adipose tissue of mice. A relationship between cancer cell proliferation and expression of *Ffar2* has been recently reported: loss of *Ffar2* expression fosters colon carcinogenesis (Pan *et al*, 2016; Sivaprakasam *et al*, 2016) whereas overexpression of *Ffar2* decreases cell proliferation (Tang *et al*, 2011; Shi *et al*, 2014). However, the mechanism of action of FFAR2 on cell growth remains unclear. We believe that the cellular model we generated here could contribute to the future elucidation of the underlying molecular pathways.

Regulation of murine *Ffar2* expression is not well understood. Senga *et al* (2003) showed that murine *Ffar2* expression in M1 leukaemia cells is associated with cell differentiation and is under the control of STAT3. Murine *Ffar2* expression also positively correlates with the expression of differentiation factors in the adipose tissue (Dewulf *et al*, 2011). It was therefore tempting to link *Ffar2* expression to cell differentiation in BaF cells. However, mRNA expression of lymphocyte B differentiation markers (early B cell factor 1 and CD19) in BaF cells was not detected after 24 h or 72 h of incubation in the presence of propionate (10 mM) or CMTB (10 μM). In BaF cells, the increased murine *Ffar2* expression occurred independently of STAT3 activation, suggesting the existence of cell type-dependent mechanisms involved in the regulation of *Ffar2*.

In the context of leukaemia treatment, our data revealed that ITF feeding to BaF-transplanted mice increases *Ffar2* expression specifically in BaF cells residing in the liver. However, it remains to be determined whether the modulation of *Ffar2* expression by ITF (likely through the production of propionate) constitutes one of the pathways by which ITF reduces leukaemic cell proliferation. Beside specific nutritional tools, pharmacological agents aiming at increasing the expression of *Ffar2* in leukaemic cells could

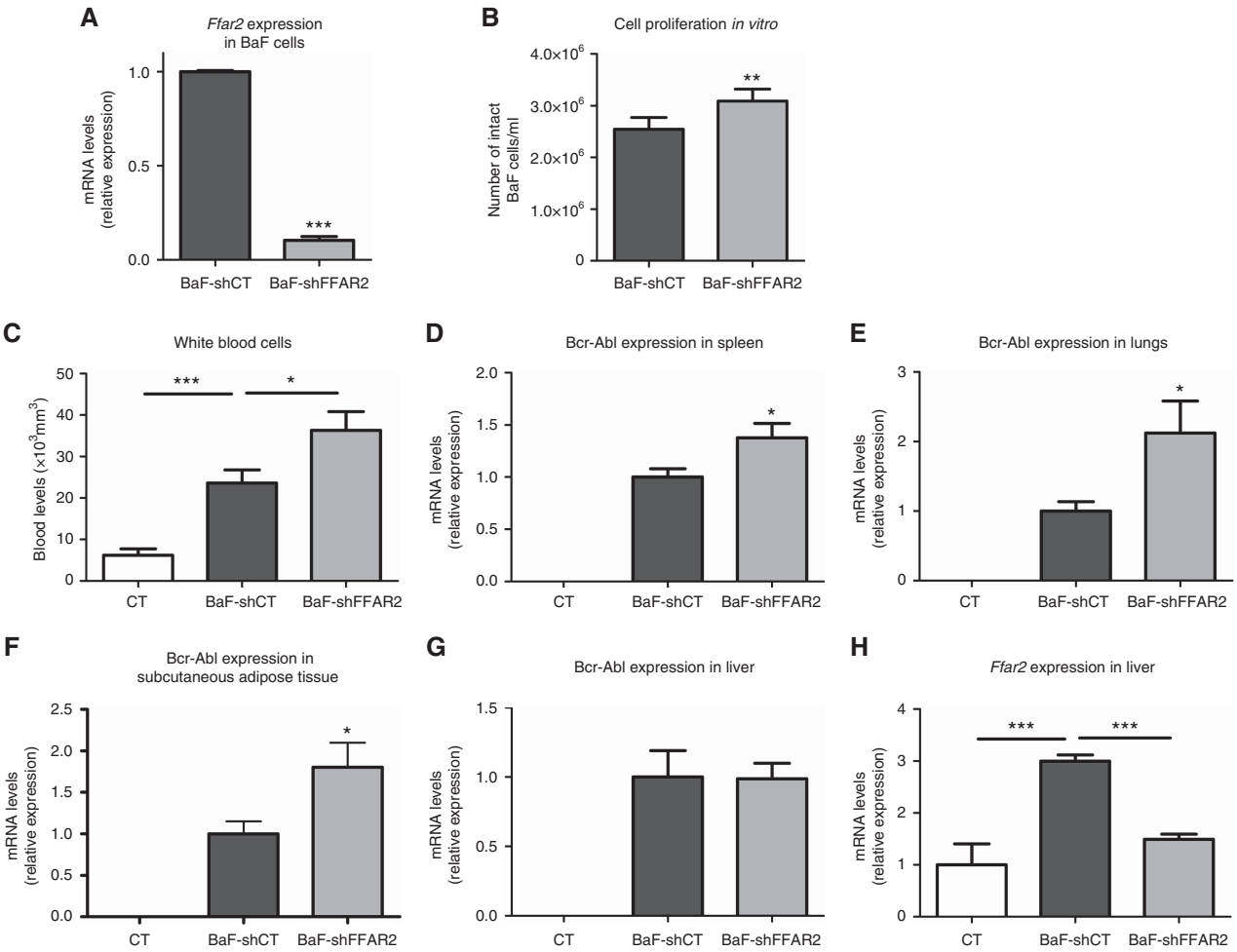

Figure 3. Decreasing *Ffar2* expression increases leukaemic cell accumulation *in vivo*. *Ffar2* expression in BaF cells *in vitro* (five independent experiments performed in triplicate) (**A**). Number of intact BaF cells per ml 48 h after incubation (four independent experiments) (**B**). White blood cell count (**C**). Bcr-Abl expression in spleen (**D**), lungs (**E**), subcutaneous adipose tissue (**F**) and liver (**G**). *Ffar2* expression in liver (**H**). For **C**–**H**, CT: control mice ($n=4$), BaF-shCT: mice transplanted with BaF cells transduced with a control shRNA ($n=8$), BaF-shFFAR2: mice transplanted with BaF cells transduced with a shRNA targeting FFAR2 ($n=9$). *$P<0.05$, **$P<0.01$, ***$P<0.001$ *vs* BaF-shCT. Paired *t*-test for **B**.

constitute an alternative approach to control leukaemic cell proliferation. Ang *et al* (2015) have recently shown that basal human *Ffar2* expression is mainly under the control of x-box binding protein 1 (XBP1). However, one should avoid to increase *Ffar2* expression through activation of the XBP1 pathway, as inhibiting this pathway has been recently proposed as a promising therapeutic approach for acute myeloid leukaemia (Sun *et al*, 2016). Ang *et al* (2015) also suggested that several transcription factors may be involved in upregulating the gene under the appropriate external stimuli. Therefore, these alternative pathways need to be considered to increase human *Ffar2* expression pharmacologically.

To conclude, our findings expand and clarify the current knowledge on the role and the therapeutic interest of FFAR2. Our data highlight that reduced cell proliferation is associated with increased *Ffar2* expression *in vitro* and *in vivo*, and that modulation of *Ffar2* expression regulates leukaemic cell growth *in vitro* and *in vivo*. Nutritional tools or pharmaceuticals targeting *Ffar2* expression or activity might thus constitute an attractive therapeutic approach to tackle leukaemia progression in humans.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)