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Src family kinase inhibitor PP2 accelerates differentiation in human intestinal epithelial cells

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ABSTRACT

The proto-oncogene Src is an important protein tyrosine kinase involved in signaling pathways that control cell adhesion, growth, migration and survival. Here, we investigated the involvement of Src family kinases (SFKs) in human intestinal cell differentiation. We first observed that Src activity peaked in early stages of Caco-2/15 cell differentiation. Inhibition of SFKs with PP2, a selective SFK inhibitor, accelerated the overall differentiation program. Interestingly, all polarization and terminal differentiation markers tested, including sucrase-isomaltase, lactase-phlorizin hydrolase and E and Li-cadherins were found to be significantly up-regulated after only 3 days of treatment in the newly differentiating cells. Further investigation of the effects of PP2 revealed a significant up-regulation of the two main intestinal epithelial cell-specific transcription factors Cdx2 and HNF1α and a reduction of polycomb PRC2-related epigenetic repressing activity as measured by a decrease in H3K27me3, two events closely related to the control of cell terminal differentiation in the intestine. Taken together, these data suggest that SFKs play a key role in the control of intestinal epithelial cell terminal differentiation.

1. Introduction

Src family kinases (SFKs) are membrane-associated non-receptor protein tyrosine kinases that play pivotal roles in regulating various cellular processes including proliferation, adhesion, migration and survival [1]. Currently eight SFK members have been identified in mammals, namely Src, Fyn, Yes, Lyn, Lck, Hck, Fgr and Blk. Among these, Src, Fyn and Yes are ubiquitously expressed, whereas the others are expressed mainly in the immune system [2]. SFKs are regulated by growth factors, cytokines, cell adhesion and antigen receptor activation [1,3]. Src is generally maintained in an inactive conformation by phosphorylation at ^{527}Tyr. Activation requires the dephosphorylation of this residue by phosphatases which leads to intramolecular autophosphorylation at ^{416}Tyr, promoting kinase activity [4]. Src signaling has been shown to play an important role in the regulation of enterocytic anoikis [5], migration and the oxidative stress response in intestinal epithelial cells [6]. In colon cancer, Src has been associated with unregulated proliferation and tumor progression [6,7]. Src functions are also associated with the development of metastasis. Indeed, Src is a critical regulator of migration and $Src^{-/-}$ cells are deficient in this process [8]. Trevino et al. have reported that the inhibition of Src expression and activity inhibits tumor progression and metastasis of human pancreatic adenocarcinoma cells in an orthotopic nude mouse model [9]. Src kinase was also shown to be essential for osteoclast activation and osteoblast inhibition [10]. Recent studies have reported that Src kinase plays a positive role in osteoclast differentiation [11]. SFK members have also been implicated in the differentiation of other cell types such as T cells [12], myoblasts [13] and chondrocytes [14].

PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-d]pyrimidine) has been identified as a potent and selective inhibitor of the Src family of protein tyrosine kinases. It was originally used as a treatment for patients with chronic myeloid leukemia [15]. Recent studies have shown that PP2 efficiently inhibits the proliferation and growth of cervical cancer cell lines [16] and it has been reported that PP2 stimulates hormonal differentiation of human trophoblast cells [17,18]. Furthermore, PP2 treatment has been shown to strongly promote differentiation of primary mouse osteoblasts isolated from mouse calvaria [19].

The epithelium of the small intestine is characterized by rapid and constant renewal. This process involves highly hierarchized proliferation and differentiation processes all occurring in the crypt in order to generate the functional cells that will migrate onto the villus [20–22]. The stem, transient amplifying and terminal differentiating cells are located in the lower, middle and upper thirds of the glands, respectively. Cell migration from the "stem cell zone" to the "transit amplifying zone" triggers the irreversible process

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of cell determination. However, committed cells need to divide several times in this compartment before undergoing terminal differentiation in the upper gland and the mechanisms responsible for the transient repression of absorptive cell differentiation in the transit amplifying zone remain incompletely understood. The Caco-2/15 cell line has proven to be an invaluable model for studying absorptive cell differentiation. These cells are unique in that upon reaching confluence they spontaneously initiate a differentiation program that is complete by 20–30 days post-confluence where cells are non-proliferative and display a highly polarized morphology as well as the expression of specific biochemical markers that are comparable to those of the enterocytes of the villus [23–26]. This model has been one of the most used human intestinal cell models [27]. We recently used Caco-2/15 cells to shown that epigenetic modulation *via* the polycomb PRC2 complex, which catalyzes the trimethylation of lysine 27 in histone 3 (H3K27me3) to silent gene expression, can participate in the repression of enterocytic terminal differentiation in the transit amplifying cell population [28].

In the present study, we evaluated the role of Src kinase on enterocyte differentiation by treating early differentiating Caco-2/15 cells with the SFK inhibitor PP2. The impact of SFK inhibition on the first steps of differentiation was evaluated on both cell polarity and terminal differentiation markers.

2. Materials and methods

2.1. Cell culture

The Caco-2/15 cell line, provided by Dr. Quaroni (Cornell University, Ithaca, NY), has been characterized elsewhere [23,25,27].

2.2. Cell signaling inhibitor treatments

Caco-2/15 cells were grown as described [23,25,27]. At day 0 (confluence) 20 μM PP2, a specific Src signaling pathway inhibitor, was added to the culture medium. Controls consisted of DMSO only. The medium was renewed daily for 72 h. Total RNA and protein were extracted from the cell monolayer for each condition tested at 3 days post confluence.

2.3. Antibodies

Mouse primary antibodies used in this study were: actin (WB: 1/75,000) (Santa Cruz Biotechnology, Santa Cruz, CA), villin (WB: 1/3000) (BD Biosciences Pharmingen, Mississauga, ON) and the monoclonal antibody against sucrase-isomaltase (HSI-14), produced and characterized as previously described (WB: 1/10, IF: 1/100) [26]. Rabbit primary antibodies used in this study were: the anti-phosphorylated form of Src (WB: 1/1000) (Y416, Cell Signaling Technology, Beverly, MA), total Src (WB: 1/1000) (Cell Signaling Technology), E-cadherin (WB: 1/20,000, IF: 1/500) (BD Biosciences Pharmingen) and ZO-1 (IF: 1/1000) (Zymed, Burlington, ON). Secondary antibodies used were AlexaFluor 488 goat anti-mouse and goat anti-rabbit (Invitrogen, Burlington, ON).

2.4. Western blot

Western blots were performed on SDS–PAGE gels under denaturing conditions as previously described [28]. Primary antibodies were incubated overnight at room temperature or 4 °C and detected using horseradish peroxidase-conjugated secondary antibodies (anti-mouse, anti-rabbit, GE Healthcare-Amersham Bioscience, Baie d'Urfe, QC) and developed using the Immobilon Western® kit (Millipore, Billerica, MA).

2.5. Quantitative RT-PCR

RNA extraction, quality verification, reverse transcription and cDNA amplification by real-time PCR were performed as previously described using an Mx3000P (Stratagene, La Jolla, CA) [29,30]. Assessments of gene expression were established according to the Pfaffl mathematical model using RPLPO for normalization [31]. Human sucrase-isomaltase (SI), lactase-phlorizin hydrolase (LCT), aminopeptidase-N (ANPEP), dipeptidylpeptidase IV (DPP4), Cdx2 (CDX2), HNF1α (HNF1A), E-cadherin (CDH1), Li-cadherin (CDH17), cingulin (CGN), zonula occludens 2 (TJP2) and RPLP0 were evaluated as previously described [28–32]. Primers used to detect human apolipoprotein A1 (APOA1) were forward 5′-TGGATGTGCTCAAAGACAGC-3′ and reverse 5′-AGGCCCTCTGTCTCCTTTTC-3′, for Src (SRC), forward 5′-GCTGACTGAGCTCACCACAA-3′ and reverse 5′-CGGTGGACGTGAAGTAGTCC-3′, for Fyn (FYN), forward 5′-ACGGGAGGTTCACAATCAAG-3′ and reverse 5′-AGGGTCCTTTTTCCAGCAGT-3′, for Lyn (LYN), forward 5′-TGATGTGTGGTCCTTTG-GAA-3′ and reverse 5′-CTGCCTTTTCTTTCCAGCAC-3′ and for Yes (YES), forward 5′-ATGCCAGAAGCTTTCCTTCA-3′ and reverse 5′-CAGCAATCTGAGCAGCCATA-3′.

2.6. Indirect immunofluorescence

Cells were seeded onto glass cover slips and processed as previously reported [25,33]. Both primary and secondary antibodies were diluted in 10% Blotto-PBS. Samples were viewed with a DMRXA microscope (Leica, Concord, ON) equipped for epifluorescence and digital imaging (RTE/CCD Y/Hz-1300 cooled camera). Images were acquired using MetaMorph software (Molecular Devices, Sunnyvale, CA) with 20× and 40× objectives.

2.7. Data presentation and statistical analyses

All experiments were performed at least three times. Student's unpaired *t*-test and ANOVA using Bonferroni's Multiple Comparison Test used to analyze the results and data were considered to be significantly relevant at $p < 0.05$ and are presented respectively as mean ± SEM. Statistical calculations were performed using Prism 3.0 software (GraphPad Software, San Diego, CA).

3. Results

3.1. Src kinase is activated during human intestinal epithelial cell differentiation

The Caco-2/15 cell line has been widely exploited for its unique ability to spontaneously undertake a differentiation program upon reaching confluence which is reflected by robust expression of specific biomarkers [23,25] such as SI, that increases with confluence. The analysis of Src mRNA expression in Caco-2/15 cells showed significant increase between 0 and 30 days of post-confluence (PC), which correlated with a rise in SI levels (Fig. 1A). The activation status of Src evaluated by measuring the level of Tyr 416 phosphorylation ($pSrc^{Y416}$) showed a significant increase upon attaining confluence and peaked at 3 days PC (Fig. 1B). Peak levels of Src activity occurring between 0 and 3 days coincided with the beginning of SI accumulation (Fig. 1B, right panel). These results suggest that SFKs could be involved with early events of intestinal epithelial cell differentiation.

3.2. Inhibition of SFKs accelerates the enterocytic differentiation program

To further investigate the potential role of SFKs in intestinal epithelial cell differentiation, we evaluated the impact of its inhibition

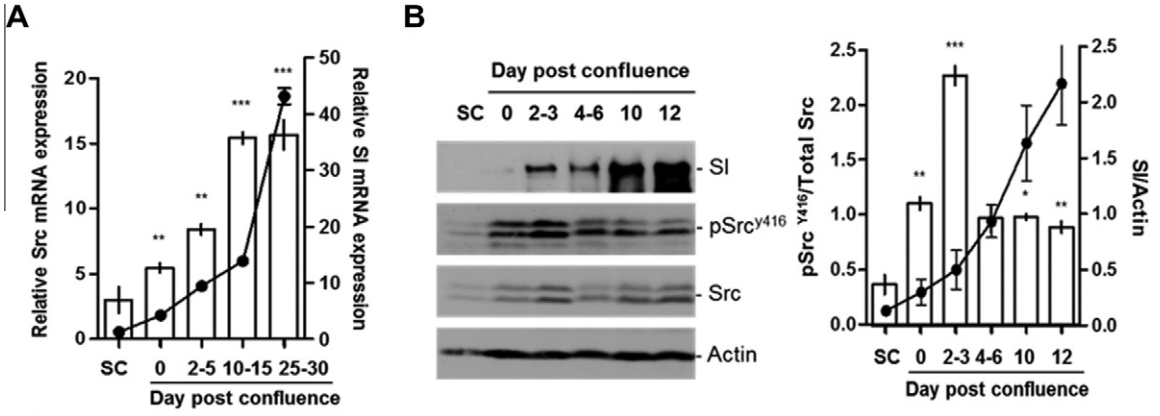

Fig. 1. Src kinase is activated during Caco-2/15 human intestinal epithelial cell differentiation. Whole cell protein and total RNA were isolated from Caco-2/15 cells at different times of confluence. (A) The mRNA expressions of Src (bars) and SI (curve) were determined by qPCR. (B) Protein extracts were analyzed by Western blot for detection of SI, phosphorylated Src on tyrosine 416 ($pSrc^{Y416}$) and total Src as well as actin. Bar graph shows $pSrc^{Y416}$ normalized to total Src and the curve shows SI expression relative to actin. ($n = 3$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *versus SC Ctrl*). SC: sub-confluence.

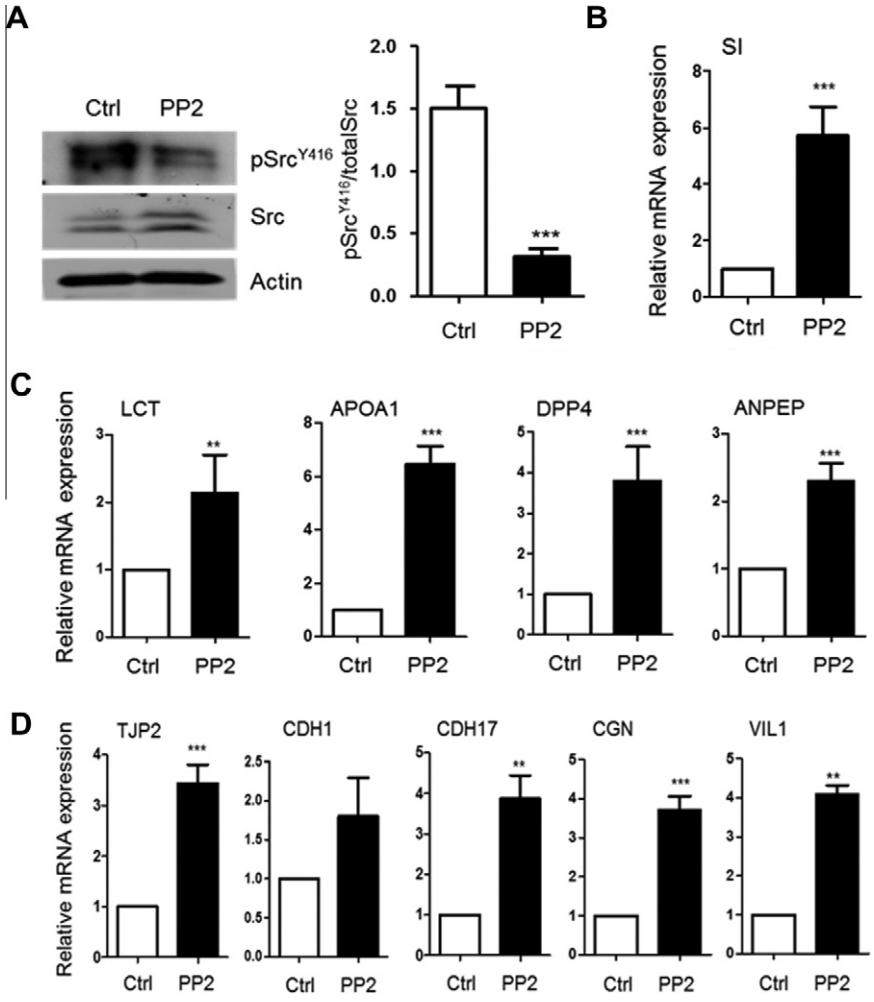

Fig. 2. Effect of PP2 on Src phosphorylation and gene expression in Caco-2/15 cells. Newly confluent cells were treated for 3 days with PP2 or control (DMSO only; Ctrl) in complete culture medium. (A) Cell lysates were separated by SDS–PAGE, transferred to membranes and immunoblotted for $pSrc^{Y416}$ detection. After stripping, membranes were reprobed with anti-Src and actin. Histogram illustrating the ratio of $pSrc^{Y416}$ on total Src. (B–D) qPCR analysis of intestinal cell markers. (B) SI. (C) Other terminal differentiation markers. (D) Polarization markers. ($n = 3$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *versus Ctrl*).

on newly differentiating Caco-2/15 cells. Cells were treated with the Src inhibitor PP2 at 20 uM [5] for 3 days beginning at confluence. Src activity was found to be significantly decreased as evaluated by measuring the levels of $pSrc^{Y416}$ as an indicator of Src kinase activity (Fig. 2A). However, surprisingly, we observed a sharp increase (~6×) in SI transcript levels (Fig. 2B) suggesting that Src and/or other SFKs act as a repressor of the early steps of intestinal cell differentiation. Quantitative PCR of mRNA of SFKs confirmed that Caco-2 cells express significant levels of various SFKs namely Src, Fyn, Lyn and Yes (not shown). PP2 treatment caused

a significant decrease in relative amounts of transcript levels of Src (0.49 ± 0.01, mean ± SEM) and Fyn (0.31 ± 0.06).

To further investigate the effect of SFK inhibition on intestinal cell differentiation, transcript levels of other well-characterized intestinal cell terminal differentiation markers were analyzed by qPCR. Interestingly, PP2 treatment during the first 3 days of post-confluent culture induced significant increases of all tested markers (Fig. 2C) suggesting that the effect is general and affects the entire intestinal terminal differentiation program. Previous studies had pointed towards differentiation and polarization being uncoupled events in newly differentiating Caco-2/15 cells [25,33]. Expression of a number of epithelial polarity markers, such as E-cadherin, Li-cadherin, villin, cingulin and ZO-2, was therefore investigated. PP2 treatment induced significant up-regulation of all tested markers at the transcript level (Fig. 2D). To determine whether the observed increases in mRNA translated into increased protein levels, we monitored protein expression in PP2 treated and control cell cultures by Western blot analyses for SI and E-cadherin. Both markers showed significantly increased expression at 3 days PC in PP2 treated cultures (Fig. 3A). Indirect immunofluorescence for SI and E-cadherin confirmed increased expression levels and extended these results by showing that sucrase-isomaltase was efficiently and correctly inserted into the apical membrane while E-cadherin was massively targeted to the lateral membranes (Fig. 3B).

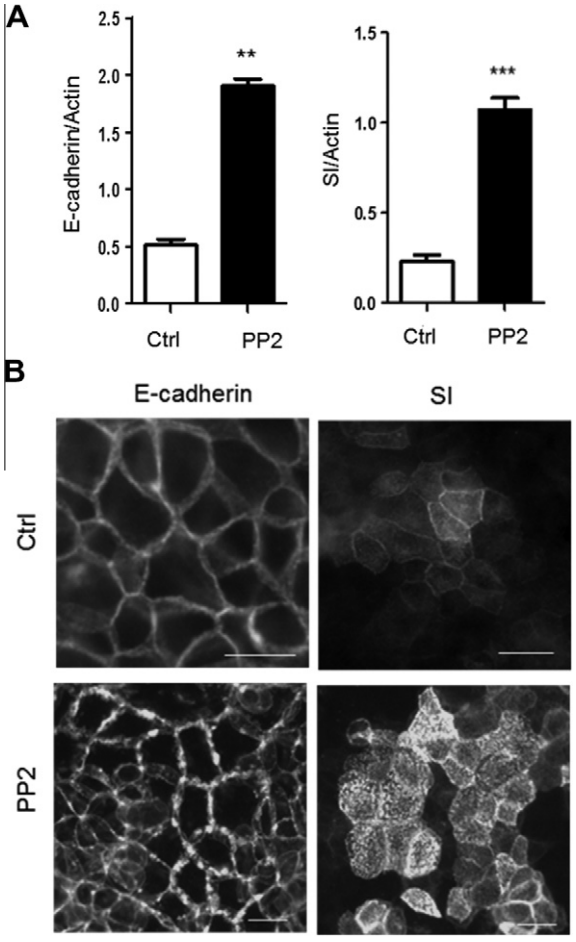

Fig. 3. Effect of PP2 on differentiation and polarity markers at the protein level. Newly confluent cells were treated for 3 days with PP2 or control (DMSO only; Ctrl) in complete culture medium. (A) Cell lysates were prepared and analysed by immunoblot for detection of SI and E-cadherin. Bar graphs show the amounts of both proteins relative to actin. ($n = 3$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *versus Ctrl*). (B) Caco-2/15 cells were cultured on sterile chamber slides until confluence then treated with or without PP2 as above. Cells were then immunostained for E-cadherin and SI then processed for immunofluorescence and picture acquisition. Exposition times were similar.

3.3. SFKs regulate expression of enterocyte-specific transcription factors

The ubiquitous effect of PP2 treatment on both cell polarization and terminal differentiation suggested that SFKs may act on a fundamental mechanism of enterocytic maturation. Recent work from our laboratory has shown that the transcription factors Cdx2, HNF1α and GATA4 cooperate in the initiation of the enterocytic differentiation program [30]. Since GATA4 is not expressed in Caco-2 cells [32], the influence of PP2 on the expression of Cdx2 and HNF1α was investigated. Interestingly, inhibition of SFK activity by PP2 treatment in 3 day PC cell cultures revealed a significant up-regulation of both HNF1α and Cdx2 at both transcript (HNF1α: 7.73 ± 0.74 times relative to ctrl; Cdx2: 3.23 ± 0.28 times relative to ctrl) and protein levels (Fig. 4A). These results suggest that Src activity in early differentiation events acts as a negative modulator of these two regulatory genes associated with the transcription of both polarity and terminal differentiation markers.

3.4. PP2 treatment reduces PRC2-related activity in intestinal cells

Epigenetic modulation *via* the polycomb PRC2 complex has recently been shown to be involved in the repression of terminal differentiation in intestinal cells [28]. The potential implication of SFKs on PRC2 activity was therefore tested. As shown in Fig. 4B, PP2 treatment caused a significant decrease in H3K27me3 levels.

4. Discussion

A coordinated balance between proliferation and differentiation is essential for the intestinal epithelium to preserve its constant renewal capacity. A key question in intestinal cell biology is what regulates the initiation of the terminal differentiation process. Recent work from our laboratory has shown that intestine-specific transcription factors such as Cdx2 and HNF1α cooperate in the initiation of the differentiation program while the polycomb PRC2 complex acts as a transient repressor. The SFKs control multiple cellular events such as adhesion and spreading, migration, apoptosis, cell cycle progression and gene transcription [34]. SFKs have been shown to also be involved in the differentiation of different cell types such as T cells [12], myoblasts [13], trophoblasts [17,18], chondrocytes [14] and osteoclasts [10]. However, little is known regarding the role of SFKs in the regulation of differentiation in human intestinal epithelial cells. In this study we assessed the effects of the SFK inhibitor PP2 on the initiation of enterocytic differentiation in human intestinal Caco-2/15 cells and disclosed that this signaling pathway plays an unsuspected role in the control of differentiation events. Indeed, we show for the first time that SFK inhibition results in the triggering of intestinal polarization and terminal differentiation. The effects are quantitatively significant as illustrated by SI levels in 3 day PC cells treated with PP2 reaching comparable levels as those found in Caco-2 cells after 20 days at confluence.

In is noteworthy that in addition to SI expression, which is a major enterocyte terminal differentiation marker [23,25,26], up-regulated expression of several other terminal differentiation markers such as LCT and DPP4 as well as cell polarization markers such as CDH1 and CHD17 [28,30] was observed after PP2 treatment. Although there is evidence that these events are not necessarily coordinated at the cellular level [25,33], they may be functionally related since Src has been shown to regulate the integ-

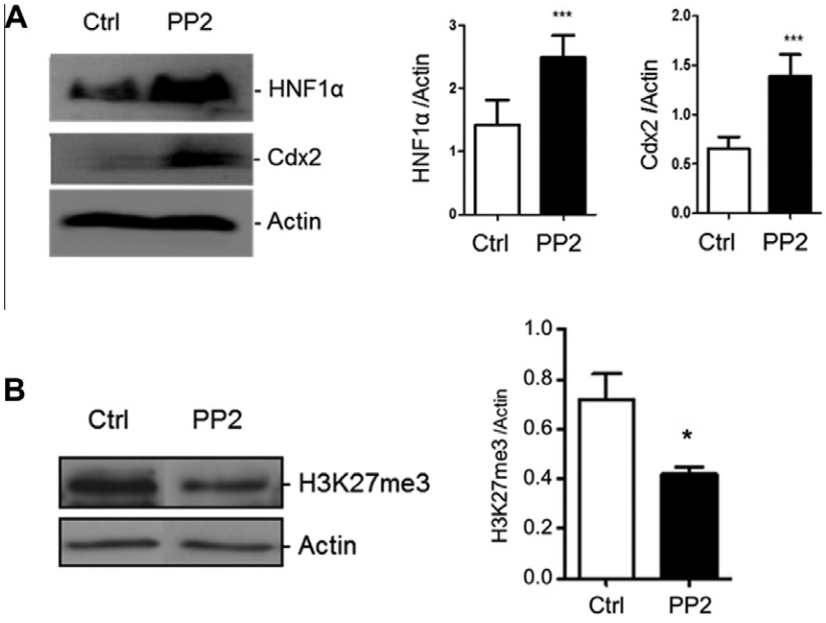

Fig. 4. Effect of PP2 on Cdx2 and HNF1α expression and H3K27 trimethylation. Newly confluent cells were treated for 3 days with PP2 or control (DMSO only; Ctrl) in complete culture medium. (A) Cell lysates were prepared and analysed by immunoblot for detection of HNF1α and Cdx2. Bar graphs show the amounts of both proteins relative to actin. ($n = 3$, $^{***}p < 0.001$ *versus Ctrl*). (B) Cell lysates were prepared and analysed by immunoblot for detection of the PRC2-associated epigenetic marker H3K27me3. Bar graph shows the amounts of both proteins relative to actin. ($n = 3$, $^{*}p < 0.05$ *versus Ctrl*).

rity of adherens junctions [35,36] and this junction system can play a role in the control of cell differentiation [37]. Furthermore, E-cadherin-mediated cell–cell contact can trigger p38 MAPK cascade activation in differentiating enterocytes [38] while Src regulates p38 MAPK activation [39]. Interestingly, the PI3 K product phosphatidylinositol 3,4,5-triphosphate can recruit and activate the GTP exchange factor for Rac, which is required for adherens junction formation [40].

In this context the inhibitory effect of PP2 on the polycomb PRC2 complex is interesting because it has previously been found that PRC2 mediated histone H3 methylation is active in proliferative intestinal epithelial cells where it appears to impede intestinal cell terminal differentiation [28]. Knockdown of the PRC2 component Suz12 caused accelerated expression of a subset of differentiation markers including SI and LCT but did not affect polarity markers. That inhibition of Src was found to increase expression of all terminal differentiation and polarity markers tested suggests that SFK activity acts as a negative regulator of enterocytic differentiation on a more general scale, and certainly upstream of PRC2. A similar role for Src has been identified in mammary epithelium, where pharmacological inhibition of SFK activity reduced tumor proliferation in a mouse model for breast cancer and was associated with a significant decrease in expression of another PRC2 component, Ezh2 [41].

A second distinction between Src and PRC2 activity inhibition is the effect on transcription factor expression. Inhibition of PRC2 activity did not significantly affect expression of HNF1α or of Cdx2 [28] while PP2 triggered up-regulation of both transcription factors at the mRNA and protein levels. These results are consistent with a previous study where our group identified a central role for these intestinal epithelial cell-specific transcription factors on terminal differentiation and polarization [30]. Considering the crucial regulatory role of these factors on the transcription of intestine specific genes [30,42,43], the finding that SFKs tightly regulate HNF1α and Cdx2 abundance is of significant importance.

Taken together, the present work has led to the identification of an original and major effect of SFKs on the enterocytic differentiation program. Based on our observations that SFK inhibition by PP2 leads to over-expression of a panel of markers representative of both epithelial polarization and intestinal terminal differentiation events, a phenomenon consistent with the up-regulation of the two main intestinal epithelial cell-specific transcription factors Cdx2 and HNF1α and the reduction of polycomb PRC2-related epigenetic repressing activity, our data suggest that SFKs are responsible for delayed Caco-2 cell overall differentiation.

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