The extracellular-regulated protein kinase 5 (ERK5) promotes cell proliferation through the down-regulation of inhibitors of cyclin dependent protein kinases (CDKs)

Diana Perez-Madrigal, Katherine G. Finegan, Blanca Paramo, Cathy Tournier

University of Manchester, Faculty of Life Sciences, Manchester, M13 9PT, UK

Abstract

Activation of the extracellular-regulated protein kinase 5 (ERK5) has been associated with mitogenic signal transduction. However, conflicting findings have challenged the idea that ERK5 is a critical regulator of cell proliferation. We have addressed this issue by testing the effect of the conditional loss of ERK5 in primary fibroblasts. We have discovered that ERK5 suppressed the expression of the cyclin dependent kinase (CDK) inhibitors, p21 and p27, by decreasing mRNA and protein stability, respectively. As a result, low level CDK2 activity detected in ERK5-deficient cells correlated with a defect in G1 to S phase transition of the cell cycle. Similarly, we found that the malignant MDA-MB-231 human breast cancer cell line was dependent on ERK5 to proliferate. We propose that ERK5 blocks p21 expression in MDA-MB-231 cells via a mechanism that implicates c-Myc-dependent transcriptional regulation of the miR-17-92 cluster. Together with evidence that cancer patients with poor prognosis display a high level of expression of components of the ERK5 signaling pathway, these findings support the hypothesis that ERK5 can be a potential target for cancer therapy.

Keywords: ERK5, MAPK, p21, p27, CDK, Cell cycle

1. Introduction

The extracellular-regulated protein kinase 5 (ERK5), also known as Big mitogen activated protein (MAP) kinase 1 (BMK1), is a non-redundant mitogen-activated protein kinase (MAPK) required for the maintenance of vascular integrity during development and tumorigenesis [1,2]. ERK5 activity is increased mainly in response to mitogens, oxidative and osmotic stresses upon phosphorylation by a MAPK/ERK kinase 5 (MEK5) [3]. MEK5 specifically recognizes the TEY motif present in the N-terminal half of the protein within the catalytic domain which is highly homologous to that of ERK1/2. In contrast, the large C-terminal half of ERK5 is unique and possesses both nuclear localizing and transcriptional activities [4]. The nuclear localization of ERK5 in mitosis was recently demonstrated to be independent of MEK5 phosphorylation [5,6].

In comparison to the other members of the MAPK family, little is known about the downstream targets of ERK5. The best-characterized substrates are the transcription factors of the myocyte enhancer factor 2 (MEF2) family [7,8]. The discovery that serum was a potent inducer of c-fos gene transcription via ERK5-induced MEF2C transcriptional activation provided the first evidence that the ERK5 signaling pathway was involved in regulating cell proliferation [7]. Epidermal growth factor (EGF) was subsequently found to transmit its growth promoting effect in HeLa cells via ERK5 [9]. This finding was confirmed in a variety of other immortalized cell lines [10]. More recently, a novel potent and specific ATP-competitive inhibitor of ERK5, XMD8-92, was shown to suppress tumor formation through reduced cell proliferation in mice bearing xenografts [11].

However, the idea that ERK5 is a critical regulator of cell proliferation does not appear to be consistent with the demonstration that erk5−/−, mek5−/−, and wild type fibroblasts do not display marked differences in their ability to progress through S phase [2,12–14]. This suggests that the ERK5 signaling pathway may be important for promoting or regulating the proliferation of certain cell types under certain conditions. Alternatively, null fibroblasts may have been selected for compensatory proliferative mechanisms during isolation. Furthermore, despite our detailed molecular understanding of the cell cycle, the mechanism that makes a cell dependent on ERK5 for its ability to proliferate remains largely unknown.

To clarify these issues, we decided to test the effect of the conditional deletion of the erk5 gene in primary cultures of mouse embryonic fibroblasts (MEFs) and determine the relevance of our findings in a malignant breast cancer cell line. Our results demonstrate that the proliferation of primary or cancer cells is impaired in the absence of a functional ERK5 signaling. The requirement of ERK5 to suppress the expression of inhibitors of cyclin dependent kinases (CDKs) provides a mechanism by which ERK5 promotes hyperphosphorylation of the retinoblastoma protein (Rb), thereby allowing E2F-mediated
transcriptional regulation of gene expression required for entry into
S-phase.

2. Materials and methods

2.1. Tissue culture

MEFs obtained from 13.5 day old embryos were cultured in DMEM
supplemented with 10% fetal bovine serum (FBS, Sigma), 1% penicillin/
streptomycin, and 1% glutamine [14]. Genotyping determination of the
cells was obtained by PCR on genomic DNA extracted from the embryos
using forward (5′-CCAGCGCAGATAAGGCTA-3′) and reverse (5′-AGAG
TGGAGAATCAGGAGGG-3′) primers for the flox allele and forward (5′-CG
GTCGATGCAACGAGTGATGAGG-3′) and reverse (5′-CCAGAGCAGGAA
ATCATCCGCTG-3′) primers for the creERT2 transgene. Genetically
modified MEFs were mock treated (ETOH) or incubated with 4-OHT
(500 nM, diluted in 95% ethanol) for 24 h to induce gene deletion. The
media was changed and the cells were harvested immediately after
or 24 and 48 h later. All experiments were performed with cells at passage
2 and 4. Similar data were obtained in experiments with independently
isolated MEFs. The mouse strains were maintained in a pathogen-free
facility at the University of Manchester. All animal procedures were
performed under license in accordance with the UK Home Office
Animals (Scientifc Procedures) Act (1986) and institutional guidelines.

2.2. Preparation of cell lysates and immunoblot analysis

Proteins were extracted from cells in RIPA buffer: 50 mM Tris pH
7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium-deoxycholate,
10% glycerol, 1 mM orthovanadate, 1 mM phenylsulphonyl fluoride
(PMSF), 0.1% sodium dodecyl sulfate (SDS), 10 μg/ml leupeptin, and
10 μg/ml aprotinin. Extracts were clarified by centrifugation (14,000 g
for 20 min at 4 °C). 20 to 50 μg of protein was subjected to immuno-
 blot analysis following SDS-polyacrylamide gel electrophoresis (PAGE)
with antibodies against ERK5 from Millipore, ERK1/2, cyclin E, CDK2,
CDK4, p21 and p27 from Santa Cruz, P-ERK1/2, cyclin D1, c-Myc from
Cell Signaling, tubulin from Sigma and Rb from BD Pharmingen.
Immunocomplexes were detected by enhanced chemiluminescence
with anti-rabbit or anti-mouse immunoglobulin G coupled to horserad-
ish peroxidase as the secondary antibody (GE HealthCare).

2.3. Protein kinase assay

Endogenous ERK5, CDK2 and CDK4 activities were measured following
immunoprecipitation in kinase buffer (25 mM HEPES pH 7.4, 25 mM
β-glycerophosphate, 25 mM MgCl2, 2 mM DTT, 0.1% orthovanadate)
containing 50 μM [γ-32P] ATP (10 Ci/mmol) and 1 μg of GST-MEF2C,
Histone H1 (Roche) or Rb (Santa Cruz), respectively. The reactions
were terminated by the addition of Laemmli sample buffer. The radioac-
tivity incorporated into the recombiant proteins was quantified after
SDS-PAGE by PhosphorImager analysis.

2.4. Immunofluorescence

Cells were fixed in 4% paraformaldehyde (PFA) prior to being incubat-
ed with a specific antibody to p27 (Santa Cruz). Immune complexes were
detected with a secondary antibody conjugated to fluorescente (Jackson
ImmunoResearch). Nuclei were stained with DAPI (5 μg/ml). Fluores-
cence images were viewed with an Olympus Widefield microscope.

2.5. Reporter gene expression assay

MEFs were transiently transfected using Lipofectamine 2000 re-
agent (Invitrogen) with the p21 promoter-luciferase reporter plasmid
(Addgene plasmid 16451). A PRL-Tk plasmid encoding Renilla lucifer-
ase was employed for monitoring transfection efficiency. Aliquots of
cell lysates were assayed for firefly and Renilla luciferase activities
according to the manufacturer’s instructions (Promega).

2.6. Cell viability and death assays

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,
5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were incu-
bated with 2.5 mg/ml MTT (Sigma) for 2 h at 37 °C. MTT crystals
were solubilized in DMSO and samples were aliquoted to measure
their absorbance at 570 nM. Caspase activity was measured by spect-
trofluorometry using the DEVD-AMC caspase 3 specific fluorogenic
substrate (Alexis Biochemicals). Senescence was measured using the
senescence β-galactosidase staining kit (Cell Signaling).

2.7. Cell proliferation assays

For BrdU analysis, 70% confluent cells were labeled with 1 μM
BrdU (Sigma) for 1 h, collected by trypsinization and stained with an
anti-BrdU antibody (DAKO) and propidium iodide (Sigma). For
crystal violet staining, cells were washed in PBS and fixed with 4% PFA. The fixed cells were stained with 0.1% crystal violet and subse-
quently washed 3 times with distilled water. Dried cells were incubated
with 10% acetic acid to solubilize the crystal violet. Absorbance was read
at 590 nm.

2.8. Real time PCR

Total RNA was isolated using the Trizol™ reagent and cDNA
synthesis was carried out as previously described [12]. Real-time
quantitative PCRs were performed using the SYBR Green I Core Kit
(Eurogentec). Forward and reverse primers for p27 (Sigma) were
published before [15]. For p27, forward primer, 5′-ACCGTCTTTTATCCGGGC
and reverse primer 5′-CAAGACCTGCTTCCGCTGT-3′ were
designed from the published mouse sequence (NM_009875.4) to
generate an ampiclon of 155 bp. PCR products were detected in the
ABI-PRISM 7700 systems (Applied Biosystems). Results were ana-
alyzed using the 2−ΔΔCt methods. actin mRNA was used for normaliza-
tion [12]. For the detection of mature miRNAs, cDNA was synthesized
using the TaqMan® MicroRNA Reverse Transcription kit according
to manufacturer’s instructions. Real-time quantitative PCRs were car-
rried out using the TaqMan® Universal Master Mix II in conjunction
with TaqMan® Assays (hsa-miR-17-5p ID: 002308, hsa-miR-20a ID:
000580). PCR products were detected in the CFX-96 systems (Biorad).
Results were analyzed using the 2−ΔΔCt methods. RNU6B was used
for normalization.

2.9. Statistical analyses

All p values were generated using an unpaired student’s t-test.

3. Results

3.1. The loss of ERK5 impairs cell proliferation

To assess the requirement of ERK5 in cell proliferation, we tested
the effect of the functional loss of ERK5 signaling in primary MEFs
carrying homozygous erk5−/− allele and a transgene expressing the
recombinase Cre fused to a mutated form of the estrogen receptor (ER$^{27}$). Incubation of the cells with 4-hydroxytamoxifen (4-OHT) triggers the nuclear translocation of Cre where it specifically recombines the fl allele. Immunoblot analysis confirmed that the inactivation of the erk5 gene only occurred in Cre expressing erk5$^{fl/fl}$ MEFs treated with 4-OHT (Fig. 1A). The maximal loss of ERK5 expression was detected 48 h after incubation of the cells with 4-OHT for 24 h (i.e. 72 h time point). The very low residual amount of the protein detected under these conditions is consistent with previous knowledge that the deletion of floxed genes following 4-OHT-induced Cre recombination does not occur with 100% efficiency.

ERK5 expressed in mock treated (ETOH) MEFs displayed an electrophoretic mobility shift characteristic of the phosphorylated form of ERK5 (Fig. 1A). Like ERK5, the phosphorylated forms of ERK1 and ERK2 (ERK1/2) were detected in proliferating MEFs incubated in serum (Fig. 1A). The level of phosphorylation and expression of ERK1/2 was not significantly affected by Cre-mediated recombination of the erk5 gene. To compare the effect of the functional loss of ERK5 to that of ERK1/2, cells were incubated with PD0593901 (PD) to inhibit ERK1/2 activity without affecting ERK5 (Fig. 1A).

In the absence of ERK5 the number of viable cells decreased to a level comparable to that associated with ERK1/2 inhibition (Fig. 1B). The data correspond to the mean±SD (N=2). (D) DNA synthesis was monitored by BrdU incorporation. The data expressed as percent of maximum correspond to the mean±SD (N=3). *, P<0.05 and **, P<0.005 indicate a significant difference compared to mock treated MEFs.

3.2. ERK5 is required for cell cycle re-entry

Previous studies have implicated ERK5 in entry into S phase but also G2/M regulation [9,16]. To distinguish between these two possibilities, we tested the ability of serum starved MEFs blocked at G0 to re-enter the cell cycle in the absence of ERK5. The number of cells in S phase was monitored by BrdU incorporation after serum stimulation. Interestingly, ERK5 was detected as a doublet in serum starved cells, indicating that starvation did not prevent ERK5 phosphorylation (Fig. 2A). Nonetheless, a transient activation of ERK5 was detected by protein kinase assay following serum stimulation with a maximum at 10 min (Fig. 2B). As expected, Cre expressing erk5$^{fl/fl}$ MEFs incubated with 4-OHT did not display increased ERK5 activity under this condition (Fig. 2B). This defect reduced the capability of the cells to re-enter S phase (Fig. 2C).

To confirm the requirement of ERK5 in the G1/S phase transition of the cell cycle, Cre expressing erk5$^{fl/fl}$ MEFs were synchronized at the G1/S boundary by a double thymidine–aphidicolin block. In parallel,
the cells were mock treated or treated with 4-OHT so that ERK5 expression was lost by the end of the synchronization process (T = 0, Fig. 3A). Progression through the cell cycle following the re-addition of serum was monitored by propidium iodide staining. No significant change was observed in ERK5 phosphorylation between cells arrested in late G1 and cells re-entering the cell cycle, as indicated by similar patterns of electrophoretic mobility retardation of ERK5 (Fig. 3B). As expected, the majority of the cells were arrested at the G0/G1 phase at the time of the block and a large proportion of mock treated cells incubated in serum had re-entered S phase at 4 h (Fig. 3C, black circle). This correlated with a decreased number of cells in G0/G1. The number of ERK5-deficient MEFs that left G0/G1 to enter S phase was much lower (Fig. 3C, white circle). We suggest that the small proportion of Cre expressing erk5fl/fl MEFs that left G0/G1 to enter S phase was no longer able to inactivate E2F by ERK5 activity (fold) correlated with a significant decrease in the level of expression of p21 and mock treated cells (Fig. 4A). The expression level of cyclin E or of CDK2 was not compromised by ERK5 gene deletion (Fig. 4B). Instead, the gradual disappearance of ERK5 expression is required for entry into S phase [18].

We found no evidence that ERK5 was required for regulating the cyclin D1/CDK4 complex activity (Fig. 4A). In fact, 4-OHT treated erk5fl/fl MEFs expressing Cre displayed an even higher level of CDK4 activity than mock treated cells (Fig. 4A). This is likely to be a consequence of increased cyclin D1 expression. In contrast, maximal loss of ERK5 detected at 72 h (Fig. 4C) correlated with a significant decrease in the cyclin E/CDK2 complex activity (Fig. 4B). Reduced CDK2 activity was associated with decreased Rb phosphorylation (Fig. 4B). The expression level of cyclin E or of CDK2 was not compromised by erk5 gene deletion (Fig. 4B). The gradual disappearance of ERK5 caused a gradual increase in the level of expression of p21 and p27 (Fig. 4C). p21 and p27 are CDK inhibitors (CKIs) and as such, they are critical regulators of the G1 to S phase transition of the cell cycle [19]. Increased p27 expression, but not p21, was also detected in cells treated with PD to inhibit ERK1/2 activity (Fig. 4C).

Together, these data indicate that increased CKI expression caused by erk5 gene deletion leads to the inhibition of cyclin E/CDK2 activity and the dephosphorylation of Rb, which results in growth arrest in the late G1 phase of the cell cycle.

### 3.4. ERK5 regulates p21 and p27 via two independent mechanisms

To determine how ERK5 controls p21 and p27 expression, the level of the transcripts was measured by quantitative real time (RT) PCR...

Fig. 3. The loss of ERK5 impairs the re-entry of late G1-arrested MEFs into the cell cycle. (A) Schematic representation. Cre expressing erk5−/− MEFs were mock treated (ETOH) or treated with 4-OHT for 24 h. The media was changed and 8 h later the cells were incubated in thymidine and aphidicolin as indicated. (B) Protein lysates were analyzed by immunoblot with an antibody against ERK5. Tubulin was used as a loading control. Similar results were obtained in three independent experiments. The electrophoretic mobility shift associated with ERK5 phosphorylation is indicated by an arrow. (C) Cell cycle profile of MEFs stained by propidium iodide was analyzed by FACS. The data correspond to the mean ± SD (N = 3). *, P < 0.05 indicates a significant difference between 4-OHT and mock treated MEFs.

We found that p21 mRNA was up-regulated in cells treated with 4-OHT with a kinetic consistent with the gradual increase in p21 and decrease in ERK5 protein expression (Fig. 4C and D). The transcriptional regulation of p21 by ERK5 was examined by luciferase assay using the full-length p21 promoter reporter (Fig. 5A). Cisplatin, which has been shown to induce p21 transcription via up-regulation of p53 [20], was used as a positive control. The loss of ERK5 did not increase p21 luciferase activity (Fig. 5A). Instead, the stability of the p21 transcript measured in cells incubated with actinomycin D1 for various amounts of time, was significantly elevated in ERK5-deficient cells (Fig. 5B; compare a half life of 4 h in mock treated cells with a half life of almost 8 h in 4-OHT treated cells). In contrast to p21, the level of p27 mRNA expression was not affected by the loss of ERK5 (Fig. 4D). However, we found that erk5 gene deletion increased the half-life of the p27 protein measured in cells incubated with cycloheximide (CHX) (Fig. 5C). The stabilization of p27 in the absence of ERK5 resulted in the accumulation of the protein in the nucleus (Fig. 5D). Increased nuclear localization of p27 was also detected in cells incubated with the ERK1/2 inhibitor, consistent with the requirement of ERK1/2 to suppress p27 expression (Figs. 4C and 5D). Together, this study suggests that ERK5 is a critical regulator of CKI expression.

3.5. Pharmacological inhibition of ERK5 prevents cell proliferation of breast cancer cells

The level of p21 is down-regulated in many human cancers. Interestingly, XMD8-92, a novel ERK5 inhibitor, has been identified as a good candidate for cancer therapy due to its potent anti-tumor activity with low level toxicity [11]. Therefore, we explored the possibility that pharmacological inhibition of ERK5 using XMD8-92 could restore p21 expression in the malignant breast cancer cell line MDA-MB-231.

In contrast to MEFs, MDA-MB-231 cells displayed multiple shifted bands of ERK5, suggesting the expression of distinct phosphorylated forms of the protein (Fig. 6A). Most of them had disappeared in lysates of cells treated with 25 μM XMD8-92 (Fig. 6A). This concentration did not affect the phosphorylation of ERK1/2 (Fig. 6A) and was sufficient to prevent ERK5 activation by sorbitol in MDA-MB-231 cells (D.P.M., unpublished data). Consistent with our previous finding using genetically modified MEFs, the pharmacological inhibition of ERK5 caused a dramatic increase in p21 expression in MDA-MB-231 cells, which display undetectable level of the protein under basal condition (Fig. 6A). This resulted in severe cell cycle defect (Fig. 6B), without inducing apoptosis (Fig. 6C).

Recently, miR-17-5p and miR-20a have been shown to negatively regulate p21 expression by targeting two sequences in the 3’UTR region of the transcript [21]. These miRNAs belong to the miR-17-92 cluster which can be transcriptionally activated by c-Myc [22]. Interestingly, c-Myc can be phosphorylated by ERK5 at Ser 62 [23], a phosphorylation site associated with increased c-Myc protein stability [24]. Based on these findings and consistent with genetic evidence that ERK5 controls p21 mRNA stability (Fig. 5B), we proposed that ERK5 suppressed p21 expression in breast cancer cells by promoting the transcription of miR-17-5p and miR-20a via c-Myc. Consistent with our hypothesis, we found that inhibition of ERK5 following treatment of MDA-MB-231 cells with XMD8-92 decreased the level of c-Myc (Fig. 6A). This was accompanied by an approximately 30% decrease in the level of miR17-5p and miR-20a (Fig. 6D). The link between ERK5 signaling and miRNA-dependent regulation of p21 expression was demonstrated by evidence that transient transfection of miR17-5p and/or miR-20a mimics significantly lowered the level of p21 in MDA-MB-231 treated with XMD8-92 (Fig. 6E). The remaining expression of p21 detected under these conditions suggests that other mechanisms are likely to contribute to mediating the effect of ERK5 on p21 mRNA stability.

Together, this study suggests that ERK5 promotes breast cancer cell proliferation by suppressing p21 expression via a mechanism that implicates the up-regulation of miRNAs via cMyc.

4. Discussion

This study genetically establishes the requirement of ERK5 in normal cell proliferation. The cell cycle defect caused by the conditional loss of ERK5 in primary MEFs was not associated with increased
cell death, a phenotype previously observed using a similar genetic approach in immortalized MEFs [25]. These distinct phenotypic abnormalities can be explained by increased sensitivity of cells that have gained immortality, a well known phenomenon in cancer biology that constitutes the rational for chemotherapy. In addition, none of these findings could have been predicted from our analyses of erk5−/− and mek5−/− fibroblasts [12,14]. The absence of noxious effect associated with the conventional deletion of the erk5 or mek5 underscores the complex organization of signaling pathways that enables cells to develop compensatory mechanisms during the isolation process. Cross talks between cascades are likely to be responsible for this well-known phenomenon of “signal transduction plasticity”. Consistent with impaired proliferation, we found that ERK5-deficient MEFs displayed reduced phosphorylation of Rb and low level CDK2 activity. Based on the evidence that ERK5 does not control cyclin E or CDK2 expression, we proposed that this defect is caused by increased p21 and p27 expression.

Unlike CDK2, no marked difference was observed in the level of CDK4 activity between mock- and 4-OHT treated MEFs. The distinct contribution of CKIs in the regulation of CDKs is supported by the demonstration that p21 and p27 are more effective inhibitors of CDK2 than of CDK4 in vitro [26–29]. The ability of p27 to selectively inhibit cyclin A/CDK2 and cell cycle progression, without affecting cyclin D2/CDK4 was confirmed in Mv1Lu cells [30]. The phosphorylation of p27 by Abl at Tyr 88 or 89 in proliferating cells is thought to be responsible for preventing p27 to inhibit cyclin D/CDK4 activity [31]. Similarly, stress-induced G1 arrest in fibroblasts was shown to be associated with the accumulation of partially phosphorylated Rb caused by increased p21 level and the specific inhibition of CDK2, but not CDK4 [32,33]. The distinct affinity of p21 for different cyclin kinases

---

**Fig. 4.** The loss of ERK5 inhibits CDK2 activity and increases p21 and p27 expressions. Cre expressing erk5fl/fl MEFs were mock treated (ETOH), treated with 4-OHT or with PD, as indicated in Fig. 1. (A, B) Upper panels, protein lysates were analyzed by immunoblot with antibodies against cyclin D1, CDK4, cyclin E, CDK2 and Rb. Tubulin was used as a loading control. Similar results were obtained in three independent experiments. Bottom panels, endogenous CDK4 and CDK2 activities were measured by in vitro protein kinase assay. Radioactivity incorporated in recombinant Rb or in Histone H1 was quantified by PhosphorImager. The data expressed as fold correspond to the mean ± SD (N = 3). (C) Protein lysates were analyzed by immunoblot with antibodies against ERK5, ERK1/2, P-ERK1/2, p21 and p27. Tubulin was used as a loading control. Similar results were obtained in three independent experiments. The electrophoretic mobility shift associated with ERK5 phosphorylation is indicated by an arrow. (D) Total RNA was extracted and the amount of p21 and p27 transcripts was measured by RT-PCR. The data expressed as fold correspond to the mean ± SD (N = 4). * P<0.05 indicates a significant difference between 4-OHT and mock treated MEFs.
may be determined by the stoichiometry of the p21 subunit within the complexes [34]. Based on these studies we conclude that increased expression of p21 and p27 caused by the loss of ERK5 is sufficient to inhibit CDK2, but not CDK4, activity.

The accumulation of p27 in the nucleus of ERK5-deficient MEFs leads us to suggest that ERK5 may decrease p27 protein stability by controlling its sub-cellular localization. This model is supported by our preliminary data showing that ERK5 can phosphorylate p27 at Ser10 [35]. p27 expression was also reported to be increased in macrophages and in acute myelogenous leukemia (AML) cells displaying a reduction in ERK5 activity [36,37]. However, in contrast to our preliminary findings, increased ERK5 phosphorylation in AML cells over-expressing the Cot1 oncogene correlated with decreased p27 phosphorylation at Ser 10 concomitant with decreased protein expression [37]. These results are consistent with evidence that phosphorylation at Ser10 stabilizes p27 in resting cells (G0) [35]. Overall, further studies will be required to rigorously address the functional consequence of the post-translational modification of p27 by ERK5 on p27 stability in a cell cycle dependent manner.

Like in MEFs, the loss of a functional ERK5 pathway in MDA-MB-231 cells led to a cell proliferation defect, without causing cell death. This indicates that breast cancer cells are not dependent on ERK5 for their survival. Nonetheless, our previous genetic evidence for a role of ERK5 signaling in stress-induced cell survival [25] suggests that inhibition of ERK5 may render MDA-MB-231 cells more sensitive to genotoxic compounds. This will remain to be tested.

Fig. 5. The loss of ERK5 increases p21 mRNA and p27 protein stability. (A) Cre expressing erk5fl/fl MEFs were transiently transfected with a p21 reporter luciferase plasmid and a pRL-Tk plasmid 24 h after being mock treated (ETOH) or treated with 4-OHT. The cells were harvested 48 h later. Treatment with 25 μM cisplatin for 24 h was used as positive control. The transcriptional activity was measured by the Dual-Luciferase reporter assay system. The data expressed as fold correspond to the mean±SD (N=3). (B) erk5fl/fl MEFs expressing Cre were treated with 500 ng/ml actinomycin D1 for the indicated times 72 h after being subjected to ETOH or 4-OHT treatment. Total RNA was extracted and the amount of the p21 transcript was measured by RT-PCR. The data correspond to the mean±SD (N=4). The half-life (HL) of the p21 transcript indicated in parentheses was calculated using a nonlinear regression analysis. (C) 72 h after being exposed to ETOH or 4-OHT, erk5fl/fl MEFs expressing Cre were treated with 30 μg/ml of cycloheximide (CHX) for the indicated times. Upper panel, protein lysates were analyzed by immunoblot with an antibody against p27. The band corresponding to p27 is indicated by an arrow. * indicates a non specific band. Tubulin was used as a loading control. Bottom panel, immunoblot signals were quantified with the ImageQuantifier software (BioImage, Jackson MI). The data correspond to the mean±SD (N=3). The half-life (HL) of the p27 protein is indicated in parentheses. (D) Cre expressing erk5fl/fl MEFs were subjected to the ETOH or 4-OHT protocol for 72 h or were treated with PD for 24 h. Immunofluorescence was performed with a specific antibody to p27. The immune complex was detected with a secondary antibody conjugated to fluorescein (green). DNA was stained with DAPI (blue). Scale bar, 50 μm.
been found to bind to the 3′UTR region of the p21 mRNA causing its degradation [21]. These miRNAs belong to the miR-17-92 cluster which can be transcriptionally activated by c-Myc [22]. Based on evidence that XMDS-92 decreased c-Myc expression in MDA-MB-231 cells and that c-Myc is a substrate of ERK5 [23], we hypothesize that ERK5 suppresses p21 expression by mediating c-Myc-dependent transcriptional regulation of miR-17-5p and miR-20a. The role of miR-17-92 in breast cancer appears complex with some data suggesting that miR-17-92 acts as a tumor suppressor, while others pointing to a pro-oncogenic role for miR-17-92. Nevertheless, evidence that ERK5 contributes to mediating the oncogenic potential of various tyrosine kinases in human breast cancer cell lines [38-40] together with the link between ERK5 overexpression and decreased disease-free survival of breast cancer patients [41], indicate that ERK5 could be a potential target for breast cancer therapy.

Recently, this hypothesis was supported by the demonstration that XMDS-92, a novel potent and specific ATP-competitive inhibitor of ERK5, suppressed tumor formation through reduced cell proliferation in mice bearing xenografts [11]. Interestingly, HeLa cells incubated with XMDS-92 displayed increased p21 expression [11]. However, unlike in MDA-MB-231 cells, the phosphorylation of the promyelocytic leukemia protein (PML) by ERK5 is thought to be the mechanism by which ERK5 prevents PML to increase p21 level. However, the depletion of PML in tumor cells did not completely abrogate their response to XMDS-92. It is possible that the ability of XMDS-92 to decrease pml−/− tumor is a consequence of its inhibitory effect on angiogenesis. Alternatively, this may reflect the ability of ERK5 to control p21 expression via miRNA, independently of PML. Further studies using mouse models of human cancer will be required to test this hypothesis and confirm the potential use of XMDS-92 to treat breast cancer patients.

5. Conclusions

This study provides evidence that ERK5 sustains cell proliferation by suppressing the expression of inhibitors of CDKs. In particular, we found that ERK5 down-regulates p21 level in breast cancer cells via a mechanism that implicates c-Myc-dependent transcriptional regulation of miRNA. Overall, the link between ERK5 and CKIs underscores the essential role of ERK5 in promoting the G1 to S phase transition of the cell cycle.

Acknowledgments

We are indebted to Drs Donald Ogilvie and Allan Jordan (Drug Discovery Unit, Paterson Institute for Cancer Research, Manchester UK) for the synthesis of XMDS-92. We thank A. Whitmash (University of Manchester, UK) for critically reviewing the manuscript and the technicians at the animal facility for looking after the mice. This work was supported by a scholarship from the CONACYT to DPM and BP and by a grant from the Association for International Cancer Research to CT. The authors declare no conflict of interest.

References