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Curcumin regulates miR-21 expression and inhibits invasion and metastasis in colorectal cancer

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Running title: Curcumin regulates miR-21 regulation

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Abstract

Curcumin has promising potential in cancer prevention and therapy by interacting with proteins and modifying their expression and activity, which includes transcription factors, inflammatory cytokines, and factors of cell survival, proliferation, and angiogenesis. miR-21 is overexpressed in many tumors, promoting progression and metastasis. In the present study, we examined the potential of curcumin to regulate miR-21, tumor growth, invasion, and *in vivo* metastasis in colorectal cancer. In Rko and HCT116 cells, we identified two new transcriptional start sites of the miR-21 gene and delineated its promoter region. PMA stimulation induced miR-21 expression via motifs bound with AP-1 transcription factors. Curcumin treatment reduced miR-21 promoter activity and expression in a dose-dependent manner by inhibiting AP-1 binding to the promoter, and induced expression of tumor suppressor Pdcd4, which is a target of miR-21. Curcumin treated Rko and HCT116 cells were arrested in the G2/M phase with increasing concentrations. Furthermore, curcumin inhibited tumor growth, invasion and *in vivo* metastasis in the chicken–embryo–metastasis assay (CAM). Additionally, curcumin significantly inhibited miR-21 expression in primary tumors generated *in vivo* in the CAM assay by Rko and HCT116 cells ($p<0.00006$; $p<0.035$ respectively). Taken together, this is the first report to show that curcumin inhibits the transcriptional regulation of miR-21 via AP-1, suppresses cell proliferation, tumor growth, invasion, and *in vivo* metastasis, and stabilizes expression of tumor suppressor Pdcd4 in colorectal cancer.

Key words: CAM, Characterization, Curcumin, miR-21, Promoter, Tumor-progression

Abbreviations: miRNA, microRNA; pre-miR-21, primary transcript containing miR-21; AP-1, activation protein 1, ChIP, chromatin immunoprecipitation; RT-PCR, Reverse transcription PCR; PMA, phorbol 12-myristate 13-acetate

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Introduction

Curcumin (diferuloylmethane), a yellow colored polyphenol, is an active component of the spice turmeric (Curcuma longa) and has been used for many centuries as a food additive and a traditional medicine in Asian countries. Curcumin has chemo-preventive and therapeutic properties against many tumors in *in vitro* and *in vivo* models and has been included in clinical trials.[1] It suppresses inflammation, cell proliferation and induces apoptosis, and sensitizes tumor cells to cancer therapies.[2-4] It also suppresses invasion and angiogenesis, in cancer.[2, 5, 6] Curcumin physically binds to different proteins like thioredoxin reductase, COX2, protein kinase C (PKC), and 5-lipoxygenase (5-LOX) and modulates different molecular target molecules like transcription factors (AP-1, NF-κB, STAT-3 and β-catenin), growth factors and their receptors, cytokines and enzymes.[5, 7] Curcumin is also known to inhibit Akt/mTOR signalling in different cancer cells.[7-9] Curcumin has been administrated up to 10g/day in humans without toxicity, and serum concentrations of 1.77 ± 1.87 mol/L were observed in patients treated with 8g/day.[10] Many diseases, especially cancer, are the results of deregulation of more than 500 different genes.[11] Since curcumin is known for being non-toxic at higher doses, and for its role in inhibiting tumor progression by modulating a vast number of molecules and mechanisms,[2, 5, 6] it has been suggested to be used as a potential drug in cancer therapy.

MicroRNAs are a class of ~22 nt endogenous, non-coding RNAs generated from a larger stem loop structure, which can be expressed in a cell and tissue specific manner, influencing mRNA stability and translation. They control a wide range of biological functions such as cellular proliferation, differentiation and apoptosis.[12-14] Disruptions of miRNA–target gene regulation, including genomic instability and impaired miRNA processing, have been associated with a growing range of cancers.[13, 15] Recent reports show strong evidence that miRNAs are key molecules involved in cancer initiation and progression.[16, 17]

miR-21 shows a strong evolutionary conservation across a wide range of vertebrate species in mammalian, avian and fish clades, suggesting a conserved role for miR-21 in gene regulation.[18] Gene expression studies across tumor samples and cancer cells showed that miR-21 is the only miRNA upregulated in a large-scale profiling of 540 human samples derived from 363 specimens representing six types of solid tumours (breast, colon, lung, pancreas, prostate, and stomach) and 177 respective normal control tissues.[19] Other reports also showed that it is upregulated in other cancer types like glioblastoma, oesophageal cancer, ovarian carcinoma, cholangiocarcinoma, B-cell lymphoma, hepatocellular carcinomas, cervical cancer, uterine leiomyomas, head and neck cancer, chronic lymphocytic leukaemia, squamous cell carcinoma of the tongue and papillary thyroid carcinoma.[18] This extensive miRNA profiling in different cancers compared with normal tissues/cells identified miR-21 as a key molecule associated with neoplastic transformation and as a biomarker. Functional studies of miR-21 either with sense or antisense transfection experiments in colorectal,[20] breast,[21, 22] glioblastoma,[23] hepatocellular,[24] and neuroepithelial[25] cancer cells determined its role in inducing cell proliferation, anti-apoptosis, migration, invasion and metastasis by differentially regulating molecules like Pdcd4, PTEN, and Bcl-2. These studies strongly suggest its role in oncogenesis/tumor progression.

Pri-miR-21 is one of the first human miRNA genes for which the structure was determined as located on chromosome 17 residing within the tenth intron of the gene TMEM-49 (Transmembrane protein-49).[26, 27] *Pri-miR-21* and TMEM-49 are overlapping genes, and in the same direction of transcription are independently transcribed and terminated with their respective poly (A) signal.[27] In human promyelotic HL-60 cells, miR-21 expression is induced after phorbol 12-myristate 13-acetate (PMA) stimulation.[27],[28] Two reports in cervical cancer, and leukemia cell lines suggest that the *miR-21*-gene has two different transcription start sites (TSS): one (**C**ATT) is at intron 10 of TMEM-49[27] and the second one (**A**TAAC) is at 891 bp downstream of the TSS reported by Fujita S et al., 2008, which is

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in intron 11 of TMEM-40.[26] *Pri-miR-21* promoter regulation is controlled by AP-1, PU.1, the SWI/SNF complex, and δEF1.[27, 29, 30] BMP-6 downregulates miR-21 expression by inhibiting the expression and binding of AP-1 and δEF1 to the miR-21 promoter.[29] c-Jun and c-Fos are the major regulators of miR-21 among AP-1 family members after PMA-stimulation.[27]

Combining the promising data on curcumin's function as an anti-tumorigenic, and the role of miR-21 in tumorigenesis and progression, we performed this study to determine the *pri-miR-21* maximal promoter region and TSS sites in colorectal cancer, and especially to study the effect of curcumin on the transcriptional regulation and expression of miR-21, cell cycle regulation, tumor growth, invasion and *in vivo* metastasis in this cancer entity. As a result, we show that the traditional medicine compound curcumin inhibits miR-21-transcriptional regulation and expression, tumor growth, invasion, and *in vivo* metastasis, and stabilizes the miR-21 target Pdcd4 in colorectal cancer. Furthermore, we identified multiple TSS for *pri-miR-21*, among them two new TSS being the major ones, one at 35 bp and the other at 28 bp upstream to the TSS reported by Fujita S et al., 2008. Moreover, we report on a new additional AP-1 site (-663/-659 bp) in the upstream region of *pri-miR-21*. Finally, we observed that the TSS previously reported by Cai et al., 2004, which is located in the intron 10 of TMEM-49, is spliced from the *pri-miR-21* promoter in colorectal cancer cells.

Materials and methods

Cell culture, drug, treatments and Materials

Cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA), and grown at 37°C with ATCC recommended media supplemented with 10% fetal calf serum (FCS). Curcumin was purchased from Sigma Aldrich (Germany) and dissolved in DMSO. Mock-treated control (DMSO) cells were handled identically to curcumin treated cells. Control-miR and pre-miR21 were from Ambion (USA), Taqman primer-probes for the quantification of miR-21 and RNUB6 were from Applied Biosystems (USA), oligonucleotides were obtained from Metabion (Germany).

Preparation of protein extracts and Immunoblotting

Cells were washed with phosphate-buffered saline (PBS) after curcumin treatment and lysed in extraction buffer (Biosource, CA). Protein concentrations were determined by BCA kit (Pierce, Rockford, IL, USA). For immunoblotting, samples (40 µg/lane) were boiled for 5 min, separated via SDS-PAGE, and transferred to PVDF membranes. After transfer, the membranes were blocked with 5% non-fat milk containing Tris-buffered saline with 0.1% Tween (TTBS) for 3 h at room temperature and then probed with the indicated primary antibodies versus Pdcd4 (Abcam, Germany), p-c-Jun (#sc 822x, Santa Cruz) and GAPDH (#sc 1616, Santa Cruz) for 2 h at room temperature. After three washes with TTBS, blots were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase. After final washes with TTBS, the membranes were exposed to film after enhanced chemiluminescence (ECL) (Amersham Biosciences).

RNA isolation and Reverse Transcriptase-PCR

Total RNA was extracted from tissue culture cells and CAM tumours with TRIZOL (Invitrogen) according to the manufacturer's instructions. The RNA samples were quantified using a spectrophotometer and visualized on a MOPS–formaldehyde gel for quality assurance. 1 µg of total RNA was reverse transcribed by random hexamer primer using SuperScript II reverse transcriptase (Invitrogen), or 100 ng of total RNA was reverse transcribed by gene specific primers (Applied Biosystems (AB)). Expression of pre-miR-21 was determined by Syber green PCR (Primer pairs: pre-miR-21(sense: CAT TGT GGG TTT TGA AAA GGT TA, antisense: CCA CGA CTA GAG GCT GAC TTA GA) and R18 (RN18S1) normalizing control (sense: CGCCGCTAGAGGTGAAATTC, antisense:

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CATTCTTGGCAAATGCTTTCG), and mature miRNA was determined by the TaqMan miRNAassay using RNUB6 as the internal normalizing control. All TaqMan-PCRs were performed in triplicates.

MTT assay

$3*10^3$ cells/well were seeded in a 96-well plate in a total volume of 100 µL medium with 10% FBS. After 24h, cells were treated either with DMSO or curcumin at concentrations between 0-28 µM. After 48h, the growth inhibitory effect was evaluated using 20µL/well of CellTiter96® AqueousOneSolution (Promega-USA) according to the manufacturer's instructions.

Cell cycle analysis

Rko and HCT116 cells were treated for 24 hours with DMSO or 2.5 µM and 20 µM of curcumin. After 24 hours cells were washed with PBS twice and harvested by trypsinization. The cells were washed again with PBS and fixed with cold 70% ethanol for 1 hour. The cells were washed with PBS once and then incubated with 4 µg of ribonuclease A (Roche Applied Science) for 30 min at room temperature. Propidium iodide was added to the cell suspension at a final concentration of 20 µg/ml and incubated for 30 min. The cells were then analyzed by flow cytometry using FACScan (Becton Dickinson). The results were quantified by using the software Cell Quest (Becton Dickinson).

RACE and generation of luciferase reporter constructs

RACE and luciferase reporter constructs were generated as described previously.[31, 32] Briefly, the 5' RACE-ready first-strand cDNA was synthesized using 1µg of total RNA from Rko cells and human placenta. The 5' flanking sequence of miR-21 was amplified using a universal primer (UMP, Clontech), and a specific primer: 5'-GTAGGCTCCTGTTGGTCAAAAGAGG -3'. The resulting PCR products were gel-purified, cloned into the pGEM-T easy vector (Promega, WI), and 10 different positive clones of each were sequenced. RT-PCR was performed with a panel of colorectal, cervical (HeLa) and breast (MCF-7) cancer cells to confirm the pre-miR-21 transcription start site and splicing of intron 11 of TMEM-49 from the pre-miR-21 promoter, with sense 5'-GCCATACCAGAGTACAGTATCAGC-3' and antisense 5'-CATCCAGGACAACCAGTTTTCTCC-3'.

Approximately 2.0 kb of upstream sequence of the pre-miR-21 promoter was amplified by PCR from human genomic DNA and cloned into the pGL3-basic vector (Promega) using *Kpn* I and *Mlu* 1 restriction sites. The 1060 bp construct was generated from the 2 kb construct by Pvu II and Mlu I digestion, backbone blunted using T4 DNA pol I, and ligated. The 448 bp construct was made from the 1060 bp construct by Kpn I and Sac I digestion, backbone blunted using T4 DNA pol I, and ligated. Mutant constructs were generated by the QuikChange XL site-directed mutagenesis kit from Stratagene (La Jolla, CA) using the 1060 bp maximum promoter activity-containing construct as a template. The sequences of oligonucleotides used for cloning and mutation are depicted in Supplementary Table 1. Successful incorporation of the mutations was confirmed by automated sequencing.

Transfection and Luciferase Assays

For luciferase assays, 0.5×10^6 Rko or HCT116 cells were plated in 24 well plates, and after 24 hours transfected with Lipofectamin 2000 (Invitrogen). pRL-TK (25 ng, Renilla Luciferase; Promega) was transfected along with Luciferase reporter plasmids (1 µg) to normalize transfection efficiency. After 12 hours of transfection, cells were either mock treated with DMSO or treated with increasing concentrations of curcumin (2.5 µM to 20 µM).

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After 24 hours of treatment, cells were washed twice with PBS and lysed with 100 µl passive lysis buffer (Promega) for 20 min. Luciferase activity of 20 µl of cell lysate was measured via the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Assays for all samples were performed in triplicate, and the results were averaged.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assays were performed as described previously,[33] using either anti-p-c-Jun (sc-822 X), anti-c-Fos (sc-44 X) or anti-IgG (as control, sc-2338 X) antibodies. Rko cells were either mock treated with DMSO or treated with curcumin (10 µM) 1 hour before PMA stimulation. After 24 hours post stimulation cells were processed as described.[32] P-c-Jun and c-Fos binding to the *pri-miR-21* promoter was measured using Syber green PCR with two sets of specific primers. Set 1, sense 5′- GCCTCCCAAGTTTGCTAATG-3′; antisense 5′- TGTACTCTGGTATGGCACAAAGA -3′ and Set 2, sense 5′- GAGATCAGGCCATTGCACTC-3′; antisense 5′- GCAACACTGCCTAATGCTTG -3′.

Cell migration and invasion assay

Rko and HCT116 cells were pretreated with curcumin (10µM and 16µM, respectively). After 24h, cells were trypsinized, and $3x10^5$ cells were plated on Boyden chambers either coated with 10µg matrigel/well (for invasion assays) or uncoated (for in vitro migration assays) in serum-free medium containing 0.1% BSA (Bovine serum albumin) and respective curcumin concentrations. 10% FBS medium in the lower chamber served as the chemoattractant. After 13h, non-invading cells were removed with cotton swabs. Invaded cells were trypsinized and counted using the ATP-luminescence-based motility-invasion assay (Promega) as previously described.[34]

Chorionallantoic membrane (CAM) assay

The CAM assay was performed as described,[33, 34] with a few modifications. Briefly, $2*10^6$ cells were inoculated on the CAM of 10-day old chicken embryos. On day 12 and 14, chicken embryos were treated intravenously with curcumin or DMSO, respectively. Untreated embryos served as an additional control. On day 17, the embryos were sacrificed. Metastasis was determined by harvesting lungs and liver, and processing the tissue for genomic human DNA by quantitative *alu*-PCR.

Statistical analysis

Statistical analyses were performed using the SPSS statistical software (statistical package for social sciences, SPSS, Inc., Chicago, IL). The Student's t-test was performed for data with normal distribution, the Wilcoxon-test for all other analysis. P-values of ≤ 0.05 were considered statistically significant.

Results

Race analysis and transcription initiation site of *pri-miR-21*

Pri-miR-21 is located in intron 10 of the *TMEM-49* gene, and both of these genes are transcribed in the same direction. TMEM-49 expression (Supplementary figure 1) did not correlate with miR-21 expression levels published by Asangani et al. 2008 in the screened panel of cancer cell lines,[20, 27] suggesting that both genes have different promoters. Cai et al., 2004 and Fujita et al., 2008 reported two different TSS for miR-21 and both are located in different introns of the TMEM-49 protein coding genes with a distance between of 891 bp. To define the transcription initiation site(s) for *pri-miR-21*, 5'-RACE was performed using a TMEM-49 specific primer anchored within exon 12 (Supplementary Fig 2a), with total RNA from Rko cells and human placenta. 5'-RACE amplification from RNA of both samples

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generated two amplicons with an approximate size of 1200 bp and 500 bp (Fig 1a). Interestingly, no specific amplification of the 500 bp fragment was observed in the normal human placenta. This result was not surprising since miR-21 might not be expressed in human placenta. The 1200 bp fragment size corresponds with the TMEM-49 transcript. Cloning and sequencing of the 500 bp PCR products revealed multiple 5'-start sites, with the majority of clones including a region consisting of 35 bp upstream to the TSS reported by Fujita et al., 2008 (Supplementary Figure 2b, 3a). Interestingly, no specific band or sequence corresponding to the TSS reported by Cai et al., 2004 was observed (data not shown). Furthermore, RT-PCR performed with the specific primers binding to TMEM-49 intron 10 (*pri-miR-21* promoter) and exon 12 in a panel of cancer cells (Supplementary Fig 2a) resulted in a 477 bp fragment (Fig 1b). These observations suggest a splicing mechanism of intron 11 of TMEM-49 from the *pri-miR-21* promoter, which is a common mechanism of classical genes (Supplementary Fig 3b), and two novel major TSS at 35 bp and at 28 bp upstream to the previously reported TSS by Fujita et al., 2008, for the *pri-miR-21* promoter.

PMA induces *pri-miR-21* transcriptional activation and expression

To determine an impact of PMA-stimulation on miR-21 expression, Rko and HCT116 cells were stimulated with 100 nM of PMA in a time-dependent manner. miR-21 was upregulated after 12 hours and reached a maximum upregulation after 24 hours PMA stimulation (Supplementary Fig 4a,b). These results suggest that PMA induces miR-21 expression in colorectal cancer cells. Furthermore, to delineate the miR-21 promoter region required for constitutive and PMA-inducible activity, different luciferase constructs were made either with, or without, the TSS reported by Cai et al., 2004, and our new TSS, and transfected into Rko and HCT116 cells (Fig 1c). Cells were stimulated with DMSO or 100 nM PMA 12 hours post transfection, and after 24 hours of stimulation cells were harvested and analysed. Deletion of 1000 bp downstream of the novel 35 bp TSS determined by us and the TSS reported by Fujita et al., showed a significant increase in the promoter activity either with or without PMA-stimulation, and deletion of 500 bp from the 5' flanking region of these two TSS decreased the promoter activity in both conditions when compared with the 1 kb fragment (Fig 1c). These results suggest that the maximum miR-21 promoter is spanning 1000 bp upstream to the novel TSS determined by our study as well as the TSS reported by Fujita et al., which is 35bp downstream to our novel TSS.

Curcumin inhibits *pri-miR-21* promoter activity

Rko and HCT116 cells were treated with curcumin for 48 hours to determine the IC_{50} values for cell proliferation (Fig 2a). MTT assay determined the cell lines' IC_{50} as 10μM (Rko) and 16 μM (HCT116). To determine the activity of curcumin to regulate *pri-miR-21* promoter activity, the maximum promoter activity-giving construct was transfected into Rko and HCT116 cells. 12 hours post transfection, cells were treated for 24 hours with curcumin in different concentrations. Curcumin significantly reduced the promoter activity in both cell lines (Fig 2b). To determine the functional relevance of an upstream new AP-1 binding motif (at -663/-659 bp: AP-1 IV) in the promoter, a mutational construct specifically for this site was generated and luciferase assays performed. Either with or without PMA-stimulation, the promoter activity was decreased. Especially, with PMA-stimulation, the promoter activity of this AP-1 IV mutant construct was significantly reduced, when compared with the PMA-stimulated wild type construct. Additionally, combinatorial AP-1 mutation constructs (AP-1 binding motifs -58/-55 bp: AP-1 I; -165/-162 bp: AP-1 II; and -226/-223 bp:AP-1 III, proximal to the TSS reported by Fujita S et al., 2008) were generated along with the new upstream AP-1 binding motif and transfected into Rko and HCT116. Mutation of all four AP-1 motifs reduced the basal promoter activity significantly when compared with the wild type construct, the activity of this mutant being significantly reduced compared with other

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combinational constructs mutated for different AP-1 motifs. Moreover, PMA-induced promoter activity was most significantly reduced by the construct mutated for all four AP-1 motifs, with an activity comparable to basal promoter activity. The curcumin-inhibited promoter activity was not reduced further when compared with its DMSO control with the four AP-1 mutant construct. However, curcumin pre-treatment inhibited PMA-induced promoter activity in both cell lines (Fig 2c). ChIP analysis was performed to determine the *in vivo* relevance of AP-1 transcription factor binding to the promoter for curcumin treatment, PMA-stimulation, and the combination of both. PMA-stimulation increased the binding of p-c-Jun/AP-1 and c-Fos/AP-1 to the *pri-miR-21* promoter (Fig 1d). Under similar conditions, curcumin decreased the binding of p-c-Jun and c-Fos. Additionally, 1 hour pre-treatment with curcumin followed with PMA stimulation could not increase the binding of p-c-Jun and c-Fos to the promoter (Fig 2d). Similar results were observed in Western blot analysis; curcumin inhibited c-Jun activation in terms of phosphorylation (Fig 2e,f; Supplementary Fig 4c). These results suggest that curcumin inhibits *pri-miR-21* transcriptional regulation, and also its PMA induction, through AP-1 family members.

Curcumin represses miR-21 expression and induces Pdcd4 protein expression

To demonstrate the effect of curcumin on pre-miR-21 and miR-21 expression and function, quantitative PCR and Western blot analysis were performed for pre-miR-21, miR-21, and Pdcd4, which has been shown to be a miR-21 post-transcriptional target,[20] with 24h of curcumin treatment. Rko and HCT116 cells showed a significant reduction in pre-miR and miR-21 expression after curcumin treatment in a dose-dependent manner (Fig 3a, c). Moreover, Curcumin inhibited the PMA-induced miR-21 expression, but not completely (Supplementary Fig 4d). In parallel, Pdcd4 protein amounts were increased significantly ($p<0.05$) in both cell lines (Fig 3b, d). These results suggest that curcumin is inhibiting miR-21 expression and function in colorectal cancer, as shown by a reduction of its target Pdcd4, an important tumor suppressor in colorectal cancer.

Curcumin arrests tumor cells in the G2/M phase of the cell cycle

Curcumin has been reported to inhibit cell growth and to induce apoptosis in cancer cells.[2, 5, 35] Dose-dependent treatment of curcumin inhibited the growth of Rko and HCT116 cells (Fig 4 a, b). Fluorescence-activated cell sorting (FACS) was carried out to analyze the cell cycle profile of curcumin-treated cells. The proportion of cells with actively replicating DNA, which represents the S-phase, did not change significantly with 24 h of curcumin treatment. However, the proportion of cells containing 2N DNA, which represents the G1-phase, decreased significantly in curcumin-treated Rko (74–36%) and HCT116 cells (67-22%). Similarly, the fraction of cells with 4N DNA, which represents the G2/M-phase, increased in Rko (13–47%) and HCT116 (17-67%) cells in a dose-dependent manner. These results suggest that treatment of Rko and HCT116 cells with curcumin results in the arrest of cells in the G2/M phase of the cell cycle.

Curcumin inhibits migration and invasion

To demonstrate an ability of curcumin to inhibit migration and invasion in colon cancer, Rko and HCT116 cells were pretreated with DMSO or with curcumin (10μM and 16μM, respectively). After 24 hours, cells were either seeded in Boyden-chambers for migration, or in 10μg matrigel-coated chambers for invasion. Curcumin-treated cells showed significantly decreased migration (Rko:$p=0.01$; HCT116:$p=0.04$) and invasion (Rko:$p=0.05$; HCT116:$p=0.02$) in both cell lines when compared with DMSO-treated cells (Fig 4c, d). These results suggest that curcumin inhibits migration and invasion in colorectal cancer cells.

Curcumin inhibits tumor and metastasis formation *in vivo*

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To study the ability of curcumin to regulate *in vivo* metastasis, we employed the chorionallantoic membrane (CAM) assay of the chicken embryo, which is able to differentiate primary tumor growth and metastasis, among other phenomena, in a highly sensitive and quantitative *in vivo* setting.[33, 34] For investigating regulation of primary tumor growth, Rko and HCT116 cells were inoculated on the upper CAM of 10-day old chicken embryos either with, or without, 24 hours pre-treatment of DMSO or curcumin (20µM). On day 12, the embryos with pretreated cells were treated on the upper CAM with DMSO or curcumin. The primary tumors on the upper CAM were removed from the experiments performed with the pretreated cells, weighed and quickly frozen in liquid nitrogen. For the metastasis assay, on day 12 and 14 the embryos with plain cells were treated intravenously with 20µM curcumin or DMSO. On day 17, the intravenously treated embryos were sacrificed and their lungs and livers harvested for DNA. The number of cells metastasized into the liver and lungs was measured with a Taq-Man-based PCR amplifying human *alu*-sequences on the chicken background.[33, 34] We found that curcumin was able to significantly reduce primary tumor growth of Rko (p=0.02) and HCT116 (p=0.016) cells (Fig 5a, c), and also distant metastasis of Rko cells (liver p=0.01, lungs p=0.011). In HCT116 cells, we observed a trend for metastasis inhibition when compared with respective DMSO controls (Fig 5b, d). These data suggest that curcumin inhibits tumor growth and *in vivo* metastasis of colorectal cancer cell lines.

Curcumin inhibits miR-21 expression in primary tumors *in vivo* in the CAM assay

To support the *in vitro* data on curcumin downregulation of miR-21, total RNA was harvested from the frozen resected tumors isolated on the upper CAM from both Rko and HCT116 cells. Quantitative PCR was performed for pre-miR-21 and miR-21 expression, with DMSO- or curcumin-treated samples from both cell lines. We observed that all samples/tumors (n=5) treated with curcumin showed reduced pre-miR-21 and miR-21 expression when compared with DMSO treated samples/tumors for both cell lines. Pre-miR-21 expression in individual samples is shown in Supplementary Figure 5. The mean expression of pre-miR-21 was significantly downregulated in curcumin-treated samples in Rko (p=0.0002) and HCT116 (p=0.041). Likewise, mean miR-21 expression in Rko (p=0.00006) and HCT116 (p=0.0035) was significantly downregulated when compared with the mean of DMSO treated samples/tumors (Fig 6 a, b). These results confirm the *in vivo* relevance of our *in vitro* findings. Taken together, these data show that curcumin significantly inhibits tumor growth and *in vivo* metastasis, and that this is, in part, mediated through miR-21.

Discussion

Cancer is a complex disease occurring as a result of a progressive accumulation of genetic aberrations and epigenetic changes that enable cells to escape from normal endogenous and environmental controls.[36] Cancer arises as a localized disease, progresses to an invasive primary tumor and finally becomes metastatic.[37, 38] Colon cancer is one of the major causes of cancer related death worldwide, representing the third most common and second leading cause of cancer death. However, still too little is known about the mechanisms of cancer metastasis and especially strategies to inhibit metastasis. miRNAs have been shown to have an important role in tumor biology, including oncogenesis, progression, invasion, metastasis and angiogenesis. miRNAs are now recognized as key players of post-transcriptional control of gene expression. It has been shown that miRNAs are aberrantly expressed or mutated in cancer, suggesting that they may function as a novel class of oncogenes or tumor suppressor genes, and ongoing investigations have been starting to implicate specific miRs in metastasis.[18, 39]

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miRNA-genes were reported to be transcribed by RNA polymerase II to produce pri-miRNAs with a terminal modification of a 5'- 7-methyl guanylate (m7G) cap and a 3' poly (A) tail, like traditional protein coding genes. This process has also been reported to be regulated by known transcription factors. miR-21 is one of the first human miRNAs whose structure was determined,[26, 40] and gene expression studies on different human tumor samples reported that miR-21 is upregulated especially in lung and colorectal tumor tissues.[18-20] However, the transcriptional regulation of miR-21 is not completely understood and also not clear in terms of TSS. Cai et al., 2004[26] reported a transcriptional start site (TSS) 2445 bp upstream to the *pri-miR-21* gene, in the TMEM-49 intron 11, which is constitutively active, in 293 embryonic kidney cells. Loffler et al., 2007[41] reported a different TSS 50 bp upstream to the TSS reported by Cai et al., which is regulated by conserved enhancer binding motifs of signal transducer and activator of transcription 3 (STAT3), in melanoma cells. A third TSS was reported for *pri-miR-21* by Fujita S et al., 2008 in cervical cancer (HeLa) and leukemia (HL-60) cell lines[27], to be located in intron 10 of TMEM-49. However, the previously reported transcriptional start sites were not detected in the study by Fujita et al.[27] Moreover, the TSS reported by Fujita et al., minimally overlaps about 60 bases with the TSS of Cai et al., and both are functioning separately, suggesting that each is an independent promoter.[26, 27] Our present study with 5' RACE analysis in colorectal cancer with the specific primer in exon 12 of TMEM-49 determined multiple TSS for *pri-miR-21*, which are either identical to, or even as novel TSS located around, the TSS reported by Fujita S et al.[27] No specific bands were observed to support the previously reported TSS by Cai et al., and Loffler et al., in our data. Interestingly, intron 11 of TMEM-49 was observed to be spliced from the *pri-miR-21* gene to support RNA polymerase II transcription, which is a common mechanism in the processing of normal protein coding genes,[18, 26] and correspondingly, the TSS reported by Cai et al., and Loffler et al., were located within TMEM-49 intron 11. In our study, the construct which included the Cai-TSS was not able to induce promoter activity as opposed to the TSS by Fujita el al., and the novel TSS presented by us, suggesting that the TSS by Cai et al. is not essential for the *pri-miR-21* promoter in colorectal cancer cells. Our findings of the *pri-miR-21* TSS within intron 10 support the findings of Fujita S et al. in colorectal cancer. Certainly, this TSS is close to the core promoter, with a typical structure for most of the human coding genes, suggesting the general biological importance of this TSS. Still, we think that the most likely explanation for the discrepancies found for diverse TSS in the other studies is that many TSS might in part be cancer entity-specific.[27, 42] Ozsolak et al., 2008[43] identified and assayed the transcriptional activity of 4 regions upstream of *pri-miR-21*. Two of them are very similar to the promoters reported by Cai et al., and Fujita et al. They observed a superior induction of promoter activity with the region identified by Fujita et al, and also found that the promoter reported by Cai et al., is more active in HeLa cells, however not active in melanoma cells. These observations support the notion that there may be cell lineage and/or tumor type specificity for the usage of the respective TSS and the transcriptional regulation of *pri-miR-21*.

Subsequently, our promoter analyses on *pri-miR-21* revealed that PMA-induced AP-1 binds to the pri-miR-21 promoter and triggers the transcription of the *pri-miR-21* gene. Additionally, we found a novel AP-1 binding motif (-663/-659 bp) in the upstream sequence of the *pri-miR-21* promoter, which is also important for core promoter activity and PMA-stimulated promoter activity in colorectal cancer. Transcriptional regulation of *pri-miR-21* has been shown to be controlled by AP-1, PU.1, the SWI/SNF complex, STAT3 and δEF1.[27, 29, 30, 41] Enhancement of endogenous activity of AP-1 can be induced through various signalling axes, and is associated with oncogenic transformation.[44, 45] It has been shown that enhanced miR-21 expression in several cancers is due to transcriptional regulation by an enhanced activation of AP-1.[16, 18, 44, 45] PMA induces miR-21 expression through AP-1

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family transcription factors, mainly c-Jun and c-Fos,[27] as we also have shown here. Curcumin physically binds and modulates different target molecules like AP-1, NF-kB.[2, 5] Correspondingly, we now showed that curcumin reduces miR-21 expression by inhibiting the activation and binding of AP-1 to the *pri-miR-21* promoter. Taken together, data suggest that curcumin reduces miR-21 expression by inhibiting AP-1 function.

This is the first study to show that curcumin inhibits invasion and *in vivo* metastasis by reducing miR-21 expression. Pdcd4 is a tumor suppressor shown to inhibit phorbol ester-induced neoplastic transformation,[46] tumor promotion and progression,[47] also suppressing tumor progression in human colon carcinoma cells by downregulating MAP4K1 transcription, and inhibiting c-Jun activation and AP-1-dependent transcription.[48,49] Recently, we showed that Pdcd4 is post-transcriptionally inhibited by miR-21.[20] Pdcd4 downregulation in lung and colorectal cancers has been associated with poor patient prognosis.[50,51] Ras-induced AP-1 activity enhanced miR-21 expression and downregulated Pdcd4 by an auto-regulatory loop mechanism.[30] Based on these results, we hypothesized that curcumin might inhibit miR-21 expression by downregulating AP-1. Consistent with this, curcumin treatment increased Pdcd4 expression and inhibited AP-1 in our study, leading to a hypothetical model of interactions as depicted in Fig 6c. Our findings suggest that curcumin-induced tumor suppression, and inhibition of migration, invasion and *in vivo* metastasis, might in part be mediated by miR-21-triggered, Pdcd4-dependent mechanisms [20-22, 24]. Additionally, we found that curcumin inhibited the cell cycle in G2/M phase, which is in line with earlier findings.[9, 52, 53] This again could in part be mediated by miR-21-targeted cell cycle regulators.[18, 23] However, our present findings certainly cannot exclude other important mechanisms mediated by curcumin in the inhibition of miR-21-regulation and –induced cancer progression (Fig 6c).[2-11, 35]

Taken together, our results suggest that curcumin is an inhibitor of miR-21 and downregulates miR-21 expression by inhibiting AP-1 in colorectal cancer. Curcumin also inhibits *in vivo* tumor growth, invasion and distant metastasis in colorectal cancer. Additionally, curcumin treatment induces tumor suppressor Pdcd4 expression. Our data add to the understanding of the anti-tumorigenic activities of curcumin and the regulatory mechanisms of miR-21 that function in cancer progression. Our findings also explain a possible mechanism of Pdcd4-mediated tumor suppression induced by curcumin treatment.

Authors contributions

GM, JNG, SM, IAA, KR carried out experiments; collection and/or assembly of data by GM, JNG; data analysis and interpretation by GM, JNG, SM; Manuscript writing by GM, LDN, HA; Financial support by HA; Final approval of manuscript by all authors.

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Figure legends

Fig 1 RACE analysis to determine the miR-21 transcription start sites, and promoter characterization

a) Total RNA from Rko and normal human placenta was used for 5′ RACE PCR analysis. Two major bands, one at ~1200 bp (TMEM-49) and a second one at ~500 bp (pri-miR-21) were detected. b) RT-PCR was performed with a panel of cancer cells to confirm the splicing of introns between TMEM-19 Exon 11 and 12 and the transcription start site. c) Schematic representation of a series of deletion constructs of the promoter and reporter assay showing pri-miR-21 promoter activity. 1 μg of *pri-miR-21*-luciferase constructs, or pGL3-basic vector together with 25 ng of Renilla luciferase plasmid were transiently transfected into Rko cells. After 12 hours of transfection, cells were stimulated with 100 nM PMA for 24 hours. The firefly luciferase activity was normalized with Renilla luciferase activity. The data are presented as the mean (±S.D.) of three independent experiments performed in quadruplicates. d) The *in vivo* association of p-c-Jun and c-Fos activating the *pri-miR-21* promoter was evaluated with ChIP assay in Rko cells with and without PMA stimulation. Immunoprecipitated DNA with the specific antibody was amplified by real time PCR (Syber green) using two sets of primers (First set of primers; region I (-204/+60); Second set of primers; region II (-783/-627)).

Fig 2 Curcumin inhibits AP-1 binding to miR-21 and *pri-miR-21* promoter activity

a) MTT assays were performed with increasing concentrations of curcumin for 48 hours. Absorbance was measured at 490 nm, and the percentage of proliferating cells calculated compared to DMSO. b,c) 1 μg of the 1.0 kb promoter construct showing maximum activity was transfected along with Renilla into Rko and HCT116 cells. After 12 hours, cells were treated with increasing concentrations of curcumin (5 μM to 30 μM) for 24 hours. For mutational analysis, 1 μg of wild type/mutant construct along with Renilla was transfected into Rko or HCT116 cells. After 12 hours, Rko and HCT116 cells were pretreated in the respective wells for 45 min with curcumin (10 μM and 16 μM respectively) and stimulated with PMA (100 nM) in the respective wells for 24 hours. The firefly luciferase activity was normalized with Renilla luciferase activity. The data are presented as the mean (±S.D). d) The *in vivo* association of p-c-Jun and c-Fos with the *pri-miR-21* promoter was evaluated with a ChIP assay in Rko cells after 24 hours of DMSO, curcumin, or curcumin with PMA treatment. Immunoprecipitated DNA with the specific antibody was amplified by real time PCR (Syber green) using two sets of primers (First set of primers; region I (-204/+60); Second set of primers; region II (-783/-627)). e, f) Rko and HCT116 cells were plated and pre-treated with curcumin (10 μM and 16 μM respectively) before PMA stimulation. After 15 min or 24h, cells were lysed and Western blot analysis was performed.

Fig 3 Curcumin inhibits miR-21 expression *in vitro*

Rko and HCT116 cells were treated with curcumin for 24 hours with increasing concentrations. a, c) pre-miR-21 and mature miR-21 expression was significantly reduced

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with increasing curcumin concentration. * $p \leq 0.05$. b, d) PDCD4 protein levels were increased with increasing concentrations of curcumin treatment.

Fig 4 Curcumin inhibits the cell cycle, migration and invasion
a,b) Curcumin arrests cells in the G2/M phase. Rko and HCT116 were pretreated for 24 hours and cell cycle analysis was performed. c, d) Migration and invasion assays with curcumin treatment in Rko and HCT116 cells. After 24 hours of curcumin treatment, cells were plated on Boyden chambers with matrigel (10µg/well) for invasion and without matrigel for migration assays. Cells were collected after 13h from the top, and migrated/invaded cells were collected from the bottom of the chambers, and counted using the ATP-luminescence-based motility-invasion assay. Mock treated cells served as a control. The experiment was performed three times independently, with triplicates each time. Data represents the average (±S.D.) of one of the three independent experiments.* Statistically significant changes. $p<0.05$.

Fig 5 Curcumin reduces tumor growth and metastasis *in vivo*
$2x10^6$ Rko or HCT116 cells were inoculated on the upper chorioallantoic membrane (CAM) of chicken eggs (n=5). Cells were pretreated with DMSO or curcumin for primary tumor growth, and on the second day treated again with the respective concentration on the upper CAM. For metastasis assays, embryos were intravenously injected with DMSO, or curcumin, on 12th and 14th day. On the 17th day, the embryos were sacrificed and either tumor weights were measured, or DNA of the liver and lungs was extracted a, c) Average of tumor weights from DMSO or curcumin-treated cells grown in chicken embryos. b, d) Genomic DNA was analysed by Real-Time Alu-PCR to determine the number of metastasized Rko and HCT116 cells in liver and lungs after DMSO or curcumin treatment. The individual cell number per egg is given in the box. p values were calculated against the average of curcumin- and DMSO- treated/injected samples. * $p\leq0.05$.

Fig 6 Curcumin inhibits miR-21 expression *in vivo* (CAM tumors)
Curcumin inhibits miR-21 expression in resected tumors from chicken embryos. a, b) Relative miR-21 expression is represented in a box plot. miR-21 expression was significantly reduced in the resected curcumin-treated tumors in both cell lines (Rko: p=0.00006; HCT116: p=0.0035). c) Scheme of functional interactions between curcumin, AP-1 and miR-21 regulated genes.

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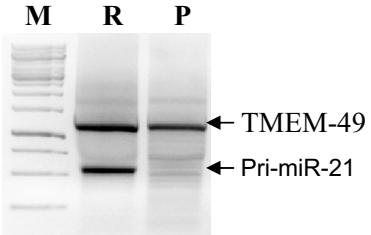
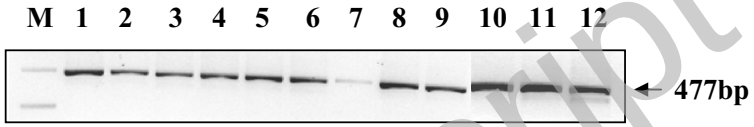
Fig 1

a)



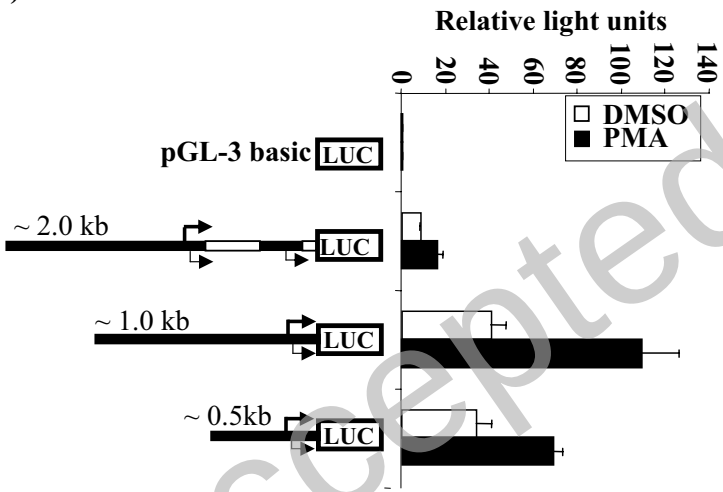
M: Marker; **R:** Rko RNA;
P: Placenta RNA

b)



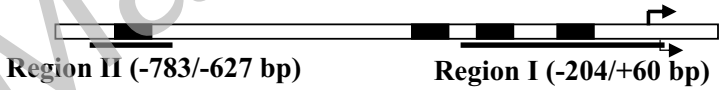
M : Marker; **1:** Rko; **2:** Caco2; **3:** HCT116; **4:** HCT15;
5: HT29; **6:** SW480; **7:** Colo206f; **8:** Colo320; **9:** Geo;
10: WiDr; **11:** HeLa; **12:** MCF-7

c)



: Exon 12 of TMEM-49
: Exon 11 of TMEM-49
: Intron 11 of TMEM-49 with Cai X at al, 2004 reported TSS
: Intron 10 of TMEM-49 with Fujita S at al, 2008 reported TSS (below forward arrow) and new TSS (up forward arrow)

d)



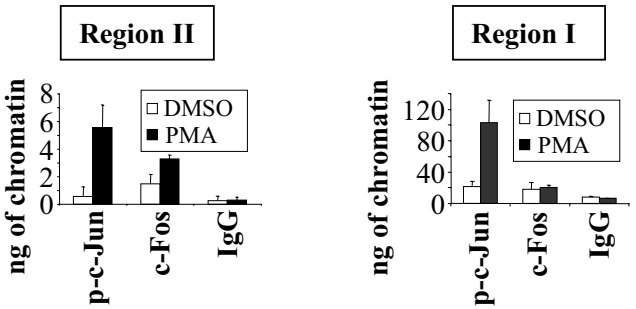


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Fig 2

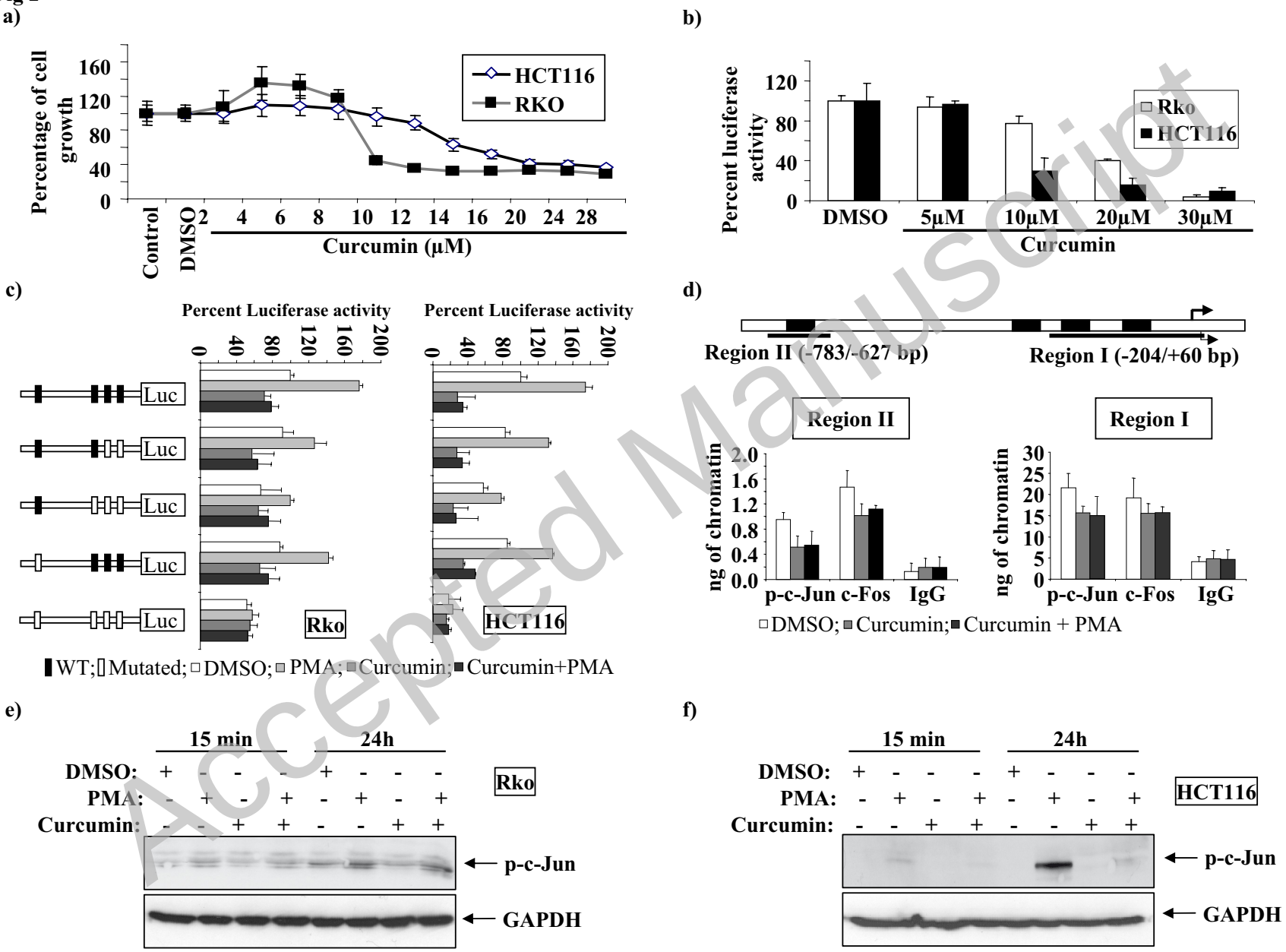

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Fig 3

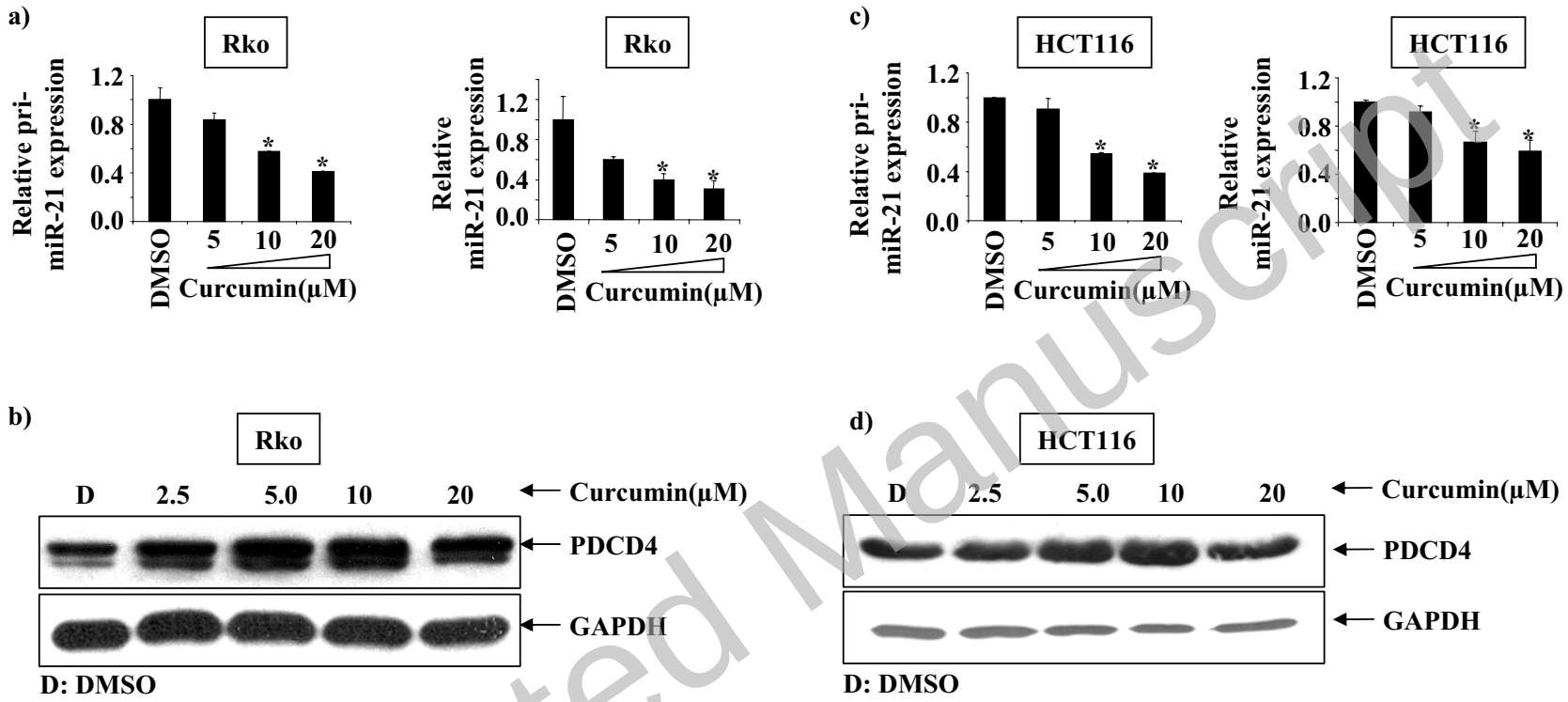

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Fig 4

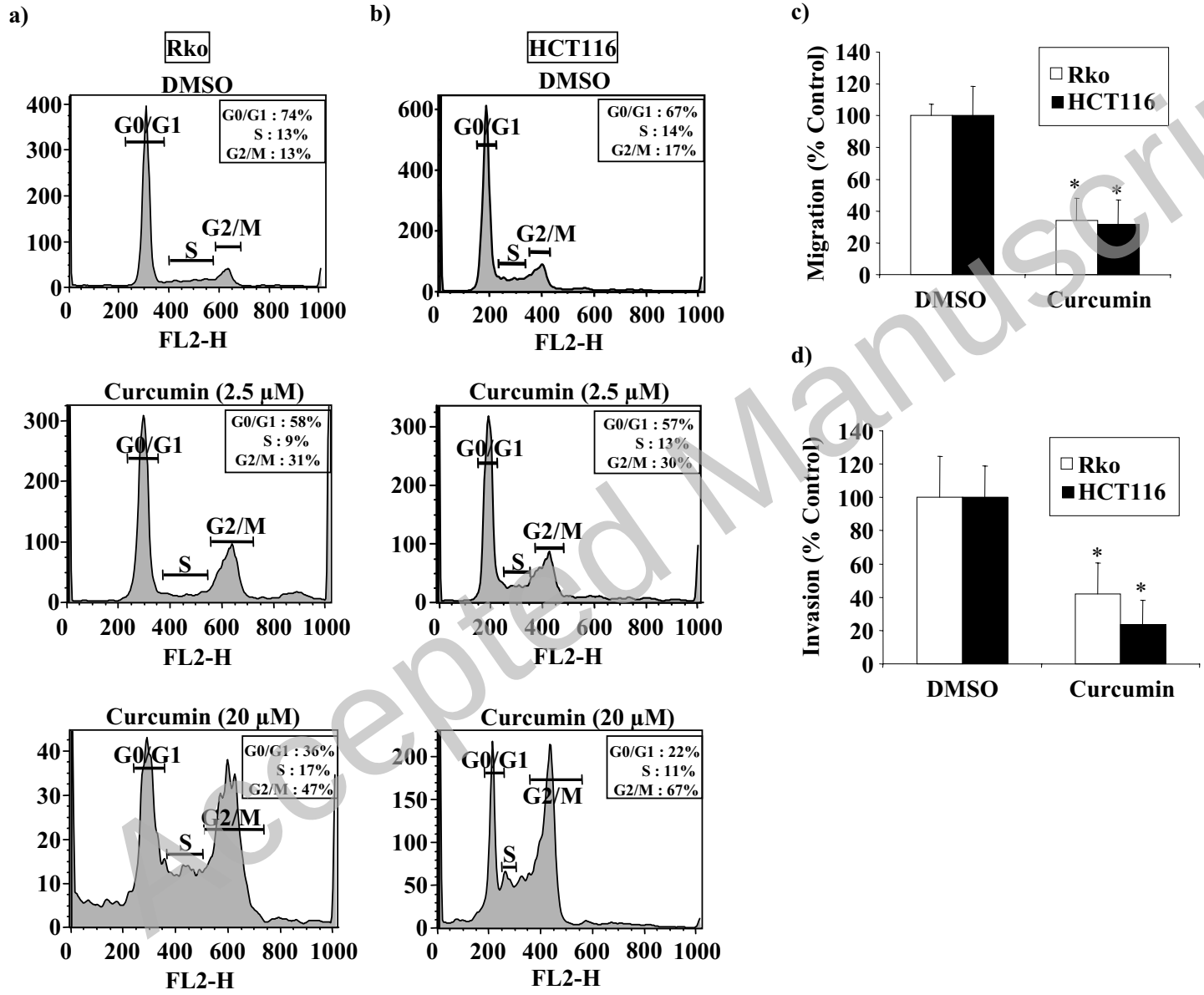

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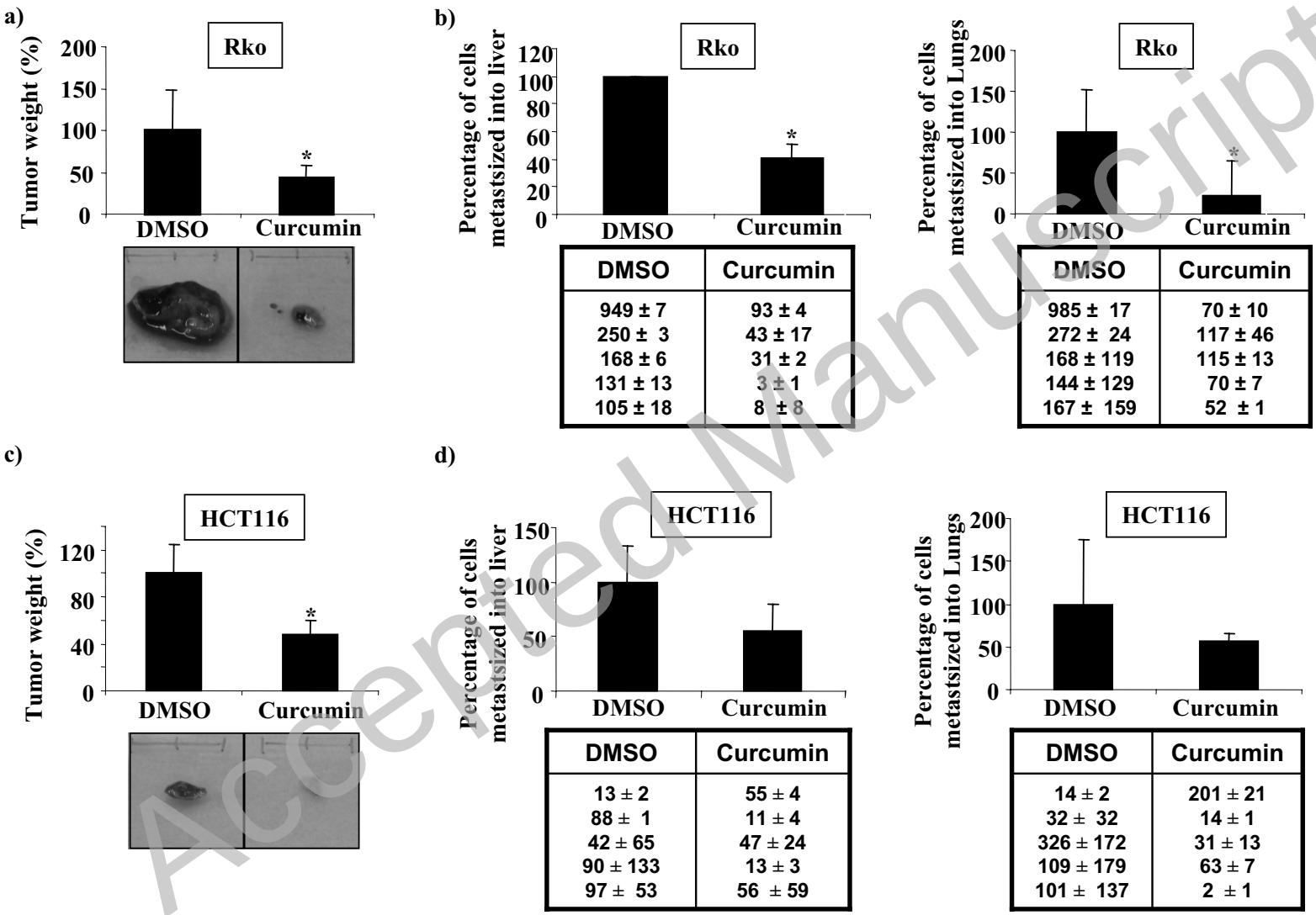
Fig 5



DMSO	Curcumin
949 ± 7	93 ± 4
250 ± 3	43 ± 17
168 ± 6	31 ± 2
131 ± 13	3 ± 1
105 ± 18	8 ± 8

DMSO	Curcumin
985 ± 17	70 ± 10
272 ± 24	117 ± 46
168 ± 119	115 ± 13
144 ± 129	70 ± 7
167 ± 159	52 ± 1

DMSO	Curcumin
13 ± 2	55 ± 4
88 ± 1	11 ± 4
42 ± 65	47 ± 24
90 ± 133	13 ± 3
97 ± 53	56 ± 59

DMSO	Curcumin
14 ± 2	201 ± 21
32 ± 32	14 ± 1
326 ± 172	31 ± 13
109 ± 179	63 ± 7
101 ± 137	2 ± 1

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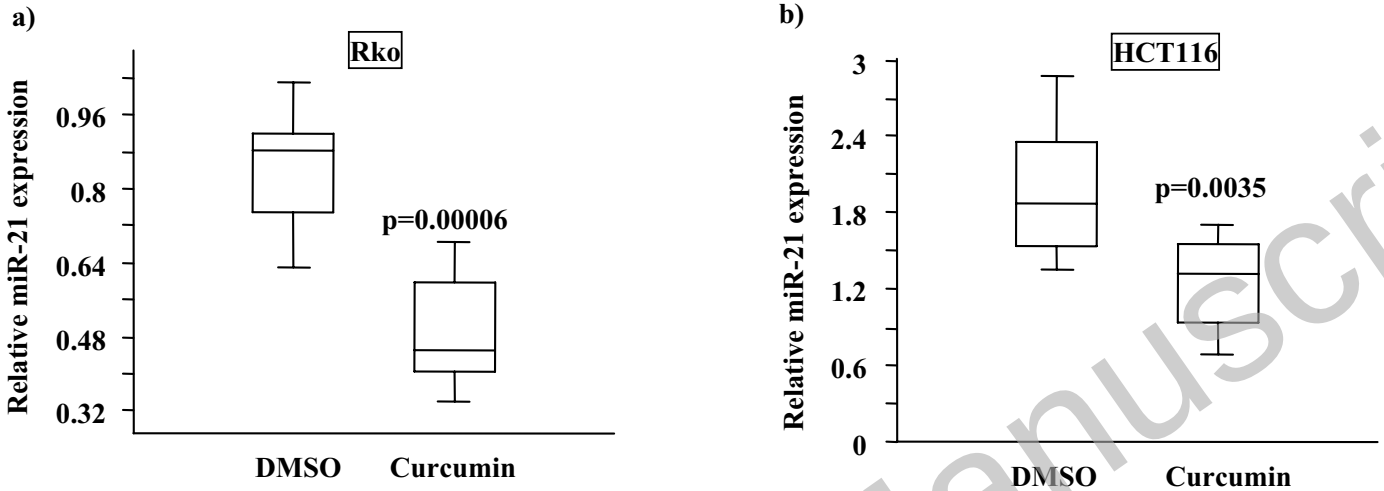
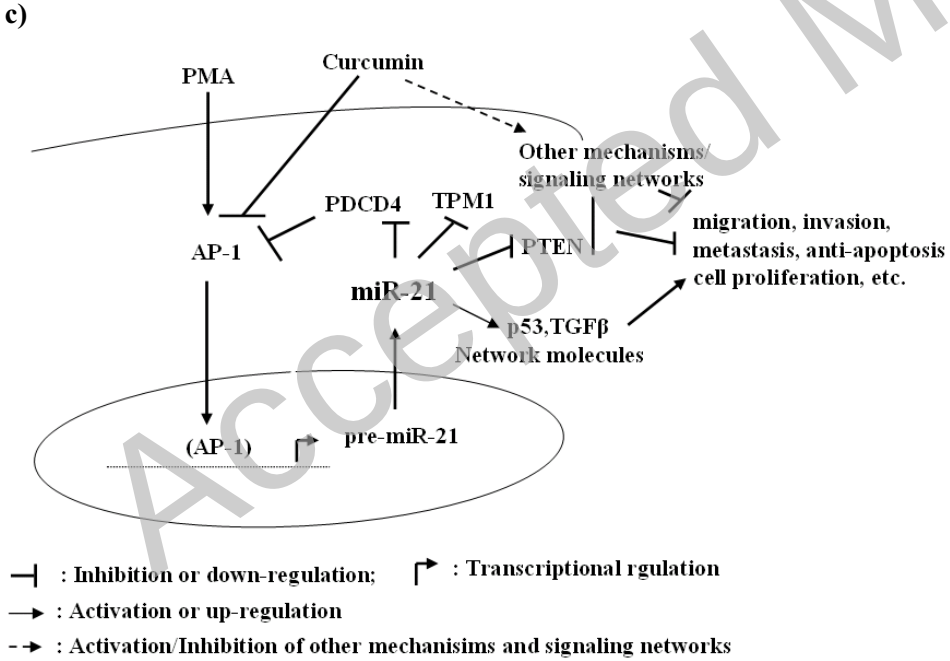
Fig 6





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