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miR-143 decreases COX-2 mRNA stability and expression in pancreatic cancer cells

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ABSTRACT

Small non-coding RNAs, microRNAs (miRNA), inhibit the translation or accelerate the degradation of message RNA (mRNA) by targeting the 3′-untranslated region (3′-UTR) in regulating growth and survival through gene suppression. Deregulated miRNA expression contributes to disease progression in several cancers types, including pancreatic cancers (PaCa). PaCa tissues and cells exhibit decreased miRNA, elevated cyclooxygenase (COX)-2 and increased prostaglandin E_2 (PGE_2) resulting in increased cancer growth and metastases. Human PaCa cell lines were used to demonstrate that restoration of miRNA-143 (miR-143) regulates COX-2 and inhibits cell proliferation. miR-143 were detected at fold levels of 0.41 ± 0.06 in AsPC-1, 0.20 ± 0.05 in Capan-2 and 0.10 ± 0.02 in MIA PaCa-2. miR-143 was not detected in BxPC-3, HPAF-II and Panc-1 which correlated with elevated mitogen-activated kinase (MAPK) and MAPK kinase (MEK) activation. Treatment with 10 μM of MEK inhibitor U0126 or PD98059 increased miR-143, respectively, by 187 ± 18 and 152 ± 26-fold in BxPC-3 and 182 ± 7 and 136 ± 9-fold in HPAF-II. miR-143 transfection diminished COX-2 mRNA stability at 60 min by 2.6 ± 0.3-fold in BxPC-3 and 2.5 ± 0.2-fold in HPAF-II. COX-2 expression and cellular proliferation in BxPC-3 and HPAF-II inversely correlated with increasing miR-143. PGE_2 levels decreased by 39.3 ± 5.0% in BxPC-3 and 48.0 ± 3.0% in HPAF-II transfected with miR-143. Restoration of miR-143 in PaCa cells suppressed of COX-2, PGE_2, cellular proliferation and MEK/MAPK activation, implicating this pathway in regulating miR-143 expression.

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1. Introduction

Small non-coding RNAs, microRNAs (miRNAs), target the 3′-untranslated region (3′-UTR) of message RNA (mRNA) to repress translation or accelerate the degradation of the mRNA in regulating gene expression [1,2]. Functional studies have demonstrated the involvement of miRNAs in diverse biological process, including cellular development, differentiation and proliferation [2,3]. Several cancers types exhibit deregulated miRNA expression resulting in disease progression, including pancreatic cancers (PaCa) [4]. miRNAs serve as oncogenes or tumor suppressors by modulating the level of critical proteins [5]. As a tumor suppressor, miRNAs has been reported to regulate aberrant cyclooxygenase (COX) expression in several cancers [6,7].

COX exists as two isoforms, a constitutive COX-1 and an inducible COX-2, and is critical in prostaglandin E_2 (PGE_2) synthesis [8]. COX-2 and PGE_2 mediates acute inflammation and in excess, promotes growth and survival of cancer [9,10]. Induction of COX-2 results from a variety of stimuli (cytokines, peptides, growth factors) and controlled at different levels by many signaling pathways [11]. Interestingly, PGE_2 induces COX-2 expression through the prostanoid receptor EP_2, with the subsequent activation of cyclic AMP response element binding protein (CREB) pathway that further increases PGE_2 production [12]. Restoration of miRNA expression has been shown to abrogate COX-2 expression in cancer cells [13]. Specifically, miR-143 has been identified to target COX-2 resulting in decreased cancer cell growth and metastases [14,15].

We previously reported increased COX-2 and PGE_2 contributes to growth and survival of both human PaCa tissue and cell lines [16,17]. Furthermore, PaCa exhibiting active K-Ras mutations [18,19] can suppress miR-143 repression of Ras responsive

Abbreviations: COX-2, cyclooxygenase-2; CREB, cyclic AMP responsive element binding protein; miR-143, microRNA-143; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PaCa, pancreatic cancer; PGE_2, prostaglandin E_2; PKA, protein kinase A; RREB1, ras responsive element binding protein.

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element binding protein-1 (RREB1) [20,21]. This study focuses on whether restoration of miR-143 expression can regulate COX-2 expression and inhibit PaCa cell proliferation. BxPC-3 and HPAF-II transfected with miR-143 resulted in diminished COX-2 mRNA and COX-2 protein expression. Loss of COX-2 corresponded with a decrease in PGE_2 synthesis and cellular proliferation in both cell lines. This report demonstrates that the down-regulation of COX-2 expression by miR-143 results in part from the destabilization of COX-2 mRNA and inhibition of RREB1 activation through MEK/MAPK suppression.

2. Materials and methods

2.1. Reagents

Actinomycin D, arachidonic acid, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), chloroform and methanol were purchased from Sigma (St. Louis, MO). COX-2 antibody and COX-2 specific inhibitor N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398) was purchased from Cayman Chemical (Ann Arbor, MI). CREB, p-CREB, MEK, p-MEK, MAPK, p-MAPK, p-p38MAPK and RREB1 antibodies were purchased from Cell Signaling (Danvers, MA). GAPDH antibody was acquired from Santa Cruz Biotechnology (Santa Cruz, CA). MEK inhibitors U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) and PD98059 (2′-amino-3′-methoxyflavone) were acquired from Calbiochem (EMD Millipore, Billerica, MA). Horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG and enhanced chemiluminescence (ECL) reagents were obtained from (ThermoFisher Scientific, Pittsburg, PA).

2.2. Cell culture

Human PaCa cell lines AsPC-1 (CRL-1682), BxPC-3 (CRL-1687), Capan-2 (HTB-80), HPAF-II (CRL-1997), MIA PaCa-2 (CRL-1420) and Panc-1 (CRL-1469) were acquired from American Type Culture Collection (Rockville, MD). AsPC-1, BxPC-3 and HPAF-II were propagated in RPMI 1640 medium supplemented with 10% FBS and 1 % PSG antibiotic mix (100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine) (Life Technologies) at 5% CO_2 and 37 °C. Capan-2, MIA PaCa-2 and Panc-1 were propagated in DMEM supplemented with 10% FBS and 1% PSG antibiotic mix at 10% CO_2 and 37 °C. For experiments, cells were propagated in 100-mm tissue culture dishes to confluence (5–7 days) and arrested in serum-free medium.

2.3. RNA extraction

RNA was extracted with 1 ml of Trizol reagent (Life Technologies) and 0.2 ml of chloroform at 12,000×g for 15 min at 4 °C, precipitated with 0.5 ml of 2-propanol at 12,000×g for 10 min at 4 °C, washed with 75% ethanol at 7,500×g for 5 min at 4 °C, dissolved in 30 μL of RNA Storage Solution containing 1 mM sodium citrate, pH 6.4 (Life Technologies) and stored at −20 °C for subsequent analysis. RNA concentration was quantified at dual wavelengths of 260 and 280 nm on a Bio-Rad spectrophotometer (Hercules, CA).

2.4. In silico miR-143 sequence verification

Targets of miR-143 were identified using TargetScan (targetscan.org) and miRanda (microRNA.org) on-line database search. COX-2 was identified as a potential target with predicted binding of hsa-miR-143 sequence 3′-GUAGAG-5′ to COX-2 3′-UTR sequence 3′-CAUCUC-5′.

2.5. miR-143 detection

Small RNAs tagged with poly A and converted to cDNA (QuantiMir, System Biosciences, Mountain View, CA) were quantified using SYBR green kit (Bio-Rad). Primers used were miR-143 (Accession MIMAT0000435) forward sequence: 5′-TGA GAT GAA GCA CTG TAG CTC-3′ and control U6 snRNA forward sequence: 5′-CGC AAG GAT GAC ACG CAA ATT C-3′ supplied by QuantiMir. Thermal cycling conditions were 95 °C for 3 min of activation, 40 cycles of 95 °C for 15 s denaturing and 55 °C for 60 s of annealing/extending and 55–95 °C at 0.5 °C increments for 5 s of melt curve analysis. Ct values were normalized to U6 control and reaction mixtures were resolved on a 1.5% agarose gel at 90 V for 60 min.

2.6. miR-143 transfection

BxPC-3 and HPAF-II were seeded at 400,000 cells/well in 6-well plates, allowed to stabilize overnight in RPMI complete media, and replenished with RPMI supplemented with 5% FBS. Cells were treated with 0, 25, 50 and 100 nM of miR-143 (C-300611-05-0005, ThermoFisher Scientific) delivered in 10 μL of Lipofectamine 2000 (Life Technologies) per well, incubated for 24 h, replenished with complete RPMI and harvested for RNA or protein after 72 h.

2.7. COX-2 mRNA by real time PCR

RNA extracts were reverse transcribed and cDNAs amplified using TaqMan Gold RT-PCR kit (Applied Biosystems, Foster City, CA). COX-2 (accession NM_000963) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were quantified by real-time PCR analysis using a Bio-Rad IQ5. COX-2 primer used was sense 5′-GCT TTA TGC TGA AGC CCT ATG A-3′ and antisense 5′-TCC AAC TCT GCA GAC ATT TCC-3′ with corresponding universal probe 2 (Cat. 04684982001, Roche, Indianapolis, IN). Human GAPDH primer and probe set was acquired from Applied Biosystems. Thermal cycling conditions were 48 °C for 15 min of activation, 95 °C for 10 min of amplification and 40 cycles of 95 °C at 15 s denaturing and 60 °C at 60 s annealing/extending.

2.8. Protein expression/Western blotting

Protein were harvested using RIPA lysis buffer (ThermoFisher Scientific), diluted with 2× LDS buffer containing SDS (Life Technologies) and denatured at 95 °C for 10 min. Protein lysates were subjected to a variable 4–20% Precise Tris–Glycine gel (ThermoFisher Scientific) for 45 min at 200 V and transferred onto a nitrocellulose membrane for 75 min at 100 V. Membranes were washed with Tris buffered saline (TBS, Sigma), blocked with 5% dried nonfat milk (Bio-Rad) in 1% tween–TBS and probed with antibody raised against p-CREB (1:1000), p-MEK (1:1000), p-MAPK (1:1000), RREB1 (1:1000), p-p38MAPK (1:1000) or COX-2 (1:1000) with GAPDH (1:2500) as a visual loading control. Bands were visualized by secondary antibody IgG-linked horseradish peroxidase conjugate (1:2500) and ECL and quantified using ChemiDoc XRS imaging software (Bio-Rad).

2.9. PGE_2 assay

PGE_2 from serum-starved, confluent cells cultured in 24-well plates challenged with 5 μM of arachidonic acid stabilized with fatty acid-free BSA (Sigma) were quantified using Prostaglandin E_2 Express EIA kit and 6-keto Prostaglandin $F_{1\alpha}$ EIA kit (Cayman Chemical). PGE_2 absorbance were measured at 405–420 nm and normalized to protein absorbance readings at 595 nm using the Bradford assay (Bio-Rad).

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