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ORIGINAL ARTICLE

MUC1 regulates cyclin D1 gene expression through p120 catenin and β-catenin

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MUC1 interacts with β-catenin and p120 catenin to modulate WNT signaling. We investigated the effect of overexpressing MUC1 on the regulation of cyclin D1, a downstream target for the WNT/β-catenin signaling pathway, in two human pancreatic cancer cell lines, Panc-1 and S2-013. We observed a significant enhancement in the activation of cyclin D1 promoter-reporter activity in poorly differentiated Panc1.MUC1F cells that overexpress recombinant MUC1 relative to Panc-1.NEO cells, which express very low levels of endogenous MUC1. In stark contrast, cyclin D1 promoter activity was not affected in moderately differentiated S2-013.MUC1F cells that overexpressed recombinant MUC1 relative to S2-013.NEO cells that expressed low levels of endogenous MUC1. The S2-013 cell line was recently shown to be deficient in p120 catenin. MUC1 is known to interact with P120 catenin. We show here that re-expression of different isoforms of p120 catenin restored cyclin D1 promoter activity. Further, MUC1 affected subcellular localization of p120 catenin in association with one of the main effectors of P120 catenin, the transcriptional repressor Kaiso, supporting the hypothesis that p120 catenin relieved transcriptional repression by Kaiso. Thus, full activation of cyclin D1 promoter activity requires β-catenin activation of TCF-lef and stabilization of specific p120 catenin isoforms to relieve the repression of KAISO. Our data show MUC1 enhances the activities of both β-catenin and p120 catenin.

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INTRODUCTION

MUC1 is a transmembrane type I mucin expressed by ductal epithelial cells. Full-length MUC1 is synthesized as a single polypeptide chain, which undergoes an early proteolytic cleavage in its SEA domain (sea-urchin sperm protein, enterokinase and agrin), creating two subunits that remain associated during intracellular synthesis, transit and expression on the cell surface. The extracellular domain of MUC1 contains an extended tandem repeat and an SEA domain, and can serve as a ligand for receptors such as E-selectin[1] or I-CAM.[2] The cytoplasmic tail (CT) of MUC1 contains 69 amino acids and includes several potential phosphorylation sites.[3] Earlier studies provided evidence that the MUC1 CT functions as an adaptor protein during morphogenetic signaling through protein–protein interactions with p53,[4,5] β-catenin[6,7] or Grb2/SOS,[8] following phosphorylation by GSK-3β,[9] c-Src,[10] EGFR,[11] PKC-δ,[12] Lyn[13] or ZAP-70.[14] MUC1 CT modulates signaling in other pathways including those that affect cell proliferation, migration and invasion.[15,16]

MUC1 is overexpressed by different adenocarcinomas including pancreatic cancer.[17] Previously, we reported that under conditions of MUC1 expression, fragments of the MUC1 CT were detected in the cytosol and nucleus of human pancreatic cancer cell lines, Panc-1.MUC1F and S2-013.MUC1F, and that the MUC1 CT associated with and stabilized the steady-state level of nuclear β-catenin.[18] A well-documented functional consequence of the nuclear translocation and accumulation of β-catenin during WNT signaling is that nuclear β-catenin binds to LEF/Tcf-4 transcription factors and changes the architectural conformation of DNA, resulting in the transcriptional activation of several genes including cyclin D1, c-myc, connexin 43 and c-jun.[19–21] Recent reports suggest that MUC1/β-catenin complexes influence the transcription of WNT target genes associated with carcinogenesis, including cyclin D1.[22]

We previously observed that MUC1 expression stabilized β-catenin and obtained preliminary results showing that this significantly enhanced cyclin D1 promoter activity in one pancreatic cancer cell line, Panc1; however, a second pancreatic cancer cell line that was evaluated (S2-013) did not show any effects by MUC1 on cyclin D1 transcription. We therefore investigated further the deficiency of activity in the second cell line and discovered that this line lacked expression of p120 catenin. Re-expression of p120 catenin in these cells restored cyclin D1 promoter activity and responsiveness to modulation by MUC1. Thus, this study provides insights into multiple aspects of molecular mechanisms whereby MUC1, P120 catenin and Kaiso modulate the activity of WNT signaling, and consequent effects on cellular proliferation of pancreatic cancer cells.

RESULTS

Expression of MUC1 promotes cyclin D1 gene promoter activity in some but not all pancreatic cancer cell lines

Expression of MUC1 stabilizes β-catenin and enhances its steady-state nuclear levels[18] in pancreatic cancer cells including those used in the studies reported here. We explored the functional influence of MUC1 expression and association with nuclear β-catenin on the regulation of cyclin D1, a gene known to be regulated by β-catenin and LEF/Tcf-4.

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As β-catenin functions in transcriptional regulation by providing a transactivation domain for forming bipartite complexes with other factors such as LEF/Tcf-4, which together serve as transcriptional coactivators,[23] we initially evaluated the steady-state levels of LEF/Tcf-4 protein in two human pancreatic cancer cell lines and confirmed that there were equivalent levels of expression of LEF/TCF4 in these cell lines (Supplementary Figure 1). Western blots of membrane/cytoplasmic and nuclear extracts of Panc-1 and S2-013 subclones expressing either high levels of recombinant MUC1 (MUC1F) or very low (Panc1.NEO) to low (S2-013.NEO) levels of endogenous MUC1 were probed with a monoclonal antibody (mAb) to Tcf-4. Irrespective of the levels of MUC1, nuclear Tcf-4 protein was expressed at relatively constant levels in each of the Panc-1 and S2-013 cell lines.

Cyclin D1-promoter/luciferase-reporter assays were used to evaluate the effect of MUC1 expression on activation of cyclin D1 gene transcription. TCF/LEF-driven promoter activity was evaluated by using a TOPFLASH construct, which contains a 1745-bp sequence of the human cyclin D1 promoter that includes a wild-type LEF/Tcf-4-binding site followed by a luciferase reporter gene. A construct that encoded the same cyclin D1 promoter region with a mutated LEF/Tcf-4-binding site (FOPFLASH) was used as a control. Each promoter/reporter construct was transiently transfected into matched sets of two different pancreatic cancer cell lines expressing high levels of MUC1 and low or no detectable expression of MUC1-Panc-1 and -S2-013 (Panc-1.MUC1F and S2-013.MUC1F expressed high levels of recombinant MUC1, and controls included S2-013.NEO (low levels of endogenous MUC1) and Panc-1.NEO cells (very low levels of endogenous MUC1)).

There was no activity of cyclin D1 promoters with mutated LEF/Tcf-4-binding sites in NEO control and MUC1-expressing cells (Figure 1). In contrast, for the construct containing wild-type sequences, there was low cyclin D1 promoter activity in NEO control cells, but there was a significant enhancement (approximately 100-fold) for cyclin D1 promoter activity in Panc-1.MUC1F (Figure 1). This supported the hypothesis that WNT signaling through β-catenin-activated TCF/LEF in these cells and that MUC1 stabilization of β-catenin dramatically increased cyclin D1 promoter activity. Curiously, however, we found that cyclin D1 promoter activity in S2-013 cells was low and unresponsive to alterations in MUC1 expression (Figure 1), either in S2-013 cells expressing high levels of MUC1 or in cells in which the low levels of endogenous MUC1 was knocked down by RNA interference. This negative result indicated that MUC1 stabilization of β-catenin is not sufficient to activate or influence cyclin D1 promoter activity and that transactivation of the cyclin D1 gene promoter in this cell line is affected by other factors.

We confirmed the consequences of expressing MUC1 on steady-state levels of endogenous cyclin D1 in the Panc1 and S2-013 pancreatic cancer cell lines. Consistent with the promoter-reporter assay results, expression of MUC1 elevated the steady-state level of cyclin D1 transcripts (data not shown) and cyclin D1 protein in Panc-1 cells, but not in S2-013 cells (Figure 2). These results suggest that expression of MUC1 contributes to the increase in cyclin D1 transcripts in the Panc-1 cells, but not in S2-013 cells. Previous studies reported that expression of MUC1 co-activated the cyclin D1 gene by transactivation in human HCT116 colon carcinoma cells.[24] A recent report suggests that MUC1 induces TCF7L2 transcription factor activation and promotes cyclin D1 expression in human breast cancer cells.[22] Taken together, these results indicate that the effects of MUC1 expression on cyclin D1 gene expression vary among different cell types.

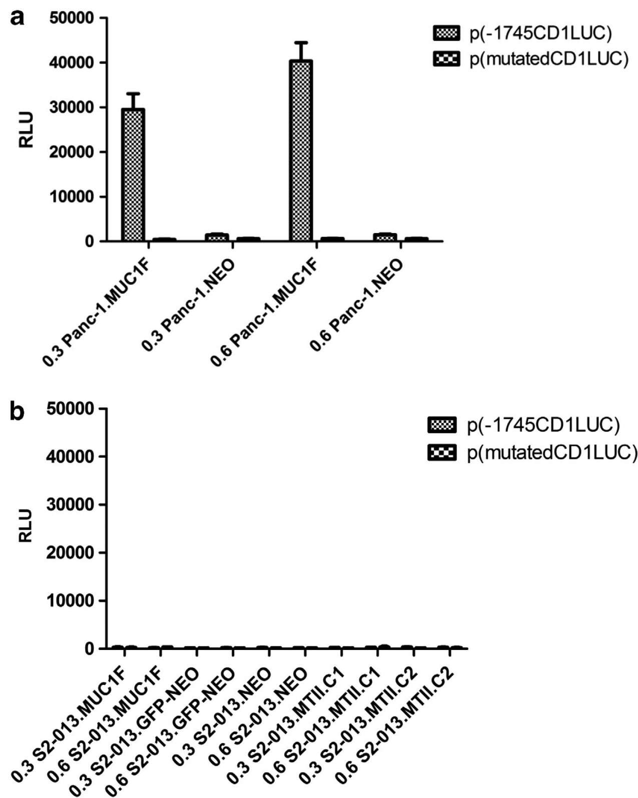

Figure 1. Luciferase assay detecting effect of MUC1 on cyclin D1 promoter activity. Cells were transfected with 300 or 600 ng of the reporter plasmids containing 1745 bp of the cyclin D1 gene promoter (−1745CD1Luc, TOPFLASH) consists of wild-type LEF-1/Tcf-4-binding site or reporter plasmids containing mutated LEF-1/Tcf-4-binding site in the cyclin D1 gene promoter (FOPFLASH), together with 400 ng of a synthetic Renilla luciferase reporter plasmids SV40 as transfection control. Each transfection was carried out in duplicate plates, and the data (relative lumenescence units-RLU) shown here are representative of three independent experiments. (**a**) Panc-1.MUC1F or Panc-1.Neo (95% confluence). (**b**) S2-013.MUC1F cells, S2-013.NEO cells, S2-013 GFP-NEO, two clones of S2-013 cells in which MUC1 was knocked down by MUC1-siRNA—S2-013.MTII.C1 and S2-013.MTII.C2 (95% confluence).

Re-expression of specific p120 catenin isoforms in S2-013 cells restore cyclin D1 promoter activity

We recently reported that the S2-013 cell line is deficient in expression of p120 catenin, and that re-expression of p120 catenin in these cells stabilized and enhanced steady-state levels of β-catenin.[15] P120 catenin is a member of the Armadillo repeat protein family that has multiple isoforms whose expression and localization varies depending on tissues and cell types.[15] P120 catenin stabilizes the cadherin–catenin adhesion complex at the plasma membrane, but also has additional roles in the cytoplasm and nucleus, where it has been reported to modulate transcription by associating with the transcriptional repressor Kaiso.[25,26] We therefore examined p120 catenin expression in the Panc1 utilized here and sought to further investigate the effects of p120 catenin re-expression in S2-013 cells on MUC1 modulation of cyclin D1 promoter activity. The western blot from Figure 3a confirms that S2-013 cells do not express p120 catenin and reveal that Panc-1 cells express different isoforms of p120 catenin. Isoforms of p120 catenin differ in amino termini because of alternative splicing and usage of alternate translation-initiating codons, each of which has a distinct start codon in the N-terminal region.[27–30] Isoform 1 contains a coiled-coil domain at the N-terminus; isoform 2 contains the entire ‘regulatory region’; isoform 3 has most of regulatory region; isoform 4 lacks the majority of the regulatory region. Panc-1 Neo cells expressed p120 isoform 3 and low levels of isoform 1. Expression of MUC1 in Panc-1 cells stabilized and

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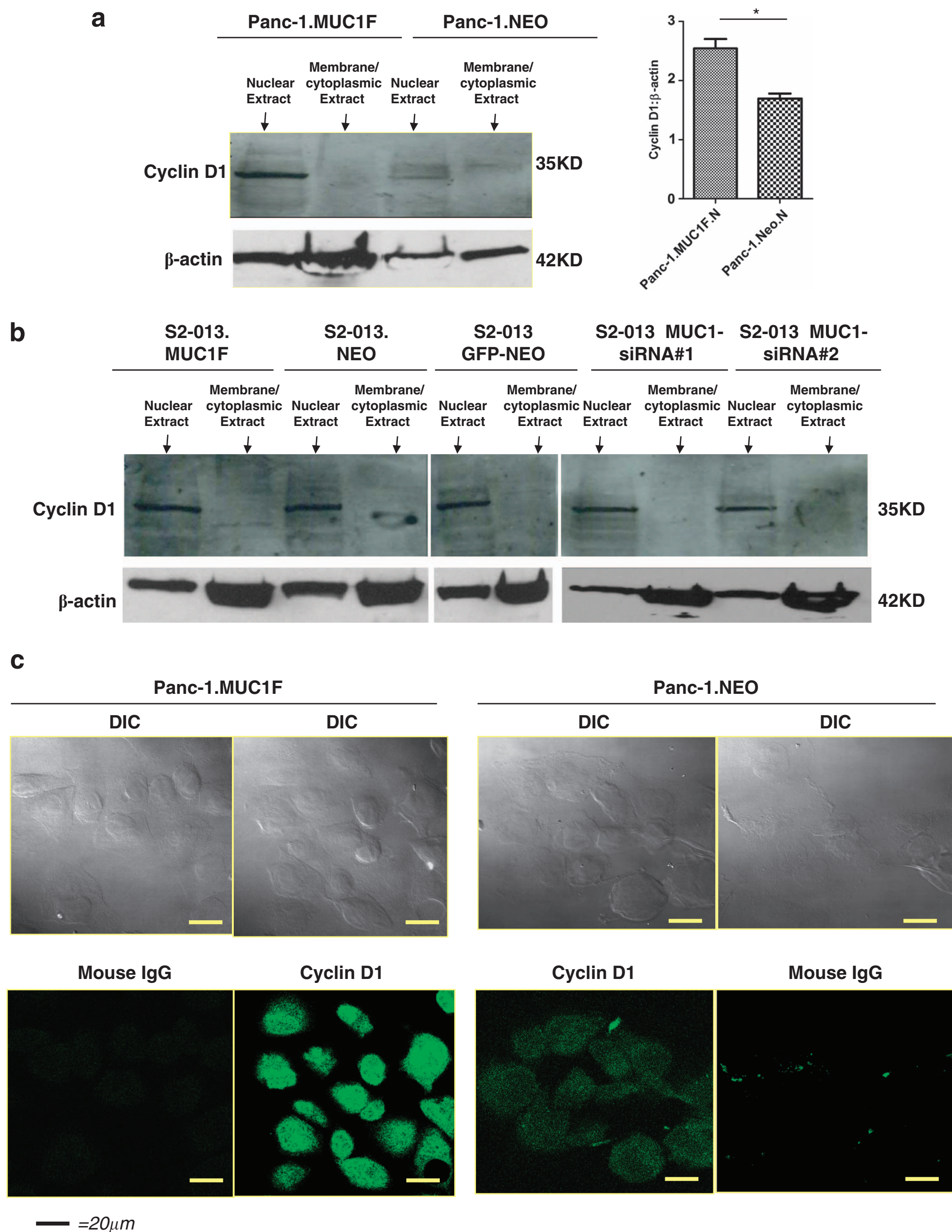

Figure 2. Western blot protein levels of cyclin D1 in pancreatic cancer cell lines. (**a**, **b**) Membrane/cytoplasmic extracts and nuclear extracts from Panc-1.MUC1F, Panc-1.NEO, S2-013.MUC1F, S2-013.NEO, S2-013 GFP-NEO, two clones of MUC1-siRNA S2-013 cells were subjected to 4–20% SDS–PAGE and analyzed by immunoblot (IB) with an anti-cyclin D1 mAb and β-actin as a loading control. Right panel in **a** shows densitometry analysis of the signal for nuclear cyclin D1 normalized to the signal for β-actin. *$P<0.01$, significant difference. (**c**) Cyclin D1 protein expression is enhanced by expression of MUC1 in Panc-1.MUC1F cells as compared with Panc-1.NEO cells. Confocal microscopy was used to determine the cyclin D1 protein expression in Panc-1.MUC1F and Panc-1.NEO cells. Cells grown above 90% confluence on coverslips were fixed with 4% paraformaldehyde and permeablized with Trixton X-100 before incubation with mAb anti-cyclin D1, which was identified with fluorescein isothiocyanate-conjugated secondary antibody and visualized as green color. Images were examined with a Zeiss LSM 410 laser scanning microscope (Bar = 20 μM; × 100 magnification; results shown here represent three or four individual cell scanning observations.).

increased expression of different isoforms of p120 catenin, especially isoforms 1 and 4.

Given the deficiency of p120 catenin expression in S2-013 cells, and the possibility p120 catenin could also influence WNT signaling through β-catenin, we elected to examine cyclin D1 promoter activity in S2-013 cells that expressed different p120 catenin isoforms in the presence of high-level and low-level expression of MUC1 (Figure 3b). Interestingly, different isoforms of p120 catenin expressed in S2-013 cells showed distinct subcellular localizations. P120 catenin 1A was mainly present on the cell membrane and cytoplasm. P120 catenin 3A showed greater localization to the nucleus compared with other isoforms. P120 catenin 4A was mostly distributed at the cell surface with a small amount detected in the cytoplasm (Figure 3c). Upon MUC1 expression, the nuclear localizations of p120 catenin 1A and 3A were slightly increased. P120 catenin 4A was mostly localized on the cell membrane in the presence of MUC1 (Figure 3d). The distinct subcellular localizations of different p120 catenin isoforms is partly associated with distinct functions related to cell adhesion, motility and metastasis.[15] The differences in subcellular localization and functional domains in the different p120 catenin isoforms were also predicted to affect WNT signaling and

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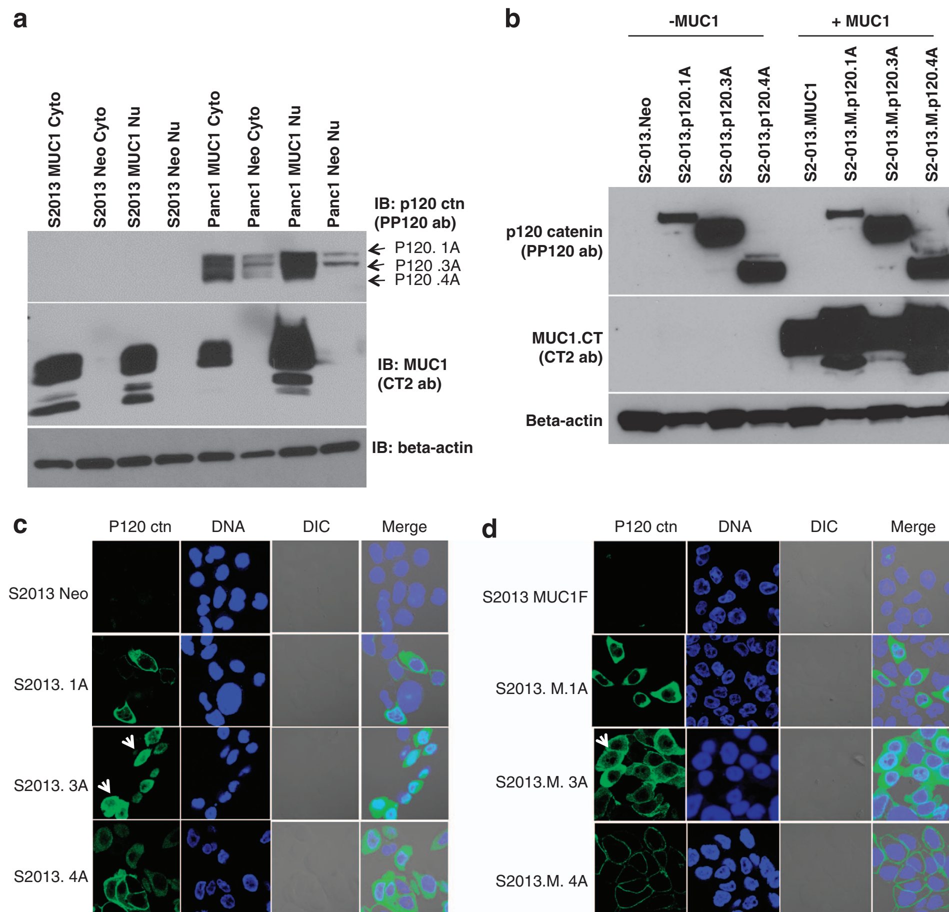

Figure 3. P120 catenin was not detected in S2-013 cells and re-expression of different p120 catenin isoforms in this cells showed distinct subcellular localization. (**a**) Membrane/cytoplasmic extracts and nuclear extracts from S2-013.NEO, S2-013.MUC1F, Panc-1.NEO, Panc-1.MUC1F were subjected to 10% SDS–PAGE and analyzed by immunoblot (IB) with an anti-p120 catenin mAb. The same blot was striped and reprobed with anti-MUC1.CT antibody. (**b**) Re-expression of different p120 catenin isoforms were confirmed by western blot using the same p120 catenin antibody and MUC1 antibody. (**c** and **d**) Immunofluorescence analysis of re-expression of p120 catenin in S2-013 cells. The green color indicates stains for p120 catenin. Blue indicates 4′-6-diamidino-2-phenylindole (DAPI) staining for nuclei. The arrowhead indicates subcellular localization of p120 catenin in the nucleus.

downstream transcriptional regulation, including cyclin D1 promoter activity. Thus, we performed luciferase assays with S2-013 cell lines expressing the different p120 catenin isoforms with and without expression of MUC1. Strikingly, re-expression of p120 catenin 1A or 4A (but not 3A) significantly increased the cyclin D1 promoter activity in S2-013 cells compared with NEO control. Interestingly, superimposing MUC1 expression in this system significantly increased cyclin D1 activity only when co-expressed with p120 catenin isoform 4A (Figure 4a). The effects of MUC1 and p120 catenin on cyclin D1 promoter activity were also reflected in levels of protein expression (Figure 4b).

These data suggest that full activation of cyclin D1 promoter activity requires β-catenin activation of TCF-lef and stabilization of specific p120 catenin isoforms. Our data show that MUC1 enhances the activities of both β-catenin and isoform 4A of p120 catenin in this system.

Different p120 catenin isoforms association with Kaiso

We sought to investigate the mechanism by which p120 catenin was enhancing TCF/Lef activity on the cyclin D1 promoter. The transcriptional repressor Kaiso has been shown to bind to the cyclin D1 promoter and repress transcription in both a sequence-specific and methylation-dependent manner,[31] and it has been reported that p120 catenin can bind to Kaiso and de-repress its effects on transcription.[25,26] We investigated the possibility that specific isoforms of p120 catenin physically associate with Kaiso and sought to establish the precise subcellular localization of these complexes, given that binding in different subcellular locales might be associated with different types of activities. Result from proximity ligation assays revealed that Kaiso was highly associated with p120 catenin 3A in the nucleus (Figure 5) with less seen in the cytoplasm. P120 catenin isoform1A showed moderate and equivalent levels of interaction with Kaiso in both the nucleus and cytoplasm (Figure 5). P120 catenin isoform 4A showed higher levels of interaction with Kaiso in the cytoplasm (Figure 5). These differences in localization may explain in part the observed effects of different isoforms on cyclin D1 promoter activity. The high levels of interaction of p120 catenin isoform 3A with Kaiso in the nucleus (Figure 5) together with the results of cyclin D1 promoter activity (Figure 4) suggest that this isoform may not relieve Kaiso repression, whereas sequestration of Kaiso in the cytoplasm by p120 catenin isoforms 1A and 4A may enable de-repression of Kasio at the cyclin D1 promoter.

MUC1 expression or p120 catenin re-expression affects cell growth properties

We evaluated growth rates of Panc-1 and S2-013 cells to investigate the functional consequences of cyclin D1 gene transcriptional activation and elevation of cyclin D1 protein in response to MUC1 expression and p120 catenin re-expression. There were dramatic and significant differences in the *in vitro*

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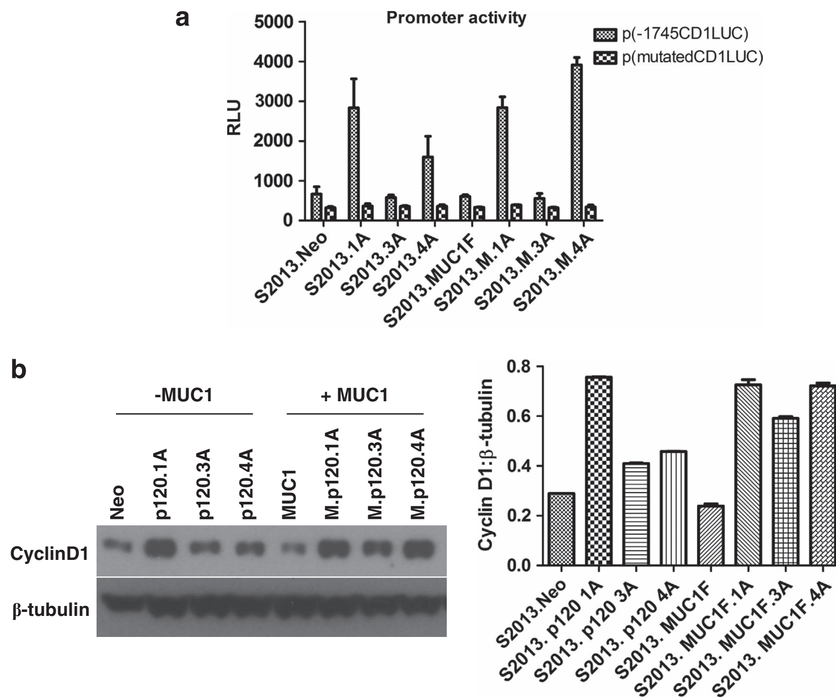

Figure 4. Re-expression of specific p120 catenin isoform restores cyclin D1 promoter activity and increases its protein expression level. (**a**) Luciferase reporter assay of cyclin D1 promoter. S2-013 cells with re-expression of different p120 catenin isoforms (with/without MUC1 expression) were transfected with wild-type cyclin D1 reporter construct, which contains wild-type LEF-1/Tcf-4-binding site or reporter plasmids containing mutated LEF-1/Tcf-4-binding site in the cyclin D1 gene promoter (FOPFLASH), together with Renilla luciferase reporter plasmids SV40 as transfection control. Each transfection was carried out in duplicate plates, and the data shown here are relative luminescence units (RLUs) derived from luminescence assays on cell extracts that are representative of three independent experiments. (**b**) Nuclear extracts from S2-013 cells with re-expression of different p120 catenin isoforms with or without MUC1 expression were subjected to 10% SDS–PAGE and analyzed by immunoblot (IB) with an anti-cyclin D1 catenin mAb or β-tubulin as a loading control. Right panel shows densitometry analysis of the signal for cyclin D1 normalized to the signal for β-tubulin.

growth rates of Panc1.MUC1F and Panc1.NEO cells (***$P<0.0001$), suggesting that expression of MUC1 and concomitant increases in cyclin D1 expression significantly enhance proliferation of Panc-1 cells (Figure 6a). The growth rate of S2013 cells expressing different isoforms of p120 catenin (S2-013.1A, S2-013.3A, S2-013.4A) were slightly but statistically significantly higher than the S2-013.Neo (**$P<0.05$; Figure 6b). In addition, the growth rate of S2013 cells expressing MUC1 Flag (S2-013.MUC1F, S2-013.M.1A, S2-013.M.3A, S2-013.M.4A) were also significantly higher than S2-013.Neo (**$P<0.05$; Figure 6c). There were no significant differences among S2-013.MUC1F, S2-013.M.1A, S2-013.M.3A and S2-013.M.4A. These results suggest that enhanced levels of cyclin D1 following MUC1 expression or p120 catenin re-expression enhanced the growth rate of Panc-1 and S2-013 pancreatic cancer cell lines.

DISCUSSION

MUC1 engages cytoplasmic signaling proteins that also engage in transcriptional regulation, such as β-catenin, directly influencing transcription of genes associated with malignant progression. The MUC1 CT associates with and stabilizes steady-state levels of β-catenin in the cytosol and nucleus.[18] One functional consequence of the cytoplasm-to-nucleus translocation of β-catenin during canonical WNT-signaling is the association of β-catenin and LEF/Tcf-4, which functions as part of a transcription factor complex that initiates expression of cyclin D1, c-Myc, connexin-43, c-Jun and other genes.[19–21,23] It has been reported that LEF/Tcf-4 complexes serve as architectural transcription factors that induce DNA conformational changes by binding to their target sequence via the HMG box.[32] However, this protein–DNA interaction is not sufficient to initiate gene transcription,[33] and requires association with other transcription factors, such as β-catenin, which provide a transactivation domain and affect DNA-binding properties.[23] Studies have indicated that nuclear β-catenin and LEF/Tcf-4 complexes were not sufficient for activation of gene expression, which suggested that at least one other component is required to activate gene expression and that this third component may vary with cell type.[34]

We initially examined the capacity of MUC1 expression to influence cyclin D1 gene promoter activity by using cyclin D1 gene-promoter-reporter assays. Two pancreatic tumor cell lines were investigated: Panc-1, a poorly differentiated pancreatic tumor cell line that expresses very low or undetectable levels of MUC1 mRNA and protein; the S2-013 pancreatic tumor cell line, a moderately differentiated tumor cell line that expresses low levels of endogenous MUC1. Expression of MUC1 significantly enhanced cyclin D1 promoter activity in Panc-1 cells, but not in S2-013. RNA interference-mediated knockdown of endogenous MUC1 in S2-013 cells did not dramatically affect the activity of the cyclin D1 promoter.

Expressing MUC1 in Panc-1, a cell line with little or no endogenous MUC1, enhanced the transcriptional co-activator status of β-catenin in association with LEF/Tcf-4, perhaps through increasing the steady-state nuclear levels of β-catenin.[18] This interpretation was supported by the detection of protein–DNA interactions between nuclear β-catenin, LEF/Tcf-4 proteins and oligonucleotides based on the cyclin D1 promoter, in both Panc-1.MUC1F and Panc-1.NEO cells, and the finding of enhanced binding of β-catenin and LEF/Tcf-4 proteins to cyclin D1-promoter-derived oligonucleotides in Panc-1.MUC1F cells as compared with Panc-1.NEO cells (data not shown).

Steady-state levels of endogenous cyclin D1 transcripts and protein were affected by MUC1 expression as evidenced by results of northern blots (data not shown) and western blots (Figure 2).

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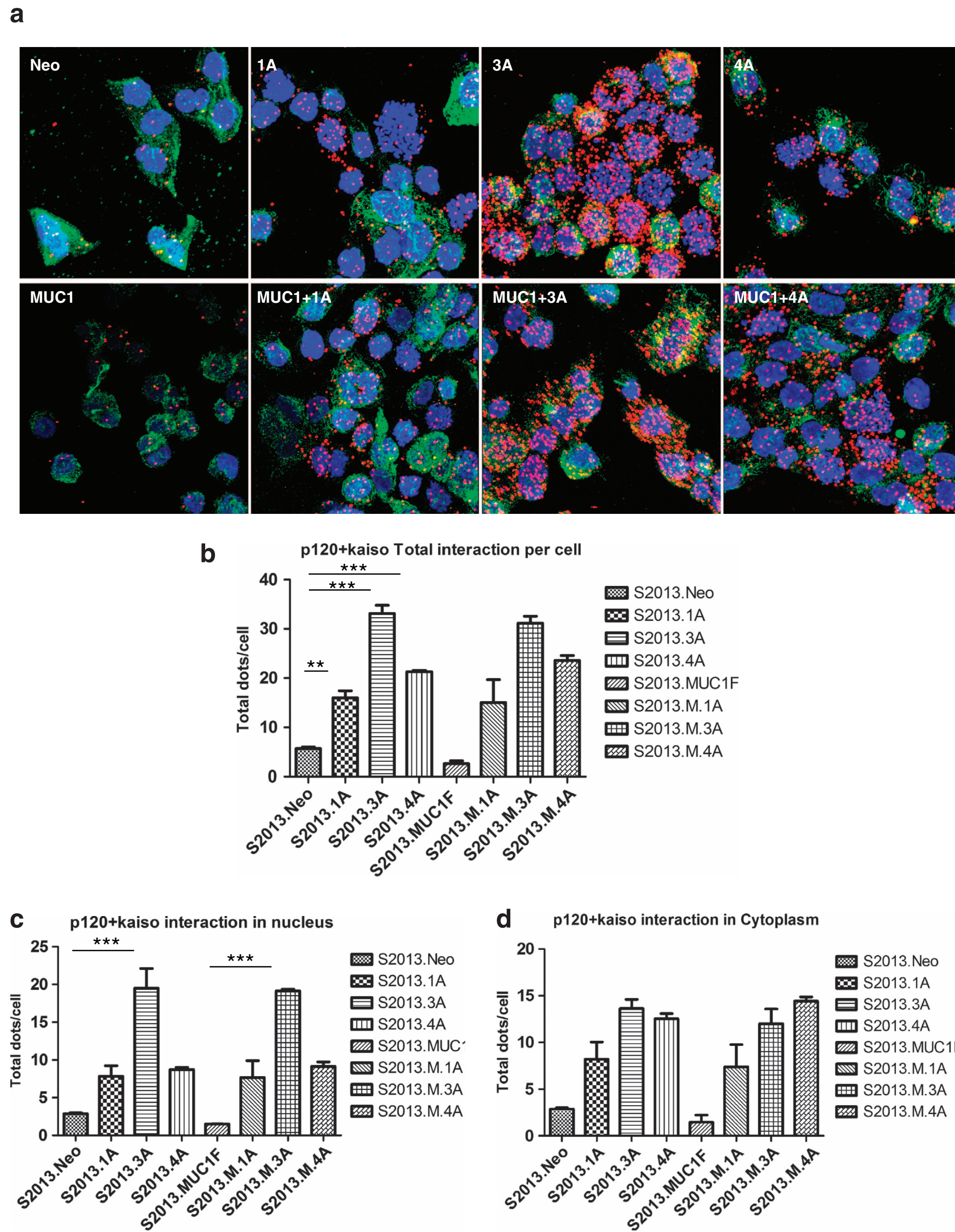

Figure 5. Different p120 catenin isoforms associate with Kaiso. (**a**) Proximity ligation assay (PLA) was used to detect interactions between p120 catenin and Kaiso in S2-013 cells with re-expression of different p120 catenin isoforms with or without MUC1. The red dots indicate interactions between p120 catenin and Kaiso. Blue (4′-6-diamidino-2-phenylindole, DAPI) staining indicates nuclei. The green indicates α-tubulin. (**b**–**d**) Quantification of results from PLAs. Results were compiled from three independent experiments.

Significantly higher levels of cyclin D1 mRNA transcripts and protein were detected in Panc-1.MUC1F cells overexpressing full-length MUC1F relative to Panc-1.NEO. We also investigated subcellular expression patterns of the cyclin D1 protein under conditions of expression of MUC1. Nuclear levels of cyclin D1 were much higher in Panc-1.MUC1F cells overexpressing recombinant MUC1 than Panc-1.NEO cells. Our results are consistent with a report that evaluated a pancreatic intraepithelial neoplasia tissue microarray and reported correlated expression of MUC1 and cyclin D1.[35] This result further supports the hypothesis that the CT of MUC1 contributed to elevated levels of steady-state cyclin D1 gene transcripts in Panc-1 cells. However, steady-state levels of cyclin D1 transcripts (data not shown) and protein (Figure 2) were not significantly altered in S2-013 cells with or without high levels of MUC1. Moreover, knockdown of MUC1 in S2-013 cells by the small interfering RNA (siRNA) strategy did not affect levels of cyclin D1 mRNA as compared with S2-013.MUC1F cells overexpressing MUC1F. A published finding with human HCT 116 colon carcinoma cells reported that expression of MUC1 cytoplasmic domain co-activated WNT-target gene transcription,[24] which is similar to our findings with Panc-1. Taken together, these results support the hypothesis that nuclear MUC1 CT can serve as co-factors for activation of cyclin D1 gene expression during the canonical β-catenin/WNT signal pathway in some cancer cells, but clearly shows that these pathways are not operative in all tumor cell lines.

The fact that MUC1 expression contributes to cyclin D1 gene regulation in some of the human pancreatic cancer cells but not others suggested that another undefined factor was required to activate reporter gene expression. We recently reported that a major difference between S2-013 cell and Panc1 cell is the absence of detectable P120 catenin in S2-013 cells,[15] and therefore hypothesized that functional p120 catenin was

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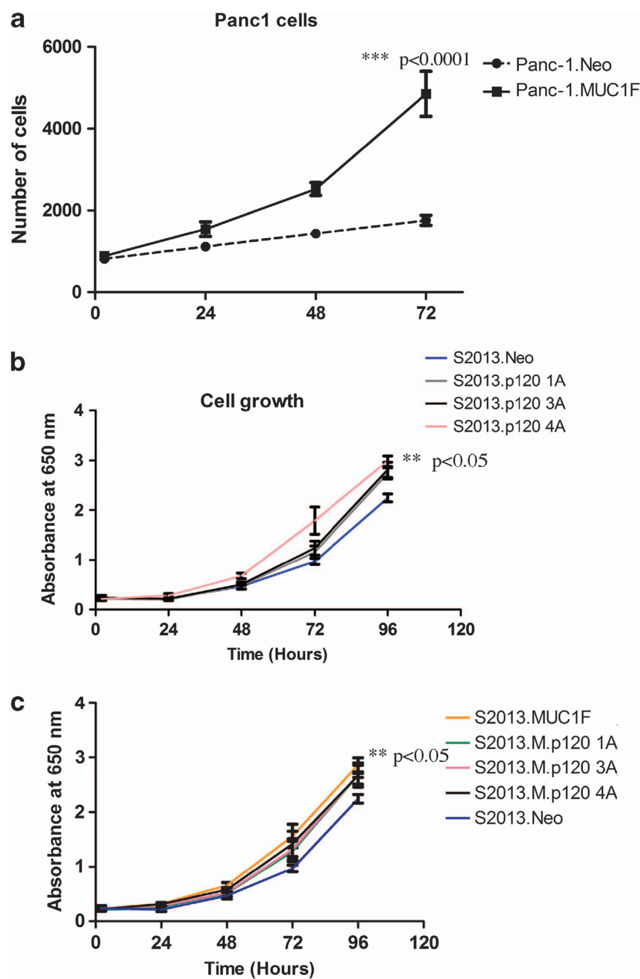

Figure 6. Effect of MUC1 expression and p120 catenin re-expression on *in vitro* cell growth in Panc-1 and S2-013 cells. (**a**) *In vitro* growth rate of Panc-1.MUC1F and Panc-1.NEO cells were evaluated by counting cells as described in the Materials and methods. (**b** and **c**) Cell growth assay using a methylene blue cell dye to measure the *in vitro* growth rate of S2-013 cells with re-expression of different p120 catenin isoforms with or without MUC1 expression.

required to fully activate cyclin D1 promoter activity. We evaluated S2-013 cells that re-expressed different p120 catenin isoforms for cyclin D1 promoter activity. The results showed that re-expression of p120 1A or 4A significantly increased the cyclin D1 promoter activity in S2-013 cells compared with NEO controls. Furthermore, p120 catenin isoform 4A plus MUC1 expression in S2-013 cells dramatically and significantly increased cyclin D1 promoter activity. The effects of MUC1 and p120 catenin on cyclin D1 promoter activity were also reflected in levels of protein expression. Thus, re-expressing isoforms 1A and 4A of p120 catenin restored responsiveness of the cyclin D1 promoter in these cells. Consistent with these findings, we note that expression of MUC1 in Panc1 cells also stabilized and enhanced expression of p120 catenin isoforms 1A and 4A (Figure 3a).

Published evidence that P120 catenin can associate with and inhibit activity of the transcriptional repressor Kasio[25] led us to hypothesize that p120 catenin activity on the cyclin D1 promoter was in part due to de-repression of Kaiso. Kaiso is a BTB/POZ-zinc finger transcription factor implicated in the development of cancer that was first identified as a specific binding partner for P120 catenin.[36] Kaiso binds to the promoter of cyclin D1 and represses gene expression.[31] An independent study reported that Kaiso/p120-catenin and TCF/β-catenin coordinately regulate canonical the WNT gene targets Siamois, cyclin D1.[26] Our results demonstrate that there are high levels of complexes in which Kaiso associates with p120 catenin (Figure 5). Interestingly, MUC1 expression enhances the cytoplasmic distribution of p120 catenin/ Kaiso complexes (especially isoform 4A in S2-013 cells). This is consistent with the concept that sequestration of Kasio by p120 catenin in the cytoplasm contributes to its functional de-repression activity. Further evidence in support of this hypothesis will require investigation of transcription factor occupancy of the cylin D1 promoter in these and other cell types.

In dividing cells, the cyclin D1 protein helps to regulate cell cycle transition from G1 to S phase.[37] The functional consequences of increased cyclin D1 protein expression under conditions of expression of MUC1 were investigated by analyzing the *in vitro* growth rate of each cell line. Results indicated that high activity of the cyclin D1 promoter and elevated cyclin D1 protein in Panc-1.MUC1F cells significantly increased cell proliferation as compared with Panc-1.NEO cells. Growth rates of S2-013 cells that re-expressed p120 catenin isoforms were statistically significantly higher than the S2-013.Neo (**$P<0.05$). The growth rates of cells expressing MUC1 Flag (S2-013.MUC1F, S2-013.M.1A, S2-013.M.3A, S2-013.M.4A) were significantly greater than S2-013.Neo (**$P<0.05$). Thus, the effect of increased cyclin D1 promoter activity and protein expression in S2-013 cells was modest but statistically significant.

In summary, results from our study demonstrate that full activation of cyclin D1 promoter activity in pancreatic cancer cell lines requires β-catenin activation of TCF-lef and stabilization of specific p120 catenin isoforms. We propose that the role of p120 catenin is to relieve repression by KAISO, a hypothesis that requires further investigation. MUC1 enhances the activities of both β-catenin and p120 catenin in this system, which results in elevation of cyclin D1 protein in human pancreatic cancer cells.

MATERIALS AND METHODS

Materials

The human pancreatic cancer cell line Panc-1 was obtained from the American Type Culture Collections (Rockville, MD, USA). The S2-013 cell line is a cloned sub-line of a human pancreatic tumor cell line (SUIT-2), which was derived from a liver metastasis.[38] Armenian hamster mAb CT-2 against the MUC1 CT was kindly provided by Dr Sandra Gendler, Mayo Clinic Scottsdale, AZ, USA. Antibodies used were Ab anti-cyclin D1 (72-13G; Santa Cruz Biotechnology, Dallas, TX, USA); mAb anti-TCF-4 (Upstate Biotechnology Inc., Lake Placid, NY, USA); anti-β-catenin (BD Transduction Laboratories, San Jose, CA, USA). Secondary antibodies used in confocal microscopy were fluorescein isothiocyanate-conjugated goat anti-mouse IgG1, goat anti-Armenian hamster IgG(H + L) and Cy^{TM5}-conjugated Affinipure goat anti-Armenian hamster IgG(H + L), purchased from Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA.

Cell culture

Panc-1 and S2-013 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum at 37 °C in 5% CO_2, and passaged at 80–90% confluence using 0.05% trypsin with 0.53 mM EDTA (Gibco BRL, Gaithersburg, MD, USA).

Expression of epitope-tagged MUC1 deletion constructs and generation of transfectant cell lines

Constructs encoding a full-length, FLAG epitope-tagged MUC1 cDNA (MUC1F) were previously described.[39] Panc-1 and S2-013 cells were stably transfected with plasmid DNA using lipofectin and appropriate protocols (Life Technologies, Grand Island, NY, USA). Cells carrying integrated constructs and expressing MUC1 were selected by culture in 600 μg/ml G418 (Life Technologies Inc, Invitrogen, Grand Island, NY USA), selected with cloning cylinders and expanded for screening. Expression of FLAG epitope-tagged MUC1 isoforms confirmed by immunofluorescence with anti-Flag mAb M2 and by immunoblotting with M2 (data not shown).

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Subcellular fractionation

Preparation of membrane/cytoplasmic extracts and nuclear extracts was as previously described.[18] Protein concentrations were determined with a Coomassie Protein Assay kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) and bovine serum albumin as a standard.

Immunoprecipitation and immunoblot analysis

Equal amounts of protein from cytoplasmic or nuclear extracts were incubated with the following mAbs: anti-β-catenin (Transduction Laboratory Co.), anti-TCF-4 (Upstate Biotechnology, EMD Millipore Corp, Chicago, IL, USA) or anti-cyclin D1 (Santa Cruz Biotechnology), or an IgG control. The immune complexes were precipitated with protein G-agarose beads (Sigma Aldrich Inc, Atlanta, GA, USA) overnight at 4 °C. After washing three times with dialyzed Nuclear Lysis Buffer containing 20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml Pefabloe SC and 1 μg/ml aprotinin, bound material were eluted from the immunoprecipitates in reducing SDS–PAGE loading buffer containing 10% SDS, 1 M Tris–HCl (pH 6.8), 50% glycerol, 10% β-mercaptoethanol (2-ME), 2% bromophenol blue at 100 °C for 5 min.

Subcellular extracts or immunoprecipitated proteins were resolved by electrophoresis on 6 or 10% denaturing polyacrylamide gels (with 3% polyacrylamide stacking gels), transferred to polyvinylidene difluoride membranes electrophoretically and blocked in 5% dry milk in Tris-buffered saline (TBS; 0.9% NaCl, 10 mM Tris, pH 7.4, 0.5% $MgCl_2$) at 4 °C overnight. Primary antibodies were diluted to 1:1000 or 1:500 in blotto (5% nonfat dry milk in TBS). Incubations were for 1 h at room temperature and were followed by three 10-min washes with TBS. Horseradish peroxidase-conjugated secondary antibodies were diluted to 1:20 000 in TBS, and incubated for 1 h at room temperature. Secondary antibody incubations were followed by three 10-min washes in TBS. Chemiluminescent reagents (Pierce, Thermo Fisher Scientific) were applied as per the manufacturer's instructions, and the membranes were exposed to film (Pierce, Thermo Fisher Scientific).[39] Film images were analyzed by densitometry in cases where relative quantification was performed and there were substantial differences in signals for loading controls.

Plasmids, transient transfections and luciferase assays

The cyclin D1 promoter-reporter constructs are based on the pA3-luc backbone and contain a 1745-bp fragment of the human cyclin D1 promoter.[21] One promoter construct, TOPFLASH, has a wild-type LEF/Tcf-4-binding site ($^{-81}$ GCTTTGATCTT^{-73}), and the other, FOPFLASH, has a mutated LEF/Tcf-4-binding site ($^{-81}$ GCTTTGGCCTT^{-73}). Both constructs were generously provided by Dr Richard G Pestell, Department of Oncology, Lombardi Cancer Center, Georgetown University, Washington, DC, USA. A dual-luciferase reporter assay (Promega, Thermo Fisher Scientific) was used to detect promoter activity in this study. A synthetic renilla luciferase reporter plasmid Phrl-SV40 (Promega, Thermo Fisher Scientific) was used as a control for transfection efficiency. Panc-1 cells expressing different MUC1 constructs were cultured in DMEM plus 5% fetal bovine serum, and 10 000 cells/well were seeded into a 48-well plate and grown to ~95% confluence. Each well was washed twice with PBS and transfected with 300 or 600 ng plasmid DNA using 2 μl Lipofectin (Invitrogen) in 200 μl Optimem (Life Technologies), respectively. After 24 h, cells were washed twice with PBS, fed with 3 ml DMEM plus 10% fetal bovine serum and cultured for another 2 days. The cells were then washed twice with PBS and harvested with 200 μl Passive Lysis buffer (Promega, Thermo Fisher Scientific). To detect firefly luciferase activity (from pA3 plasmids with cyclin D1 promoter), 20 μl of each lysate was reacted with 100 μl LARII buffer (Promega, Thermo Fisher Scientific) on ice. Relative light emission was detected using an Optocomp1 luminometer (MGM Instruments Inc, Hamden, CT, USA) reading 10 s with a 2-s delay. Subsequently, to detect the renilla luciferase activity (from SV-40 plasmids as transfection efficiency control), 100 μl Stop&Glo Reagent (Promega, Thermo Fisher Scientific) was added into each well. The solution was vortexed for five seconds, and read again with the Optocomp1 luminometer. Light emission data (mean of triplicates) were presented as relative lumenescence units for each cell line and analyzed for statistical significance using Prism and InStat software (GraphPad Software, Inc., La Jolla, CA, USA). Each experiment shown is representative of three independent replicates.

Generation of siRNAs for knockdown of MUC1

The mammalian pSUPER-RNA interference system was applied to construct the MUC1-siRNA. pSuper siRNA-human MUC1 plasmids were generated to target the sequence 5′-ACCTCCAGTTTAATTCCTC-3′ in the CT. In brief, a 19-mer oligonucleotide was selected and inserted into pSUPER gfp-neo vector using *Bgl*II and *Hin*dIII sites. pSUPER-GFP without inserts was used as a control. Cells were selected by FACS sorting. Cells transfected with pSUPER GFP-siRNA-MUC1 or GFP-neo using the Lipofectin method were plated in six-well culture plates and selected with 10% fetal bovine serum + RPMI with 600 μg/ml G418. Individual clones were selected by using cloning cylinders. Two independent clones, named S2-013.MTII.C1 and S2-013.MTII.C2, were examined. Western blotting using mAb CT-2 anti-MUC1 CT was performed to confirm knockdown of MUC1 protein expression (data not shown).

Immunofluorescence

Cells were cultured on glass coverslips (Fisherbrand Microscope cover glass, Thermo Fisher Scientific: 12-545-100 18CIR-1) at 4.5 × 105/well for 12 h. Cells were washed once in serum-free DMEM for 5 min followed by fixation for 15 min in PBS with 4% paraformaldehyde and 120 mM sucrose. Residual paraformaldehyde was neutralized with 0.1 M glycine in PBS for 15 min. For immunofluorescence, cells were permeabilized for 15 min by incubation in PBS with 0.1% Triton X-100. After washing with PBS, cells were incubated for 2 h with primary antibodies diluted in DMEM media. Cells were washed three times with PBS and then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG_1 (Southern Biotech, Birmingham, AL, USA) or indodicarbocyanine (Cy5)-conjugated goat-anti-Armenian hamster IgG (Jackson ImmunoResearch Laboratories Inc.) for 1 h. Propidium iodide was used as the nuclear dye at a 1:500 dilution with 0.1 mM RNAase for triple-color confocal laser scanning microscopy analysis.[40] After washing, cells were mounted in fluoromount-G (Southern Biotech). Isotype controls included mouse myeloma IgG_1 (Zymed, Life Technologies Inc), and CT-2 antibody blocked with CT-1 peptide (SSLSYTNPAVAATSANL, Dr Gendler, Mayo Clinic, AZ, USA) to confirm binding specificity. Cells were analyzed on a Zeiss LSM 410 dual beam laser confocal scanning microscope (Carl Zeiss, Inc., Thornwood, NY, USA).

Cell growth assays

One thousand cells/well were placed separately in 48-well culture plates, and after 2, 24, 48 and 72 h, the cells were harvested with trypsin digestion and counted using Z1 Series COULTER COUNTER Cell and Particle Counter (Beckman Coulter, Inc., Brea, CA, USA). A growth curve of each cell line was formulated from six independent replicates. Other methods in this study were performed as described previously.[41,42]

Statistical analysis

Two-way ANOVA was used to analyze the statistical difference between the groups. Turkey methods and spline regression were also used as alternative statistical method. A *P* value of less than 0.05 was considered statistically significant.

Proximity ligation assay

Proximity ligation assay methods in this study were performed as described previously.[15]

ABBREVIATIONS

GSK-3β, glycogen synthase kinase 3β; LEF-1, lymphoid-enhancing factor I; MUC1 CT, MUC1 cytoplasmic tail; SEA, sea-urchin sperm protein, enterokinase and agrin; Tcf-4, T-cell transcriptional factor 4.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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