RESEARCH ARTICLE

The Cell Death Inhibitor ARC Is Induced in a Tissue-Specific Manner by Deletion of the Tumor Suppressor Gene *Men1*, but Not Required for Tumor Development and Growth

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Abstract

Multiple endocrine neoplasia type 1 (MEN1) is a genetic disorder characterized by tissue-specific tumors in the endocrine pancreas, parathyroid, and pituitary glands. Although tumor development in these tissues is dependent upon genetic inactivation of the tumor suppressor *Men1*, loss of both alleles of this gene is not sufficient to induce these cancers. *Men1* encodes menin, a nuclear protein that influences transcription. A previous ChIP on chip analysis suggested that menin binds promoter sequences of *nol3*, encoding ARC, which is a cell death inhibitor that has been implicated in cancer pathogenesis. We hypothesized that ARC functions as a co-factor with *Men1* loss to induce the tissue-restricted distribution of tumors seen in MEN1. Using mouse models that recapitulate this syndrome, we found that biallelic deletion of *Men1* results in selective induction of ARC expression in tissues that develop tumors. Specifically, loss of *Men1* in all cells of the pancreas resulted in marked increases in ARC mRNA and protein in the endocrine, but not exocrine, pancreas. Similarly, ARC expression increased in the parathyroid with inactivation of *Men1* in that tissue. To test if ARC contributes to MEN1 tumor development in the endocrine pancreas, we generated mice that lacked none, one, or both copies of ARC in the context of *Men1* deletion. Studies in a cohort of 126 mice demonstrated that, although mice lacking *Men1* developed insulinomas as expected, elimination of ARC in this context did not significantly alter tumor load. Cellular rates of proliferation and death in these tumors were also not perturbed in the absence of ARC. These results indicate that ARC is upregulated by loss *Men1* in the tissue-restricted distribution of MEN1 tumors, but that ARC is not required for tumor development in this syndrome.

Introduction

Multiple endocrine neoplasia type 1 (MEN1) is a familial cancer syndrome characterized by tumors primarily in the endocrine pancreas, parathyroid gland, and anterior pituitary gland [1]. The genetic basis of MEN1 is loss of function mutations in the tumor suppressor gene *Men1*, with one mutant allele transmitted through the germ line and the other inactivated through somatic mutation [2–4]. *Men1* encodes menin, a nuclear protein [5] that regulates transcription, but whose precise biochemical function is not known [6].

As *Men1* is ubiquitously expressed [7], the basis for the highly tissue-restricted tumor distribution in MEN1 is not understood. The importance of *Men1* inactivation in the limited distribution of MEN1 tumors is demonstrated by the development of tumors in the endocrine pancreas, parathyroid, and pituitary of mice in which one allele has been deleted in the germ-line and the other inactivated by somatic mutation in those tissues [8, 9]. While differential loss of heterozygosity might contribute to the tissue-specific nature of MEN1 tumors, it is not sufficient to account for this phenomenon, as illustrated by the fact that forced deletion of both *Men1* alleles in certain tissues does not cause cancer. For example, *Men1* inactivation in the liver does not cause tumors in that organ [10], and deletion of *Men1* in all cells of the pancreas results in tumors in the endocrine, but not exocrine, tissues of this organ [11]. These observations suggest the existence of additional tissue-restricted factor(s) that cooperate with *Men1* loss in tumor development.

An important hallmark of cancer is the ability of transformed cells to evade apoptosis [12, 13]. Cancer cells often acquire the ability to suppress cell death through decreases in the abundance/activities of cell death promoters or increases in the analogous properties of cell death inhibitors such as Bcl-2 proteins [14], FLICE-like inhibitor protein (FLIP) [15], and inhibitor of apoptosis proteins (IAPs) [16]. Another cell death inhibitor that has recently been implicated in the pathogenesis of multiple cancers is Apoptosis Repressor with CARD (ARC). ARC inhibits cell death through its inactivation of both the extrinsic (death receptor) and intrinsic (mitochondrial/ER) apoptosis pathways. These actions are mediated through direct interactions of ARC with critical signaling components in these pathways [17–20].

In healthy mice, ARC is expressed primarily in cardiac and skeletal muscle [21], certain neuronal populations [22], and pancreatic islets, primarily β-cells [20]. However, ARC expression has been shown to be markedly induced in human malignancies and mouse models of cancer including breast [23, 24], colon [25], kidney [26], head and neck [27], and blood [28]. In human acute myelogenous leukemia, elevation of ARC levels correlate inversely with survival [28]. Further, deletion of *nol3*, which encodes ARC, in the context of a transgenic mouse model of breast cancer, ameliorates tumor growth, invasion, metastasis, and chemoresistance [29].

A previous ChIP on chip study suggesting that menin binds promoter sequences of *nol3* (NHGRI, GSE5357: Menin ChIP-chip) [30] led us to hypothesize that ARC may function as a co-factor in the tissue-restricted distribution of tumors in MEN1. Using mice in which *Men1* has been biallelically inactivated in specific tissues, we show that loss of *Men1* markedly induces ARC expression selectively in those tissues that are susceptible to MEN1 tumors. However, in contrast to its critical role in other cancers, elimination of ARC in the setting of *Men1* inactivation does not reduce β-cell tumor burden.

Materials and Methods

Mice

Pdx1-Cre; Men1 f/f mice [11] and PTH-Cre; Men1 f/f mice [31] have been described previously. We maintained the Pdx1-Cre transgene in a hemizygous state in all experiments. The

Pdx1-Cre; Men1 f/f mice, initially on a mixed FVB/129Sv background, were backbred 3–4 generations onto C57BL/6 for these experiments. ARC -/- (gene name is *nol3*, but mice referred to as ARC -/- here for simplicity) mice on a C57BL/6 background are as previously described [32]. Studies were performed using mice of ages and genders indicated. Genetic controls were age- and gender-matched littermates. Studies were approved by the Institute for Animals Studies of the Albert Einstein College of Medicine.

Laser capture microdissection (LCM)

Frozen sections (5 μM) from pancreatic and parathyroid tissues were stained using Arcturus HistoGene LCM Frozen Section Staining Kit (ThermoFisher Scientific). The stained tissue sections were microdissected using a Leica AS LMD Microsystem.

qRT-PCR

Total RNA was extracted using RNeasy (Qiagen) and used to generate cDNA. qPCR was performed with a Gene Amp 7500 Sequence Detector (Applied Biosystems) using SYBR Green (Invitrogen) for quantification. Primer sequences to amplify ARC were 5'-CAAGAAGAGGAT GAATCTGAAG-3' (forward) and 5'-TTGGCAGTAGGTGTCTCG-3' (reverse). Normalization was performed to 18S ribosomal RNA using primers 5'-GTAACCCGTTGAACCCCA TT-3' (forward) and 5'-CCATCCAATCGGTAGTAGCG-3' (reverse).

Immunoblotting

Islets were isolated from mice as previously described [20]. Approximately 200 islets (of similar size) were hand-picked for each mouse sample. Immunoblotting was performed as previously described [20]. Primary antibodies included ARC (Cayman) and α-tubulin (Sigma-Aldrich). IRDye 800 and 680 (LI-COR) were used to detect primary antibodies, and membranes scanned using a LI-COR Odyssey.

FACS

Fresh pancreas tissue was digested for 30 min with Collagenase IV (0.8mg/ml) at 37°C. Following washing, cells were incubated for 20 min with primary antibodies against ARC (Cayman) and insulin (DAKO). The cells were then washed and incubated for 30 min with secondary antibodies Alexa Fluor 488 and 647 (Invitrogen) to detect ARC and insulin respectively. Following washing, cells were suspended in 200 μl of buffer and analyzed using a FACSAria cell sorter (BD Biosciences). The data were analyzed with CellQuest software (BD Biosciences).

Metabolic Measurements

Blood glucose concentrations following an overnight fast were assayed using the OneTouch glucose monitoring system (LifeScan). Plasma insulin concentrations were measured by ELISA (ALPCO Diagnostics).

Immunohistochemistry

Immunofluorescence was performed as previously described [20]. Pancreatic tissue was fixed in 10% neutral buffered formalin and antigen retrieval was completed with Antigen Unmasking Solution (Vector Laboratories). Primary antibodies included ARC (Cayman), Ki67 (Abcam), and insulin (Abcam), and secondary antibodies were Alexa Fluor 488 or 568 (Invitrogen). Samples were counterstained and coverslipped with VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)

Cell death was detected using the Fluorescein In Situ Cell Death Detection Kit (Roche Applied Science). Tissue sections were counterstained with insulin to demarcate pancreatic β-cells. A minimum of 5 fields per pancreas section was analyzed.

Statistics

All data are presented as mean ± SEM. For comparisons between two groups, a two-tailed student's t-test was performed. Multiple comparisons were analyzed using analysis of variance followed by a Tukey post-hoc test. Statistics were calculated using GraphPad Prism 5 software. $P < 0.05$ was deemed significant.

Results

Deletion of *Men1* increases expression of ARC preferentially in cell types that are susceptible to MEN1

To assess whether ARC mediates the tissue-restricted nature of MEN1 tumors, we first asked whether the expression of ARC is induced by deletion of *Men1* selectively in those tissues that develop tumors in this syndrome. To address this, we used a Cre-loxP approach to delete *Men1* in cells of the endocrine pancreas (pancreatic islets), which develop MEN1 tumors, and then quantified the abundance of ARC transcripts specifically in this tissue using laser capture microdissection (Fig 1A). To provide an internal control, we chose to employ a Cre deletor (Pdx1-Cre), which would simultaneously excise *Men1* also in the exocrine pancreas, a tissue that does not develop MEN1 tumors. We observed that biallelic deletion of *Men1* in all cells of the pancreas resulted in marked increases in ARC expression in the endocrine pancreas (Fig 1B). In contrast, expression was increased only minimally in the exocrine tissue (Fig 1C). To
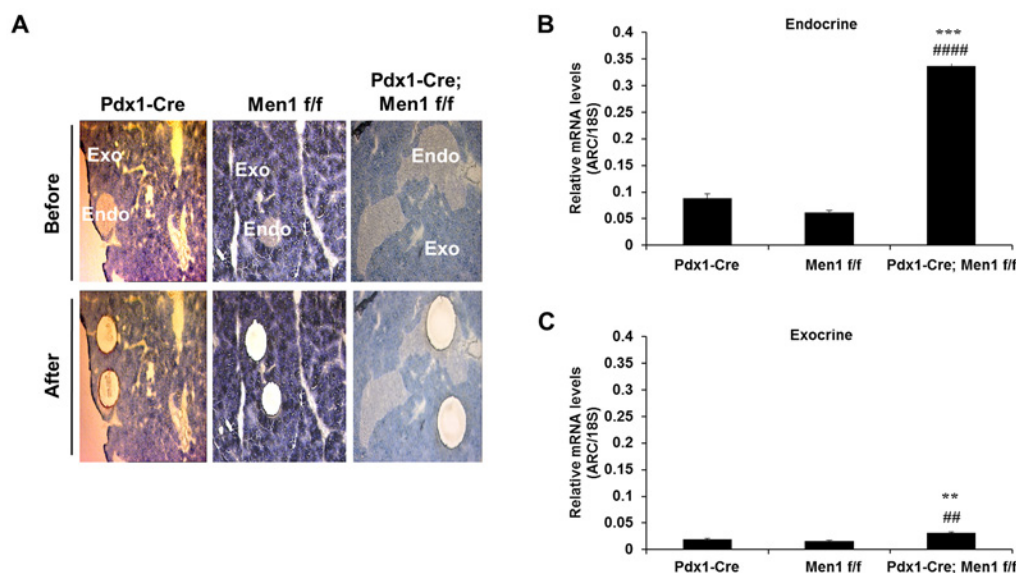
Fig 1. Deletion of *Men1* in all pancreatic cells increases ARC mRNA levels preferentially in islets. A) Endocrine (Endo) and exocrine (Exo) pancreatic tissue from 12 m old mice before (top) and after (bottom) laser capture microdissection. B) qRT-PCR for ARC in mouse pancreatic endocrine tissue from the indicated genotypes. C) qRT-PCR for ARC in mouse pancreatic exocrine tissue from the indicated genotypes. N = 3 mice per genotype. ** $P < 0.01$ versus Pdx1-Cre. *** $P < 0.001$ versus Pdx1-Cre, ## $P < 0.01$ versus Men1 f/f, #### $P < 0.0001$ versus Men1 f/f.

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further test the notion that loss of *Men1* increases ARC expression in tissues susceptible to MEN1 tumors, we deleted both alleles of *Men1* in the parathyroid gland using PTH-Cre, and again observed dramatic increases in the abundance of ARC mRNA (Fig 2A and 2B). In contrast, there were no changes in ARC expression when menin was deleted in the liver, which is consistent with this tissue not developing tumors in response to loss of *Men1* (Fig 2C).

To determine whether the increases in ARC mRNA in endocrine pancreas in response to *Men1* deletion resulted in increases in ARC protein, we first performed immunofluorescence. Consistent with our previous observations [20], ARC staining was restricted to islets and predominantly localized to the cytoplasm of these cells (Fig 3A). Importantly, under identical exposure conditions, ARC immunofluorescence was more prominent in tissue from Pdx1-Cre; Men1 f/f, as compared with Men1 f/f, mice (compare Fig 3A left and right). These increases in ARC protein were quantified using immunoblotting of isolated islets (Fig 3B). We also analyzed cells dissociated from whole pancreas tissue using FACS (Fig 3C–3E). The percentage of ARC-positive β-cells (insulin-positive) was 2–3-fold greater in pancreata from Pdx1-Cre; Men1 f/f, as compared with Men1 f/f, mice. These three approaches, in conjunction with the qRT-PCR data in Fig 1, demonstrate that deletion of *Men1* results in increased ARC mRNA and protein selectively in β-cells.

Genetic elimination of ARC does not reverse the development of insulinomas resulting from inactivation of *Men1*

The induction of ARC expression selectively in tissues that develop MEN1 tumors raises the possibility that ARC may cooperate with *Men1* loss to promote tissue-restricted tumors. To test the role of ARC in the development of insulinomas, we bred Pdx1-Cre; Men1 f/f mice with mice in which the gene encoding ARC had been deleted in the germ line to create mice lacking both menin and ARC in all cells of the pancreas. Although ARC -/- mice have no basal phenotype, we hypothesized that the absence of ARC might ameliorate the development of tumors resulting from deletion of *Men1*.

Biallelic deletion of *Men1* in the pancreas results in β-cells tumors accompanied by blunting of gains in body weight (Fig 4A) [11, 33, 34]. The tumor burden from these insulinomas is quantified clinically and in experimental models using plasma insulin concentrations [11]. As
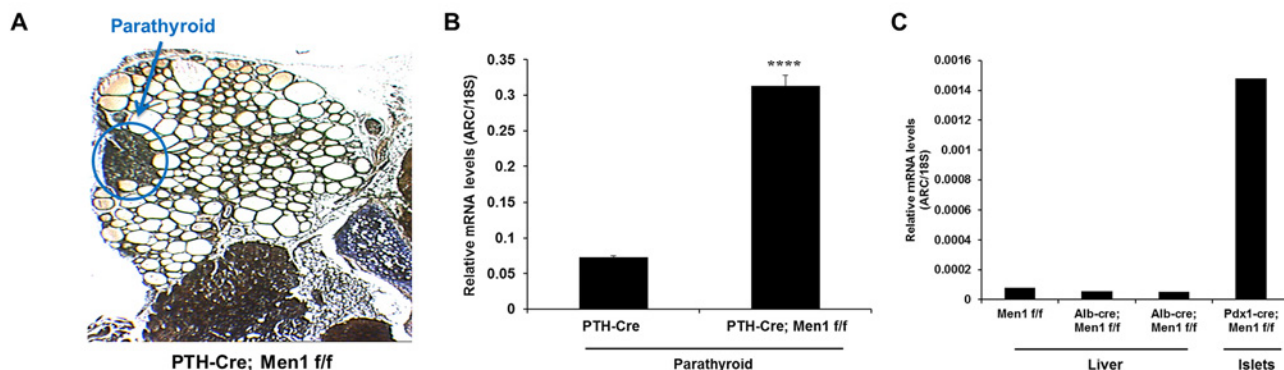

Fig 2. Deletion of *Men1* in the parathyroid hormone-secreting cells increases ARC mRNA expression. A) Parathyroid tissue (inside blue circle) from 12 m old PTH-Cre; Men1 f/f mouse subsequently subjected to laser microdissection. B) qRT-PCR for ARC in parathyroid tissue from the indicated genotypes. N = 3 mice per genotype. **** $P < 0.0001$ versus PTH-Cre. C) qRT-PCR for ARC in liver tissue from the indicated genotypes. Each bar in panel C represents mRNA samples isolated from a single mouse.

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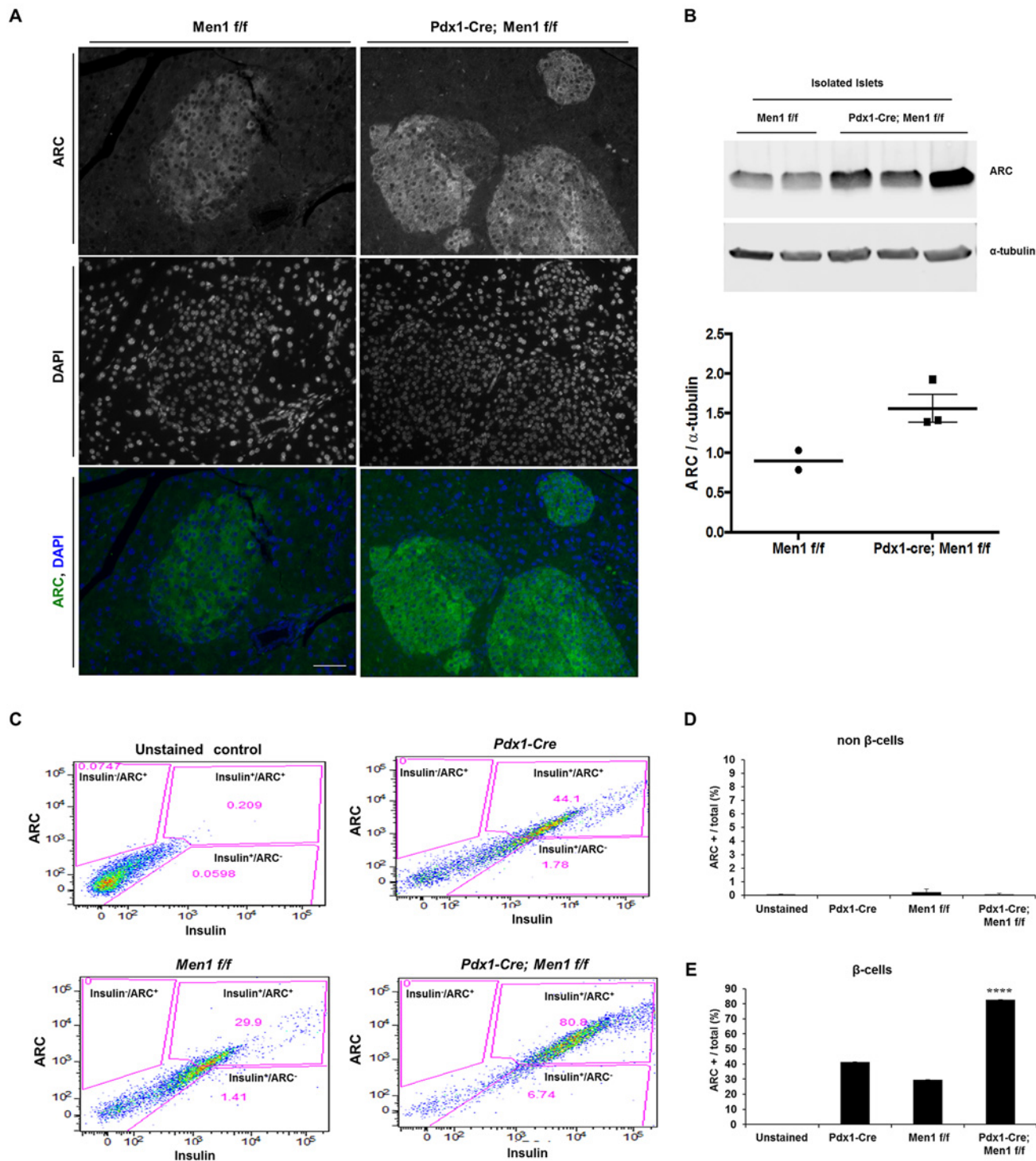
Fig 3. ARC protein is more abundant in β-cells of Pdx1-Cre; Men1 f/f mice. A) Immunostaining of pancreatic tissue for ARC from 12 m old mice. Nuclei counterstained with DAPI. Scale bar denotes 50 μM. B) Immunoblot (top) of islets isolated from Men1 f/f or Pdx1-Cre; Men1 f/f mice. Each lane represents islets isolated from an individual mouse. Quantification of ARC normalized to loading control (bottom). C) Representative FACS analysis of dissociated mouse pancreatic cells stained for ARC and insulin. D) Quantification of ARC-positive non-β-cells (as indicated by absence of insulin staining). E) Quantification of ARC-positive β-cells (insulin positive). N = 3 mice per genotype. **** P < 0.0001 versus each group.
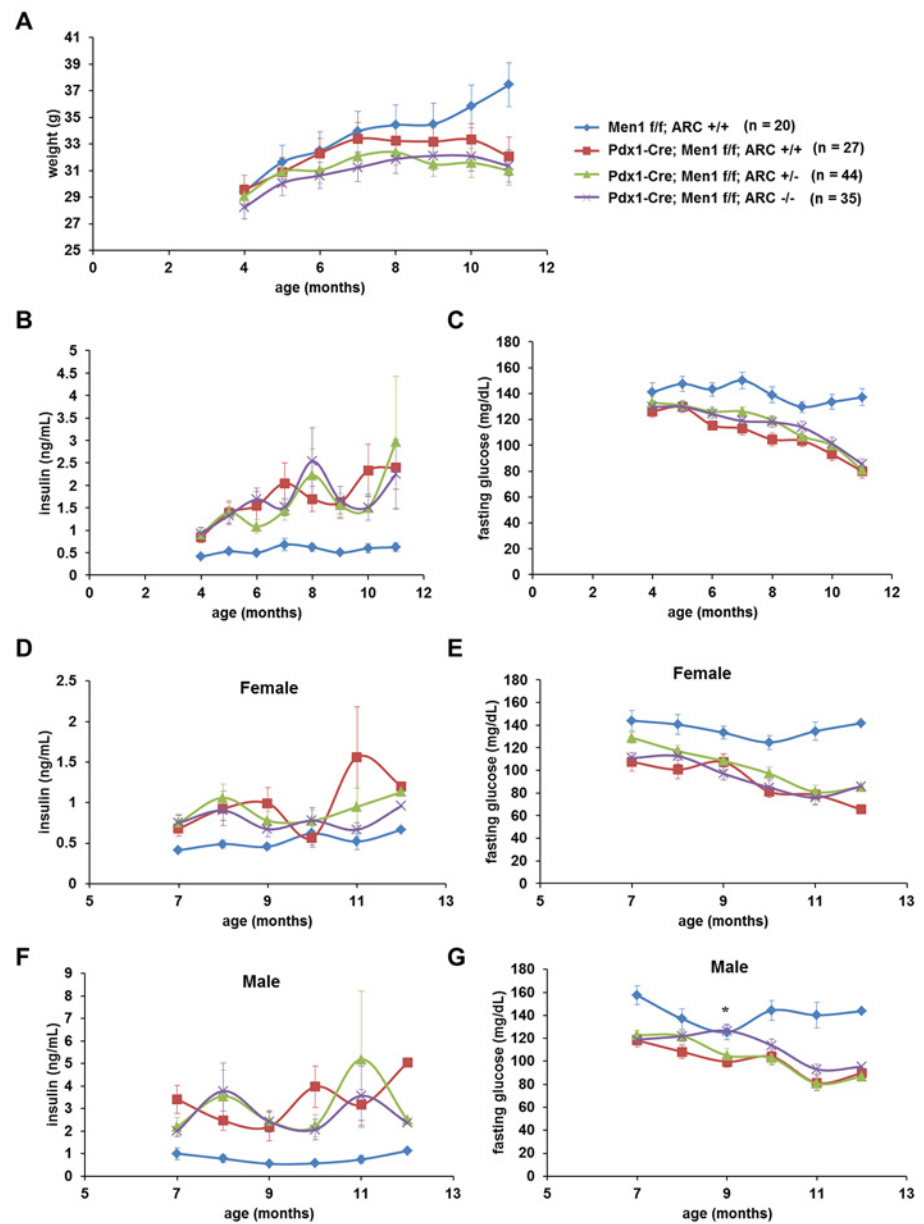
Fig 4. Generalized deletion of ARC in Pdx1-Cre; Men1 f/f mice does not significantly change islet tumor load. A) Body weights (both genders). B) Fasting plasma insulin concentrations (both genders). C) Fasting blood glucose concentrations (both genders). D) and E) Fasting insulin and glucose measurements in females. F) and G) Fasting insulin and glucose measurements in males. * $P < 0.05$ Pdx1-Cre; Men1 f/f; ARC -/- versus Pdx1-Cre; Men1 f/f; ARC +/+.

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expected, we observed age-dependent increases in plasma insulin levels in Pdx1-Cre; Men1 f/f mice compared with Men1 f/f mice (Fig 4B, red curve compared with blue curve). Since insulin stimulates the uptake of glucose into cells, Pdx1-Cre; Men1 f/f mice also exhibited reciprocal decreases in fasting blood glucose concentrations (Fig 4C). Deletion of either one or both alleles encoding ARC did not normalize insulin or glucose levels, even in the large cohort of mice with 20–44 mice of each genotype (Fig 4B and 4C, green and purple curves compared with red
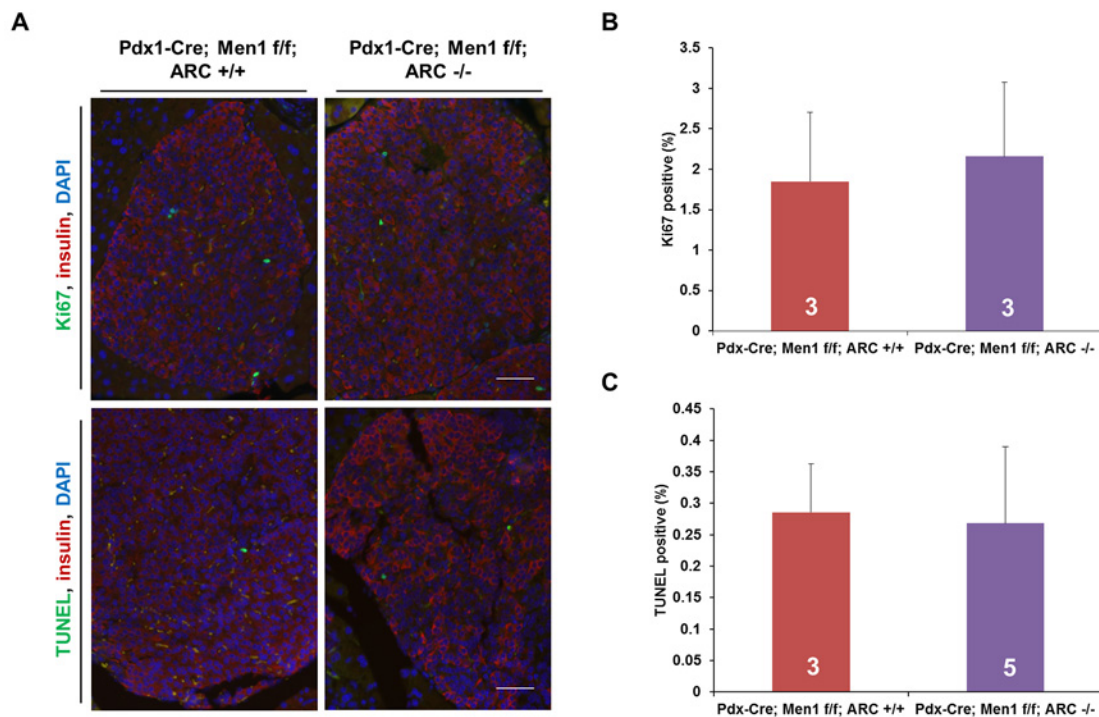
Fig 5. Deletion of ARC does not change rates of cellular proliferation and apoptosis in MEN1 insulinomas. A) Immunostaining of pancreatic tissue from 12 m old mice for Ki-67 (top, green) and TUNEL (bottom, green). β-cells are demarcated by insulin (red) and nuclei are counterstained with DAPI. Scale bar denotes 50 μM. B) Quantification of proliferating cells by Ki-67 staining. C) Quantification of apoptotic cells by TUNEL. No comparisons are significant. Number of mice in each group as indicated.

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curve). Similarly, loss of ARC had no significant effects on tumor burden when female and male mice were analyzed individually (Fig 4D–4G). To further analyze the role of ARC on β-cell tumor load, we assessed the effects of ARC deletion on β-cell proliferation and apoptosis in Pdx1-Cre; Men1 f/f mice (Fig 5A). Loss of ARC did not affect either parameter (Fig 5B and 5C). We conclude that ARC does not appear critical in the pathogenesis of insulinomas in this mouse model of MEN1.

Discussion

The mechanistic basis by which *Men1* inactivation increases ARC expression preferentially in tissues that develop MEN1 tumors is not known. While changes in ARC protein abundance may be regulated through both transcriptional mechanisms [35, 36] and at the level of protein stability [37–39], here increases in mRNA levels appear adequate to account for increases in ARC protein. Menin is a nuclear protein [5], and a previous ChIP-chip analysis suggests that it binds promoter sequences of *nol3*, which encodes ARC (NHGRI, GSE5357: Menin ChIP-chip) [30]. Thus, consistent with data suggesting that menin may function as a transcription factor [40, 41], one potential mechanism for increases in ARC mRNA following deletion of *Men1* is that menin directly represses *nol3* transcription. Other data, however, suggest that menin modulates transcription indirectly through its interactions with transcription factors, including both activators and repressors [6], and/or through promotion of epigenetic modification of histones [40, 42]. Therefore, another possible mechanism is that menin decreases ARC transcription through these mechanisms, although it is not currently known whether any of the

transcription factors that menin is known to bind/modify regulate *nol3* transcription. Another indirect mechanism by which menin may regulate *nol3* transcription is through its established inhibition of ERK signaling [43], as MEK/ERK is known to mediate increases in *nol3* transcription [35, 44]. The basis underlying the tissue-specificity of the increases in ARC expression following loss of menin is also elusive and could, in theory, be related to differences in the chromatin state of the *nol3* locus in various tissues or to any of the indirect mechanisms discussed above. For example, menin might repress *nol3* transcription through inhibition of a transcriptional activator whose presence is restricted to tumor-susceptible tissues.

Interestingly, we found that the increases in ARC levels resulting from *Men1* inactivation were not required for the development of MEN1 tumors. ARC abundance increases in multiple human cancers [23–28], and the absence of an effect of *nol3* deletion on MEN1 tumor load in this study contrasts with the amelioration of tumor growth (as well as metastasis) resulting from *nol3* inactivation in a genetic mouse model of breast cancer [29]. While the absence of an effect could reflect redundancy from another cell death inhibitor, a more likely possibility is differences in the cancer biology of MEN1 tumors in comparison to breast cancers. In the breast cancer model, the major effect of *nol3* deletion on tumor growth was to reduce rates of tumor cell proliferation (mechanism not known), while basal rates of apoptosis were not affected [29]. In contrast, *nol3* deletion affected neither basal rates of cell proliferation nor cell death in MEN1 insulinomas, which likely accounts for the absence of an effect of loss of ARC in these tumors.

Since ARC inhibits apoptosis in both transformed β-cells [20] and breast cancer cells [23], why did *nol3* deletion not affect rates of apoptosis in insulinomas and breast tumors? One possibility is that basal rates of cell death are relatively low in both of these tumors. Consistent with this hypothesis, *nol3* deletion markedly exacerbates apoptosis when mice harboring breast tumors are treated with chemotherapeutic agents [29]. Whether MEN1 tumors would behave similarly remains to be determined. Second, the ability of ARC to suppress basal levels of apoptosis may be dependent on the nature of the inciting death stimuli. For example, we have shown that ARC potently inhibits apoptosis in β-cells stimulated with ER stressors [20], but the precise nature of the death stimuli that operate in insulinomas is not known.

Acknowledgments

Author Contributions

Conceived and designed the experiments: RNK SKL WMM ZY. Performed the experiments: WMM ZY MZ. Analyzed the data: RNK SKL WMM ZY. Contributed reagents/materials/analysis tools: RNK SKL JSC. Wrote the paper: RNK SKL WMM ZY.

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