Restricted Expression of miR-30c-2-3p and miR-30α-3p in Clear Cell Renal Cell Carcinomas Enhances HIF2α Activity

Lijoy K. Mathew1,2, Samuel S. Lee1,2, Nicolas Skuli6, Shilpa Rao4, Brian Keith1,6, Katherine L. Nathanson7, Priti Lal5, and M. Celeste Simon1,2,3

ABSTRACT
Inactivation of the von-Hippel Lindau (VHL) tumor suppressor gene occurs in 90% of human clear cell renal cell carcinomas (ccRCC) and leads to the stable expression of the hypoxia-inducible factors HIF1α and HIF2α. The constitutive expression of HIF1α in a majority of VHL-deficient tumors is counterintuitive, given that HIF1α functions as a tumor suppressor in ccRCC, whereas HIF2α clearly enhances tumor growth. We demonstrate here that miR-30c-2-3p and miR-30α-3p specifically bind and inhibit expression of HIF2A transcripts, and that the locus encoding miR-30c-2-3p and miR-30α-3p is selectively repressed in “H1H2” VHL-deficient tumors expressing both HIF1α and HIF2α proteins. Inhibiting miR-30α-3p expression increases HIF2α levels in H1H2 ccRCC cells and promotes cellular proliferation, angiogenesis, and xenograft tumor growth. Our results indicate that repression of miR-30c-2-3p and miR-30α-3p repression enhances HIF2α expression and suggests a mechanism whereby the tumor-suppressive effects of constitutive HIF1α expression are attenuated in VHL-deficient H1H2 tumors.

SIGNIFICANCE: HIF1α is constitutively expressed in a majority of VHL-deficient ccRCCs, despite its tumor suppressor activity in these malignancies. This study demonstrates that repression of miR-30c-2-3p/miR-30α-3p increases HIF2α levels to promote tumor growth, thereby ameliorating the inhibitory effects of HIF1α in ccRCCs. Cancer Discov; 4(1); 53–60. © 2013 AACR.

INTRODUCTION
Clear cell renal cell carcinoma (ccRCC) is the most commonly diagnosed form of kidney cancer and accounts for the majority of renal cancer-related deaths. A characteristic feature of nearly 90% of sporadic ccRCCs is mutation or silencing of the von Hippel-Lindau (VHL) tumor suppressor gene (1). pVHL, the protein encoded by VHL, is a critical component of a ubiquitin ligase complex that regulates hypoxia-inducible factor (HIF) accumulation (1). HIFs are heterodimeric...

Corresponding Authors: M. Celeste Simon, Scientific Director and Investigator, Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, 456 BRB II/III, 421 Curie Boulevard, Philadelphia, PA 19104-6160. Phone: 215-746-5532; Fax: 215-746-5511; E-mail: celeste2@mail.med.upenn.edu; and Priti Lal, Hospital of the University of Pennsylvania, Pathology and Laboratory Medicine, 3400 Spruce Street, Philadelphia, PA 19104-4283. E-mail: priti.lal@uphs.upenn.edu
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transcription factors consisting of α and β subunits (2), and HIFα subunits (HIF1α and HIF2α) are hydroxylated by prolyl hydroxylase (PHD) enzymes, resulting in pVHL recognition, polyubiquitination, and proteasomal degradation (3). However, under low oxygen (O2) conditions, HIFα subunits are stabilized, bind the β subunit (ARNT), and activate numerous genes involved in metabolism, angiogenesis, proliferation, cellular motility/invasion, and extracellular matrix remodeling (4). Although HIF1α and HIF2α have overlapping functions, recent studies have illustrated remarkably distinct roles for each α isoform in both normal physiology and disease (4).

We have previously stratified more than 200 VHL-deficient ccRCC tumors into two subtypes: one group (~60% of all cases) expressing both HIF1α and HIF2α (designated “H1H2”) and another 30% expressing HIF2α exclusively (designated “H2”; refs. 5, 6). Although HIF2α promotes tumorigenesis (7), HIF1α has been clearly shown to be a tumor suppressor (8–10) in this disease. This differential HIFα expression pattern raises an important question: if HIF1α is a tumor suppressor, how do H1H2 ccRCCs evade the inhibitory activities of HIF1α? Delineating the mechanisms by which H1H2 ccRCCs overcome the tumor-suppressive effects of HIF1α could identify novel molecular targets for future ccRCC treatment.

Integrated genomic analyses revealed that H1H2 tumors display enhanced mitogen-activated protein kinase and mTOR signaling, whereas H2 ccRCCs exhibit increased c-Myc activity (5, 6). In addition, an important role for microRNAs (miRNA) in ccRCC progression has been suggested (11, 12). miRNAs are small, non-coding RNAs (~22 nucleotides) representing 1% to 2% of the eukaryotic transcriptome that constitute important posttranscriptional regulators of more than 30% of mammalian genes (13). miRNAs can act as either oncogenes or tumor suppressors (13), and because each miRNA potentially regulates numerous genes, they represent powerful discovery tools to identify novel pathways impacting cancer. To investigate the role of miRNAs in regulating the progression of distinct ccRCC subtypes, we performed miRNA microarray studies on H1H2 and H2 ccRCCs, along with matched normal control tissue samples. We observed preferential repression of multiple miRNAs that were differentially expressed in each ccRCC subtype, and chose miR-30c-2-3p for further analysis, as its expression was significantly more repressed in H1H2 than in H2 tumors when normalized to adjacent normal kidney RNA (Fig. 1A, arrow; B). MiR30C2 maps to human chromosome 6q13, and is closely linked to MIR30A (Supplementary Fig. S1A). Intriguingly, miR-30a-3p expression was also repressed in H1H2 tumors relative to H2 tumors (Fig. 1C and Supplementary Fig. S1B, arrows), suggesting common regulation of the genomic locus. Importantly, analysis of data from The Cancer Genome Atlas (TCGA) revealed that miR-30c-2-3p and miR-30a-3p are significantly repressed in numerous ccRCCs (n = 437) when compared with normal tissue samples (n = 68; Fig. 1D and E). Moreover, correlation analysis using TCGA data indicated that both miR-30c-2-3p and miR-30a-3p are significantly coregulated in ccRCCs (n = 437; Supplementary Fig. S1C).

As both HIF1α and HIF2α are constitutively expressed in H1H2 ccRCCs, we first investigated whether HIFs regulate the expression of miR-30c-2-3p and miR-30a-3p. Inhibition of either HIF1α subunit using short hairpin RNA (Supplementary Fig. S2A and S2B) or siRNA (Supplementary Fig. S2C and S2D) techniques demonstrated that miR-30c-2-3p and miR-30a-3p were not regulated by HIF. However, because both miRNAs are repressed in VHL-deficient ccRCCs, we reintroduced pVHL into RCC10 cells, and observed significant derepression of miR-30c-2-3p and miR-30a-3p (Fig. 1F). Altogether, these studies indicate that the preferential inhibition of miR-30c-2-3p and miR-30a-3p observed in H1H2 tumors is pVHL-dependent, but HIF-independent.

**miR-30c-2-3p/30a-3p Repress HIF2α Expression in H1H2 ccRCCs**

We employed bioinformatic tools (ref. 15; DianaMicroT) to identify specific molecular targets of miR-30c-2-3p and miR-30a-3p. Interestingly, both miRNAs were predicted to bind HIF2A transcripts, which we tested by fusing the HIF2A untranslated region (UTR) to a standard luciferase reporter gene construct. Mutating miR-30c-2-3p or miR-30a-3p seed sequences in the HIF2A 3′ UTR was sufficient to block miR-30c-2-3p/30a-3p–dependent regulation of luciferase activity (Fig. 2A and B and Supplementary Fig. S3A). We selected RCC4, RCC10, and UMRC2 ccRCC cell lines for further functional analyses, as they stably express both HIF1α and HIF2α. Ectopic expression of miR-30c-2-3p (miR-30c-2-3p EE) in RCC4 and RCC10 cells decreased HIF2A mRNA expression (Fig. 2C and Supplementary Fig. S3B), whereas miR-30c-2-3p inhibition (miR-30c-2-3p INH) increased HIF2A transcript levels (Fig. 2D). HIF2α protein levels were similarly reduced by ectopic expression of miR-30c-2-3p and increased by miR-30c-2-3p inhibition in both RCC4 and RCC10 cells (Fig. 2E), with consequent effects on the expression of HIF2α-regulated target genes, including VEGF, GLUT1, and TGFα (Fig. 2F and Supplementary Fig. S3C and S3D). To confirm that miR-30a-3p also regulates HIF2α, we stably inhibited miR-30a-3p and found elevated HIF2α abundance in RCC4 and UMRC2 cells (Fig. 2G and Supplementary Fig. S3E). In each of these studies, expression levels of HIF1α, and its transcriptional target phosphoglycerate kinase 1 (PGK1), were not altered by miR-30c-2-3p or miR-30a-3p.
miR-30a-3p (Fig. 2C–G and Supplementary Fig. S3B–S3E). Finally, our analysis of paired ccRCC tumor samples (TCGA data) revealed significant negative correlation between HIF1α or HIF2α targets (VEGF, GLUT1, TGFA) versus miR-30a-3p/miR-30c-2-3p levels in renal tumors (Supplementary Fig. S3F–S3H).

As stated above, recent studies have confirmed that HIF1α acts as a tumor suppressor in ccRCCs (10); however, 60% of ccRCCs constitutively express HIF1α (5, 6). We hypothesized that repression of miR-30a-3p and miR-30c-2-3p alleviates the suppressive effects of HIF1α by elevating relative HIF2α expression in VHL-deficient ccRCCs. To test this hypothesis, we first analyzed the basal expression of HIF2α in clinical ccRCC samples using TCGA data and observed significantly increased HIF2α levels relative to normal renal tissue (Fig. 2H). Because miR-30a-3p and miR-30c-2-3p are preferentially repressed in H1H2 tumors, we investigated whether HIF2α mRNA levels are significantly elevated in H1H2 tumors, compared with H2 tumors. To first identify H1H2 and H2 tumors among the TCGA ccRCC dataset, we first performed copy number (CN) analysis to determine the status of VHL and HIF1A. On the basis of this approach (see Methods), approximately 77% (373/470) of TCGA ccRCC samples were inferred to have VHL loss. Of these, approximately 30% also displayed a CN value for HIF1α of <1.6, signifying gene deletion (Supplementary Fig. S3H). These samples were confirmed as having hemizygous HIF1A deletions by independent analyses using cBioPortal for Cancer Genomics (Memorial Sloan-Kettering Cancer Center, New York, NY). On the basis of these criteria, we designated VHL-deficient ccRCC samples with CN <1.6 at HIF1A as the “H2” subtype (n = 110/362; Supplementary Fig. S3I), whereas VHL-deficient tumors expressing both HIF1α and HIF2α were designated as the “H1H2” group (n = 252/362). It should be emphasized that this stratification is based solely on VHL mutations/deletions and HIF1A deletions, and does not include other forms of genomic alteration that could reduce expression, such as aberrant DNA methylation or altered chromatin structure. Despite this limitation, our classification system was consistent with the stratification based on HIF1α and HIF2α immunohistochemical staining, where approximately one-third of VHL-deficient ccRCCs were designated as H2 tumors (5, 6). Importantly, our analysis of the stratified TCGA ccRCC samples revealed significantly higher HIF2A transcript levels in H1H2 tumors, relative to H2 ccRCCs (Fig. 2I). In addition, we measured HIF2A transcript levels in University of Pennsylvania (UPENN; Philadelphia, PA) ccRCC samples that were previously stratified into H1H2 and H2 subtypes by immunohistochemical staining for HIF1α and HIF2α (5, 6). Consistent with our results using the TCGA dataset, the UPENN ccRCC H1H2 tumors displayed higher HIF2A expression.
expression levels than the H2 tumors (n = 11 each; Fig. 2J), supporting our hypothesis that elevating HIF2α expression is a general mechanism whereby H1H2 ccRCCs compensate for the tumor-suppressive activities of HIF1α.

**HIF2α Is a Critical Target of miR-30c-2-3p in ccRCC**

Our data suggest that increased HIF2α protein levels are a primary functional consequence of miR-30c-2-3p/miR-30a-3p repression in H1H2 ccRCCs. To test this hypothesis directly, we transiently expressed miR-30c-2-3p/miR-30a-3p in the UMRC2 ccRCC cell line. Ectopically expressed miR-30c-2-3p inhibited UMRC2 proliferation during *in vitro* cell culture; however, no proliferative effect was observed with increased miR-30a-3p expression (Supplementary Fig. S4). Similarly, miR-30c-2-3p expression reduced the ability of UMRC2 cells to form colonies in *vitro* (Fig. 3A and B). 

**Figure 2.** Regulation and expression of HIF2α in ccRCC subtypes. A, schematic diagram depicting miR-30c-2-3p (H1) and miR-30a-3p (H2) binding sites in the HIF2α 3’UTR. B, RCC4 cells transfected with pMIR-REPORT with intact or mutated (MUT) seed sequences for miR-30c-2-3p and miR-30a-3p binding in the HIF2α 3’UTR were tested for luciferase activity in the presence of miR-30c-2-3p and miR-30a-3p mimics, respectively. C and D, expression of HIF1α and HIF2α transcripts upon stable ectopic expression (EE) and inhibition (INH) of miR-30c-2-3p in RCC4 cells. E, Western blot analysis showing the abundance of HIF1α and HIF2α protein after stable ectopic expression or inhibition of miR-30c-2-3p in RCC10 and RCC4 cells. β-tubulin was used as a loading control. The quantified value of protein expression represents the average of two experiments. F, transcript levels of HIF1α and HIF2α regulated target genes after stable ectopic expression of miR-30c-2-3p in RCC4 cells. G, Western blot analysis showing the levels of HIF1α and HIF2α protein after stable inhibition of miR-30a-3p in RCC4 and UMRC2 cells. The quantified value of protein expression represents the average of two experiments. β-tubulin was used as a loading control. H, HIF2α expression in ccRCC patient and normal renal tissue samples (TCGA). I and J, relative expression of HIF2α between H1H2 and H2 ccRCC subtypes from TCGA and UPENN human ccRCC patient samples. For all statistical analyses, *, P < 0.05; **, P < 0.005; ***, P < 0.0005. The data presented here are the average of three biologic replicates, unless specified. Data, mean ± SEM.
Regulation of HIF2α by miRNAs in ccRCCs

growth of UMRC2 cells was compromised by ectopic expression of miR-30c-2-3p (Fig. 3C). Similar results were obtained for the H2 ccRCC cell lines 786-O and 769-P (Supplementary Fig. S5C) was introduced into UMRC2 cells expressing either miR-SCR or miR-30c-2-3p. We observed that ectopic HIF2α expression significantly reversed the miR-30c-2-3p–mediated phenotypes in these assays (Fig. 3A–D). Collectively, these studies specifically illustrate that HIF2α is an important target of miR-30c-2-3p in ccRCC cells, and suggest that miR-30c-2-3p repression enhances HIF2α activity in H1H2 tumors, at the expense of HIF1α.

Modulation of miR-30c-2-3p and miR-30a-3p Expression Affects Tumor Growth and Predicts Human ccRCC Patient Survival

On the basis of our in vitro results, we first analyzed whether increased expression of miR-30c-2-3p and miR-30a-3p affects in vivo tumor growth. As UMRC2 cells reproducibly generate subcutaneous xenograft tumors (10), we injected immunocompromised (Nu/Nu) mice subcutaneously with UMRC2 cells ectopically expressing either miR-30c-2-3p, miR-30a-3p, or a scrambled control. Increased expression of either miR-30c-2-3p or miR-30a-3p significantly inhibited xenograft tumor growth when compared with the control group (Fig. 4A and Supplementary Fig. S5A and S5B). Therefore, we postulated that miR-30c-2-3p or miR-30a-3p inhibition would enhance ccRCC tumor growth in vivo. However, as noted above, the abundance of these miRNAs is already significantly repressed in ccRCC cells, making it potentially difficult to further reduce their expression. Given that basal miR-30a-3p levels are higher than miR-30c-2-3p in UMRC2 cells, we focused on the functional outcome of inhibiting miR-30a-3p in these cells. We injected immunocompromised (Nu/Nu) mice subcutaneously with UMRC2 cells expressing either a miR-30a-3p antagonist (miR-30a-3p INH) or a scrambled control (miR-SCR INH; Fig. 4B and Supplementary Fig. S7A and S7B). Importantly, tumors derived from the miR-30a-3p INH cells were significantly larger than controls (Fig. 4B and
Figure 4. Tumor-suppressive role of miR-30c-2-3p and miR-30a-3p in ccRCC tumor growth. A, growth curve of xenografts subcutaneously implanted with UMRC2 cells after ectopic expression of miR-30c-2-3p or miR-30a-3p. UMRC2 cells transduced with miR-SCR OE are used as the control (n = 8). OE, overexpression. B, xenograft tumor growth with UMRC2 cells after inhibition of miR-30a-3p, and miR-SCR INH are used as the control (n = 5). C, Ki67+ proliferating cells in UMRC2 miR-SCR INH or miR-30a-3p INH xenograft tumors. Scale bar is 20 μm. D, CD31 and smooth muscle actin (SMA) immunofluorescence in UMRC2 miR-SCR INH or miR-30a-3p INH xenograft tumor sections. Scale bar is 20 μm. DAPI, 4,6-diamidino-2-phenylindole. E, quantification of SMA+ area indicative of pericyte coverage of blood vessels in UMRC2 miR-SCR INH or miR-30a-3p INH sections. F, percentage of SMA+ area coverage of total blood vessel area (CD31+) in UMRC2 miR-SCR INH or miR-30a-3p INH tumors. G, data demonstrating CD31+ blood vessel area in UMRC2 miR-SCR INH or miR-30a-3p INH tumor sections. H, Western blot analysis showing HIF2α abundance in UMRC2 miR-SCR INH or miR-30a-3p INH xenograft tumors (n = 3 each). I and J, Kaplan–Meier survival curve based on high and low miR-30c-2-3p or miR-30a-3p expression in patients with ccRCC (TCGA). *miR-30c-2-3p low (n = 143) represents the bottom one-third of the cases, whereas *miR-30a-3p high (n = 286) is designated as the top two-thirds of the samples. Log-rank test was used to assess statistical significance. Similar classification was used for performing survival analysis based on miR-30a-3p levels. K, proposed model illustrating the role of miR-30c-2-3p and miR-30a-3p in modulating HIF2α levels in ccRCC tumors. For all statistical analyses: *, P < 0.05; **, P < 0.005; ***, P < 0.0005. Data, mean ± SEM.
Supplementary Fig. S7A and S7B). Histologic analyses also revealed mild to moderate degenerative changes in tumors from the miR-SCR INH group, a feature not observed in miR-30a-3p INH tumors (Supplementary Fig. S7C). Immuno-histochemical staining for Ki67 demonstrated increased cell proliferation in miR-30a-3p INH tumors compared with miR-SCR INH tumors (Fig. 4C). Of note, the effect of miR-30a-3p on cell proliferation is very similar to HIF2α, where increased miR-30a-3p expression (Supplementary Fig. S4) or HIF2α inhibition did not affect cell growth in vitro, but inhibited tumor growth in vivo (7). Although no significant difference in tumor blood vessel area was observed between the two cohorts, as reflected by the number of CD31+ endothelial cells (Fig. 4D and G), vessel coverage by smooth muscle actin-expressing (SMA+) pericytes was significantly increased in miR-30a-3p INH tumors (Fig. 4D and E), suggesting that these vessels are more mature (Fig. 4F). Furthermore, we observed increased HIF2α protein expression in miR-30a-3p-inhibited tumors compared with the miR-SCR INH group (Fig. 4H), suggesting that elevated HIF2α levels contribute directly to the tumor angiogenesis and cell proliferation phenotypes in miR-30a-3p INH tumors. These results are also consistent with previous reports, indicating that HIF2α expression is positively associated with ccRCC cell proliferation and tumor angiogenesis (16).

Finally, we determined whether miR-30c-2-3p and miR-30a-3p expression levels correlate with human ccRCC patient survival. Reduced expression of either miR-30c-2-3p or miR-30a-3p was significantly associated with poor prognosis in patients with ccRCC (Fig. 4I and J), suggesting that their repression is important for ccRCC growth and/or progression. Collectively, our studies illustrate a novel compensatory mechanism whereby tumor cells alter miRNA expression to increase the abundance of an oncoprotein, HIF2α.

**DISCUSSION**

Biallelic VHL inactivation leads to an increased abundance of HIF1α and HIF2α in ccRCCs, and compelling evidence suggests that HIF2α, rather than HIF1α, promotes pVHL-deficient ccRCC tumorigenesis (17). However, nearly two-thirds of VHL-mutant ccRCCs express HIF1α (5, 6), prompting us to investigate the molecular mechanisms that overcome the previously characterized tumor-suppressive activity of HIF1α in these tumors. Although the role of various miRNAs has been documented in ccRCCs (12), the major goal of our study was to identify miRNAs that are preferentially altered in H1H2 versus H2 subtypes. This analysis gave us a unique opportunity to probe novel molecular pathways that are distinct between the two ccRCC groups. We demonstrate here that HIF2α expression is elevated in H1H2 tumors, and propose that preferential repression of miR-30c-2-3p and miR-30a-3p in the H1H2 subclass contributes significantly to this phenotype.

miR-30c-2-3p and miR-30a-3p are located on chromosome 6q13, and our data suggest that their expression is pVHL dependent, but HIF independent. Similar to our results, miR-204 is also repressed in VHL-deficient ccRCCs, and positively regulated by pVHL independent of HIF activity (11). Although pVHL stimulates miR-204, miR-30c-2-3p, and miR-30a-3p expression, it is likely that additional intermediate factors are involved. pVHL clearly affects the activity of multiple transcription factors, including NF-κB (18) and β-catenin (19). Of note, GATA3 regulates miR-30c-2-3p transcription in breast tumor cells (20), and GATA3 expression is decreased in all stages of ccRCC (see Supplementary Fig. S8), due to epigenetic silencing (21). Therefore, it is plausible that loss of GATA3 in ccRCC is responsible for reduced miR-30c-2-3p and miR-30a-3p levels in VHL-deficient ccRCCs.

In addition to the modulation of HIF2α levels by miRNA repression, alternative mechanisms favoring enhanced HIF2α activity have been described previously. Factor inhibiting HIF1 (FIH1) is an oxygen-dependent enzyme that hydroxylates an asparaginyl residue in its C-terminal transactivation domain (CTAD), preventing the recruitment of coactivator proteins required for transcriptional activity (22). However, HIF2α is less sensitive to FIH1-mediated inhibition than HIF1α (23), resulting in a relative increase in HIF2α target gene expression. In addition, HIF1α undergoes preferential proteasomal degradation in the absence of pVHL, possibly via distinct ubiquitin ligases or direct interaction with the proteasome (24). These findings clearly suggest that HIF2α abundance is critical for ccRCC tumorigenesis, and miR-30c-2-3p and miR-30a-3p repression represents a novel means whereby tumor cells antagonize the tumor-suppressive role of HIF1α (Fig. 4K).

A more general role for miRNAs has been demonstrated in numerous cancers (including ccRCCs), suggesting that the development of miRNA-based therapies could ultimately be beneficial for patient care. Systemic miR-26a delivery using adeno-associated virus inhibits the progression of hepatocellular carcinoma in mice, illustrating the potential utility of these biomolecules in disease treatment (25). Although some progress has been made in miRNA delivery, the use of synthetic miRNAs for renal tumor therapy is still in its infancy. Because miR-30c-2-3p and miR-30a-3p directly regulate HIF2α, which acts as an oncoprotein in ccRCC, these miRNAs represent novel therapeutic agents of the future. In conclusion, we demonstrate a novel molecular mechanism by which HIF2α levels are elevated to compensate for the inhibitory effects of HIF1α in VHL-deficient H1H2 ccRCCs.

**METHODS**

**Cell Culture**

The human RCC cell lines RCC4 and 786-O were obtained from the American Type Culture Collection. RCC10, UMRC2, and 769-P cells were a kind gift from Dr. W.G. Kaelin (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). These cells were cultured in Dulbecco’s Modified Eagle Medium containing 10% FBS and antibiotics. All cell lines were verified for VHL and HIFα expression status using quantitative PCR and Western blot analysis within the last 6 months.

**ccRCC Patient Samples**

For performing microarray analysis, fresh tumor samples were obtained from the Hospital of UPENN. Samples were embedded at optimum cutting temperatures and sectioned for RNA extraction. The protocols used were approved by the Institutional Review Board of University of Pennsylvania.

Other information on methods is described in the Supplementary Data.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: L.K. Mathew, N. Skuli, P. Lal, M.C. Simon
Development of methodology: L.K. Mathew, N. Skuli
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.S. Lee, K.L. Nathanson, P. Lal
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.K. Mathew, S. Rao, B. Keith, K.L. Nathanson
Writing, review, and/or revision of the manuscript: L.K. Mathew, N. Skuli, B. Keith, K.L. Nathanson, M.C. Simon
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.K. Mathew, S.S. Lee
Study supervision: L.K. Mathew, M.C. Simon

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