Mammalian Target of Rapamycin Is a Therapeutic Target for Murine Ovarian Endometrioid Adenocarcinomas with Dysregulated Wnt/β-Catenin and PTEN

Pradeep S. Tanwar1, LiHua Zhang1, Tomoko Kaneko-Tarui1, Michael D. Curley1, Makoto M. Takeo2, Poonam Rani1, Drucilla J. Roberts3, Jose M. Teixeira1*

1 Vincent Center for Reproductive Biology, Department of Obstetrics, Gynecology, and Reproductive Biology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, United States of America, 2 Department of Pharmacology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, 3 Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, United States of America

Abstract

Despite the fact that epithelial ovarian cancers are the leading cause of death from gynecological cancer, very little is known about the pathophysiology of the disease. Mutations in the WNT and PI3K pathways are frequently observed in the human ovarian endometrioid adenocarcinomas (OEs). However, the role of WNT/β-catenin and PTEN/AKT signaling in the etiology and/or progression of this disease is currently unclear. In this report we show that mice with a gain-of-function mutation in β-catenin that leads to dysregulated nuclear accumulation of β-catenin expression in the ovarian surface epithelium (OSE) cells develop indolent, undifferentiated tumors with both mesenchymal and epithelial characteristics. Combining dysregulated β-catenin with homozygous deletion of PTEN in the OSE resulted in development of significantly more aggressive tumors, which was correlated with inhibition of p53 expression and cellular senescence. Induced expression of both mTOR kinase, a master regulator of proliferation, and phosphorylation of its downstream target, S6Kinase was also observed in both the indolent and aggressive mouse tumors, as well as in human OEA with nuclear β-catenin accumulation. Ectopic allotransplants of the mouse ovarian tumor cells with a gain-of-function mutation in β-catenin and PTEN deletion developed into tumors with OEA histology, the growth of which were significantly inhibited by oral rapamycin treatment. These studies demonstrate that rapamycin might be an effective therapeutic for human ovarian endometrioid patients with dysregulated Wnt/β-catenin and Pten/PI3K signaling.

Introduction

Ovarian cancer is the most deadly gynecological cancer among women in the United States with approximately 22,000 new cases diagnosed and 15,000 deaths yearly, the vast majority of which are from metastatic epithelial-derived ovarian tumors. The prognosis is poor because most patients are diagnosed during the late stage of the disease, when ovarian cancer has already metastasized and the survival rate is less than 30% [1,2]. The cell of origin and cause of disease progression is not well defined because cancer is usually diagnosed at an advanced stage [3]. The prevailing theory is that these tumors originate in the ovarian surface epithelium (OSE), a single layer of mesothelial cells covering the surface of the ovary [4], by a mechanism that might involve the formation of cortical inclusion cysts during rupture and repair after ovulation or from ovarian atrophy with aging [3].

Ovarian epithelial tumors are classified into five different major histological categories: serous, endometrioid, mucinous, clear cell, and undifferentiated [5]. It is not known whether a specific combination of genetic mutations force the OSE cells to acquire the specific lineage or whether tumors arise first and acquire more mutations leading to or commensurate with their commitment to Mullerian metaplasia later during the course of disease development. The latter hypothesis is well supported by studies in various mouse models of ovarian cancer in which initial OSE derived cancerous growths are undifferentiated [2,6,7].

Wnt signaling is essential for normal ovarian development and various members of the canonical Wnt signaling pathway are expressed in the ovary [8,9]. Mutations in the β-catenin, APC, Axin1, and Axin2 genes are associated with ovarian epithelial cancers [10,11]. Dysregulated WNT/β-catenin signaling plays an important role in the development of human ovarian endometrioid adenocarcinomas (OEs) but is rare in other types of ovarian cancer. For example, mutations in exon 3 of β-catenin, which lead to its stabilization and nuclear accumulation [12], are present in an estimated 16%–38% of human OEs [11,13]. In the present report, we have investigated the mechanisms of tumorigenesis in murine OSE cells with deletion of exon 3 of β-catenin and/or Pten. We show that the mTOR pathway is activated in mice with dysregulated WNT/β-catenin and Pten/PI3K signaling, as well as in human OEs. We also show evidence that rapamycin decreases tumor burden in allotransplants of the tumor.
cells, suggesting that human OEAs might be a good target for rapamycin therapy.

Materials and Methods

Mouse genetics and husbandry

All protocols involving animal experimentation were approved by the MGH Institutional Animal Care and Use Committee (Protocol # 2005N000195). The mice used in this study were maintained on C57BL/6J;129/SvEv mixed genetic background and housed under pathogen free standard animal housing conditions as described [14]. The following parental alleles—Ctnnb1tm1Mmt [15], Ptenfltm1Hwu [16], Amhr2tm3(cre)Bhr [17], and Ctnnb1fl(ex3) or Ctnnb1D/D—were used in the crosses and are hereafter called Ctnnb1flox/flox or Ctnnb1fl(ex3), Ptenflox/flox, or Ptenflox/flox, and Amhr2-Cre, respectively. The genotyping of mice was performed with standard PCR protocols using DNA collected from tail biopsies. The PCR conditions for Ctnnb1flox or Amhr2flox/creBlue are previously described [12,17,18]. The Ptenflox allele was detected with primers 5′-ACTCAAGGCAGGGATGAGC-3′ and 5′-GCCCGATGCAATAATATG-3′, for 35 cycles of 94°C for 9 sec, 60°C for 1 min, and 72°C for 1 min using Taq DNA polymerase (Roche). The gross images were taken with a Nikon D60 digital camera with a macro lens.

Histology and Immunofluorescence

The methods used for histology, IF, and IHC have been previously described [19]. IF and/or IHC was performed on tissues derived from minimum of three different animals per genotype. The whole tissue sections and/or at least three independent random areas of section at 10× were examined for specific markers staining and representative images were collected. The tissue blocks for human ovarian endometrioid adenocarcinomas (n = 4) and normal/benign ovarian samples (n = 3) were obtained from the Pathology Department, MGH using Institutional Review Board-approved protocols. To examine epithelial glands, whole mouse ovarian tumors (n = 6) were cut into multiple pieces. The tumors blocks were serially sectioned and are hereafter called Ctnnb1flox/flox or Ctnnb1fl(ex3), Ptenflox/flox, or Ptenflox/flox, and Amhr2-Cre, respectively. The genotyping of mice was performed with standard PCR protocols using DNA collected from tail biopsies. The PCR conditions for Ctnnb1flox or Amhr2flox/creBlue are previously described [12,17,18]. The Ptenflox allele was detected with primers 5′-ACTCAAGGCAGGGATGAGC-3′ and 5′-GCCCGATGCAATAATATG-3′, for 35 cycles of 94°C for 9 sec, 60°C for 1 min, and 72°C for 1 min using Taq DNA polymerase (Roche). The gross images were taken with a Nikon D60 digital camera with a macro lens.

Table 1. Antibodies used in this study for IHC, IF and Western blot (WB) analyses.

<table>
<thead>
<tr>
<th>ANTIGEN</th>
<th>SUPPLIER</th>
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<tbody>
<tr>
<td>p19ARF, p21</td>
<td>Abcam, Cambridge, MA</td>
</tr>
<tr>
<td>Activated Caspase 3, β-catenin</td>
<td>BD Transduction Laboratories, San Jose, CA</td>
</tr>
<tr>
<td>Vimentin, AMH, p16INK4A</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>Inhibin-α</td>
<td>Biogenes, San Ramon, CA</td>
</tr>
<tr>
<td>pH3, γ-H2AX</td>
<td>Millipore, Bellerica, MA</td>
</tr>
<tr>
<td>α-SMA-Cy3, β-catenin</td>
<td>Sigma Chemical Co, St. Louis, MO</td>
</tr>
<tr>
<td>AKT, phosphoAKT, Pten, p53, mTOR, pmTOR, S6K, pS6K, and p27kip1</td>
<td>Cell Signaling Technologies, Danvers, MA</td>
</tr>
<tr>
<td>Pan-cytokeratin</td>
<td>Neomarkers, Fremont, CA</td>
</tr>
<tr>
<td>Cytokeratin 8</td>
<td>Developmental studies hybridoma bank, IA</td>
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<tr>
<td>β-actin</td>
<td>Neomarkers, Fremont, CA</td>
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Historically, proliferation of epithelial cells was assessed by measuring the number of pH3-, active caspase 3- and TUNEL-positive cells, and DAPI stained nuclei were counted per high power fields. The images were captured with a Nikon T2000 microscope equipped with an epifluorescence attachment and a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI).

Primary tumor cell isolation, transplantation to NOD/SCID mice, and rapamycin treatments

Primary ovarian tumors were derived from the Amhr2-Cre/Ctnnb1fl(ex3)/+;Ptenflox/flox mice. Cells from the primary tumors were isolated, diluted (2×10⁶ cells/mouse) in 1:1 Matrigel (BD Biosciences), and injected subcutaneously into female NOD/SCID mice (The Jackson Laboratory, Bar Harbor, ME). Grafted mice were divided into two groups (n = 5/group) and administered 200 μl rapamycin (250 μg/dose, Rapamune, Wyeth, PA) or vehicle control (kindly donated by The American Lecithin Company, Oxford, CT) twice/week by oral gavage. After 10 weeks of treatment, tumors were excised for analysis. All animals were euthanized after 12 weeks.

Western blot analyses

Western analyses of ovarian cells collected from a minimum of 3 age-matched control and mutant mice were performed as previously described [20]. β-actin was used as a loading control. The experiments were repeated three times. Granulosa cells were isolated from mice 24 h after they were injected with pregnant mare’s serum gonadotropin by puncturing the follicles with a tuberculin syringe.

Tumor Morphometrics

At the end of study, tumor volume was calculated based on caliper measurements of the exposed tumor using the formula for an ellipsoid: 4/3 π×1/2 length×1/2 width² [21]. Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) staining was performed per the manufacturer’s instructions (Roche, Indianapolis, IN). For estimation of the total number of pH3-, active caspase 3- and TUNEL-positive cells, images from three different tumors derived from three different animals were taken at equivalent gain settings using a microscope (Nikon TE 2000-S; Micro Video Instruments, Avon, MA) equipped with a Spot digital camera. pH3-, active caspase 3-, and TUNEL-positive cells, and DAPI stained nuclei were counted with the nucleus-counting plugin of ImageJ software (v1.37, NIH).
Statistical analysis

The unpaired \( t \) test was used to test for differences between groups and \( p<0.05 \) was considered statistically significant. Statistical analyses were performed using Prism software (GraphPad Software, San Diego, CA).

Results

Deregulated Wnt/\( \beta \)-catenin signaling in human and mouse ovarian tumors

Because mutations in Wnt/\( \beta \)-catenin signaling components are frequently observed in human OEA patients [11,13], we examined \( \beta \)-catenin protein expression in human OEA tissue samples and observed nuclear accumulation of \( \beta \)-catenin, which is...
In Amhr2-Cre;Ctnnb1 mice ovaries (Panels A–C), Panels B & C are magnified views of boxed areas in Panel A. Cytokeratin 8 (CK8) immunofluorescence on a serial section (D) of panel C confirming presence of epithelial glands (arrowhead); By indicates background fluorescence from a blood vessel. (Panel E) CK8 staining in control ovary marks OSE cells (arrowheads) and oviductal (Ovi) epithelial cells. (Panel F) CK8 expression in a typical small tumor in Amhr2-Cre;Ctnnb1 mice ovaries. (Panel G) Vimentin immunofluorescence in control ovaries was present in some stromal cells but not in granulosa cells of follicles (outlined by dotted line, F). In Amhr2-Cre;Ctnnb1 mice ovaries (Panel H), vimentin expression was observed throughout the tumors. (Panels I–L) Amh and inhibin-α expression in control (I & K) and mutant (J & L) ovaries. Inhibin-α and Amh expression was present in granulosa cells of the remnant follicles (arrow, demarcated from the tumor by white dotted line) of mutant ovaries but not in tumor areas (indicated with a T). Nuclei are stained with DAPI in Panels D–L. Bars = 50 um.

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Figure 2. Histological examination of ovarian tumors formed in Amhr2-Cre;Ctnnb1 mice ovaries. H&E staining of mutant ovaries (Panels A–C). Panels B & C are magnified views of boxed areas in Panel A. Cytokeratin 8 (CK8) immunofluorescence on a serial section (D) of panel C confirming presence of epithelial glands (arrowhead); By indicates background fluorescence from a blood vessel. (Panel E) CK8 staining in control ovary marks OSE cells (arrowheads) and oviductal (Ovi) epithelial cells. (Panel F) CK8 expression in a typical small tumor in Amhr2-Cre;Ctnnb1 mice ovaries. (Panel G) Vimentin immunofluorescence in control ovaries was present in some stromal cells but not in granulosa cells of follicles (outlined by dotted line, F). In Amhr2-Cre;Ctnnb1 mice ovaries (Panel H), vimentin expression was observed throughout the tumors. (Panels I–L) Amh and inhibin-α expression in control (I & K) and mutant (J & L) ovaries. Inhibin-α and Amh expression was present in granulosa cells of the remnant follicles (arrow, demarcated from the tumor by white dotted line) of mutant ovaries but not in tumor areas (indicated with a T). Nuclei are stained with DAPI in Panels D–L. Bars = 50 um.

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Figure 3. Sustained activation of β-catenin in the somatic cells leads to the induction of p53-mediated senescence in Amhr2-Cre;Ctnnb1Δ(ex3)/+ ovaries. (Panel A–F) Colocalization of β-catenin and PTEN in serial sections of control (Ctnnb1Δ(ex3)/+), Amhr2-Cre;Ctnnb1Δ(ex3)/+ and Amhr2-Cre;Ctnnb1Δ(ex3)/+;PtenΔ/Δ ovaries. Insets in Panels B, E, & F are higher magnification images of areas marked by black rectangles. F: follicle, t: tumor.

β-catenin
Akt
pAKT
S6K
pS6K
p53
p21
p19ARF
p16INK4a
p27kip1
β-actin
expression of vimentin, a mesenchymal marker. We observed strong positive staining for vimentin in less differentiated areas of pretumoral lesions and in fully-grown tumors, suggesting that cancerous cells undergo epithelial mesenchymal transition (EMT) in these tumors (Fig. 2H). Vimentin staining was observed in stromal but not in granulosa cells of the control ovaries (Fig. 2G).

Since these tumors showed less differentiated morphology and Amhr2-cre is also expressed in granulosa cells at later stages of development [25], we examined expression of the ovarian granulosa cell tumor markers to rule out the possibility that these tumors might be derived from the granulosa cells. We analyzed the expression of inhibin-α and anti-Müllerian hormone (AMH, also known as Müllerian Inhibiting Substance [26]), which are the two markers most often used to detect murine, human, and equine granulosa cell tumors [27,28,29], in Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^ ovaries (Fig. 2I–L). Although expression of both was observed in control follicles (Fig. 2I & K) and in the remaining follicles of early tumors (Fig. 2J & L), expression of these two markers was not observed in the tumor areas.

PTEN deletion with constitutively activated (CA) β-catenin leads to development of more aggressive tumors

Interactions between Wnt/β-catenin and Akt/PTEN signaling pathways play an important role in carcinogenesis [6]. We examined the status of PTEN by performing immunohistochemical staining for β-catenin and PTEN on serial sections of Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^ ovaries and observed increased expression of PTEN in pretumoral lesions with nuclear β-catenin (Fig. 3B & E). To examine whether PTEN deletion could affect tumor progression in Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^/Pten^floxed^ ovaries, we developed another mouse model by deleting exon 3 of the β-catenin in PTEN negative cells of the mouse ovary (Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^/Pten^floxed^;Pten^floxed^) (Fig. 3C & F). These mice showed early onset of tumor development and were euthanized because of tumor-related morbidities (Fig. 3G & H). No evidence of ascites or metastases was observed by gross examination of the peritoneal cavity. The ovarian tumors in Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^/Pten^floxed^/Pten^floxed^ mice showed histopathological features similar to the tumors of Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^ mice (Fig. S2). Consistent with previous observations [22,30], deletion of PTEN alone does not result in ovarian tumor development (data not shown), suggesting that activation of the phosphatidylinositol-3′ kinase (PI3K) signaling pathway with inactivating PTEN mutations alone may not be sufficient for tumor initiation and needs to act in concert with other oncogenes to cause cancer.

PTEN has been shown to regulate P53 protein levels and this interaction plays a role in cellular senescence, growth inhibition, and cellular transformation [31]. Additionally, dysregulated β-catenin expression causes growth arrest and a senescent-like state in mouse embryonic fibroblast cells (MEFs) and activation of β-catenin stimulates proliferation of p53-deficient MEFs suggesting that the senescence observed after activation of β-catenin is mediated by p53 [32]. In vivo, p53 deficiency increases the tumorigenicity of intestinal tumors in APC-deficient mice indicating that loss of p53 plays an important role in the progression of these tumors [33]. We have observed early onset of tumor development in Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^/Pten^floxed^ mice (Fig. 3G & H). However, tumor progression has up to a year long latency period in Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^/Pten^floxed^/Pten^floxed^ mice (Fig. 3I & J). We examined whether the p53 senescence pathway might be involved in the induction of tumor progression in the Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^/Pten^floxed^/Pten^floxed^;Pten^floxed^ tumors, which could account for the discrepancy in timing of tumor progression in these two genotypes. We found positive staining for SA-β-gal, a hallmark of senescent cells [34], in pretumoral lesions present in 4-week old ovaries (Fig. 1). We examined whether the p33 senescence pathway might be involved in the inhibition of tumor progression in the Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^/Pten^floxed^/Pten^floxed^;Pten^floxed^, which could account for the discrepancy in timing of tumor progression in these two genotypes. We found positive staining for SA-β-gal, a hallmark of senescent cells [34], in pretumoral lesions present in 4-week old ovaries (Fig. 1). We examined whether the p53 senescence pathway might be involved in the inhibition of tumor progression in the Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^/Pten^floxed^/Pten^floxed^;Pten^floxed^, which could account for the discrepancy in timing of tumor progression in these two genotypes. We found positive staining for SA-β-gal, a hallmark of senescent cells [34], in pretumoral lesions present in 4-week old ovaries (Fig. 1). We examined whether the p33 senescence pathway might be involved in the inhibition of tumor progression in the Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^/Pten^floxed^/Pten^floxed^;Pten^floxed^, which could account for the discrepancy in timing of tumor progression in these two genotypes. We found positive staining for SA-β-gal, a hallmark of senescent cells [34], in pretumoral lesions present in 4-week old ovaries (Fig. 1). We examined whether the p53 senescence pathway might be involved in the inhibition of tumor progression in the Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^/Pten^floxed^/Pten^floxed^;Pten^floxed^, which could account for the discrepancy in timing of tumor progression in these two genotypes. We found positive staining for SA-β-gal, a hallmark of senescent cells [34], in pretumoral lesions present in 4-week old ovaries (Fig. 1). We examined whether the p33 senescence pathway might be involved in the inhibition of tumor progression in the Amhr2-Cre;Ctnnb1^lox/lox^/Pten^floxed^/Pten^floxed^/Pten^floxed^;Pten^floxed^, which could account for the discrepancy in timing of tumor progression in these two genotypes. We found positive staining for SA-β-gal, a hallmark of senescent cells [34], in pretumoral lesions present in 4-week old ovaries (Fig. 1).
CA β-catenin induces mTOR expression and rapamycin treatment reduces tumor burden

Mammalian target of rapamycin (mTOR) signaling is a master regulator of cellular proliferation and is an attractive therapeutic target for a variety of cancers [37]. Dysregulated Wnt/β-catenin signaling has been shown to induce mTOR expression and signaling in intestinal polyps [38] of mutant APC mice, and we have shown that mTOR expression is elevated in uteri of Amhr2-Cre;Ctnnb1Δ(ex3)/+ mice [19]. In Fig. 3X, we observed elevated expression by western analysis of pS6K, a downstream target of mTOR kinase activity, in the Amhr2-Cre;Ctnnb1Δ(ex3)/+ and Amhr2-Cre;Ctnnb1Δ(ex3)/+,PtenΔ/Δ tumors compared to control mice. Immunohistochemistry of mTOR, p mTOR, and pS6K was performed to confirm that the increased expression was isolated to the tumor cells in mutant ovaries (Fig. 4A–I). We also observed strong expression for mTOR, p mTOR, and pS6K in human OEAs (4/4) (Fig. 4J–Q), which in Fig. 1, we showed also had induced levels of nuclear β-catenin.

The upregulation of mTOR activity is commonly observed in colon, urinary bladder, and salivary gland cancers formed after dysregulated...
inhibiting mTOR activity and controlling cell proliferation and death. (Panel A) Tumor size was significantly decreased in rapamycin-treated mice compared to vehicle-treated controls. *One of the vehicle-treated mice was euthanized early because the tumor had grown too large and was excluded from morphometric measurements. (Panel B & C) pS6K immunostaining of tumors (n = 5) showed that vehicle-treated tumors were strongly positive; whereas rapamycin treated tumors (n = 5) were negative. (Panel D & E) H&E staining revealed distinct epithelial glands similar to primary tumors. Panel E is the higher magnification image of area marked by a rectangle in Panel D. Colocalization of vimentin (red) and cytokeratin (green) revealed that these tumors are strongly positive for cytokeratin (F). Occasional tumor lesions expressing both markers (G), vimentin (red, asterisk) and cytokeratin (green, arrowheads) were also observed. Bars = 50 µm.

Figure 5. Rapamycin treatment reduced tumor burden by inhibiting mTOR activity and controlling cell proliferation and death. (Panel A) Tumor size was significantly decreased in rapamycin-treated mice compared to vehicle-treated controls. *One of the vehicle-treated mice was euthanized early because the tumor had grown too large and was excluded from morphometric measurements. (Panel B & C) pS6K immunostaining of tumors (n = 5) showed that vehicle-treated tumors were strongly positive; whereas rapamycin treated tumors (n = 5) were negative. (Panel D & E) H&E staining revealed distinct epithelial glands similar to primary tumors. Panel E is the higher magnification image of area marked by a rectangle in Panel D. Colocalization of vimentin (red) and cytokeratin (green) revealed that these tumors are strongly positive for cytokeratin (F). Occasional tumor lesions expressing both markers (G), vimentin (red, asterisk) and cytokeratin (green, arrowheads) were also observed. Bars = 50 µm.

Wnt and/or PI3K signaling [38,39,40]. Since we observed increased pS6K protein levels in tumors formed in the mouse models (Amhr2-Cre;Ctnnb1 homozygous and Amhr2-Cre;Ctnnb1 heterozygous; Pten+/−/−) and in human OES, we speculated that the mTOR pathway might also be involved in the growth of these tumors. Tumor cells were collected from Amhr2-Cre;Ctnnb1 homozygous and injected into the dorsal flanks of 10 NOD/SCID mice in order control for uniformity at the start of the experiment. After the first week, five mice were randomly assigned to either a rapamycin-treated group or a vehicle-treated group. Significantly reduced tumor growth was observed in mice treated with rapamycin for 12 weeks by oral gavage, compared to the vehicle-treated controls (Fig. 5A). The tumor volume and weight were significantly lower in the rapamycin-treated group compared to the control group (Table 2). Rapamycin treatment in cancer cell lines and in vivo mouse models induces growth arrest and apoptosis [41]. We investigated the inhibitory effect of rapamycin in these tumors by analyzing the proliferation and apoptotic rate compared to control-treated tumors (Fig. S3). Rapamycin-treated tumors had 1/3rd the number of proliferating pH3-positive cells than the vehicle-treated tumors (Table 2), suggesting that rapamycin treatment decreased the proliferation rate of the tumor cells. TUNEL and activated Caspase 3 immunostaining showed that rapamycin treatment also resulted in a significant increase in cell death compared to the vehicle-treated tumors (Table 2 & Fig. S3). To confirm that rapamycin treatment inhibited the activation of mTOR signaling in the treated tumors, we performed immunohistochemical staining for pS6K. In Fig. 5B & C, we show a representative example from one of the 5 vehicle-treated tumors with strong pS6K immunostaining, whereas little pS6K immunostaining was detected in 100% (5/5) of the rapamycin-treated tumors examined. These findings indicate that rapamycin treatment significantly decreased tumor burden by inhibiting mTOR activity, mainly by reducing proliferation and increasing cell death. We also performed histological examination of vehicle- and rapamycin-treated tumors. Similar to primary tumors, grafted tumors showed epithelial glands admixed with less differentiated tumor cells (Fig. 5D & E). Colocalization of vimentin (a mesenchymal marker) and cytokeratin (an epithelial marker) in the grafted tumors showed that tumors were strongly positive for cytokeratin and some areas of tumors were positive for both markers (Fig. 5F & G), suggesting that epithelial to mesenchymal (EMT) transition might be occurring in the allotransplants.

Discussion

Most ovarian carcinomas are thought to originate from the mesothelial cells covering of the ovary known as the OSE [4]. However, the histology of the most common human epithelial ovarian cancers is more similar to the fallopian tube (serous), uterus (endometrioid) and cervix (mucinous) [4]. It is not known how this simple monolayer of cells gives rise to such a complex disease and why ovarian carcinomas are so similar to the Mullerian duct-derived tissues. During development, Mullerian duct epithelia commit to the specific lineages by acquiring a fallopian, uterine, or cervical phenotypes due, in part, to the segmental expression of Hoxa genes [42,43]. However, OSE cells maintain a primitive multipotential phenotype [44]. It has been suggested that over time these cells accumulate various genetic
mutations leading to tumor formation and subsequent differentiation to the various lineages of ovarian tumors [4,45]. Other studies have suggested alternative sites, like the distal end of fimbriae and secondary Mullerian duct system, as the points of origin for ovarian carcinomas [3,45]. Additionally, endometrioid lesions have been suggested as the precursor for OESs, particularly those with beta-catenin and PTEN mutations [46]. Our results favor the OSE origin hypothesis, at least for OEE development, since we observed tumors arising in the OSE that can differentiate into the OEE histotype.

In human colorectal cancers, tumor cells with nuclear accumulation of beta-catenin undergo growth arrest and EMT [47,48]. These cells progressively lose E-cadherin, an epithelial marker, and acquire fibronectin expression, a mesenchymal marker [47,48]. Furthermore, Wnt signaling has been shown to promote EMT and a tumor invasion of the breast and cervical cancer cells by regulating Snail activity [49]. The forced expression of Snail and Slug in ovarian cancer cell lines suppresses E-cadherin levels, which correlates with the induction of an EMT-like state and generation of cancer cells with stem cell characteristics [50]. In a previous report, it was observed that combined deletion of APC and PTEN causes formation of ovarian epithelial tumors accompanied by loss of E-cadherin expression and appearance of mesenchymal-like cells suggesting EMT [6]. Our results showing expression of both mesenchymal and epithelial markers in the tumors (Figs. 2 & 5) also suggested that EMT occurs during the development of ovarian carcinoma with dysregulated Wnt/beta-catenin signaling. Preliminary studies to investigate this possibility showed upregulated expression of Snail and Slug in tumors by immunofluorescence (data not shown) but more detailed mechanistic studies are required to determine whether dysregulated Wnt/beta-catenin expression is driving EMT, which then leads to tumor development, or whether EMT is the result of tumor development.

In previous reports [51,52], appearance of these mesenchymal cells was considered a malignant transformation of granulosa cells, which led to their classification as granulosa cell tumors of the ovary, even though the markers for granulosa cells were not reported. However, we showed that these tumors failed to express markers of granulosa cell tumors (AMH and inhibin-alpha), and express epithelial markers suggesting an epithelial origin for these tumors (Fig. 2). Consistent with our results, ovarian tumors formed in APC-PTEN knockout model were also negative for inhibin-alpha staining [6]. Recently, Fan et al. specifically activated beta-catenin in granulosa cells of the mouse ovary by conditional deleting exon3 (Ex3) of beta-catenin using Cyp19-cre and observed no tumor formation in these mutant mice (Cyp19-Cre;Clamb1<Ex3/+>). [53]. Furthermore, only membranous but not nuclear beta-catenin expression is observed in human granulosa cell tumor patient samples [28], suggesting Wnt/beta-catenin signaling does not play a role in either mouse or human granulosa cell tumor development. Alternatively, granulosa cells are thought to originate from the coelomic epithelium of the ovary during the perinatal period [54], which could classify the cells in these reports as “pregranulosa” cells that can differentiate into either granulosa cell tumors or ovarian mesenchymal tumors, depending on the context.

Lastly, our findings, along with those of others [55], provide an exciting prospect for using mTOR inhibitors together with other therapies in the treatment of ovarian carcinomas. mTOR is a critical regulator of cell growth and proliferation, and activation of this pathway occurs in many tumors including colon, ovarian, and uterine [19,38,41,55]. Activation of Wnt/beta-catenin has been shown to upregulate mTOR activity in colon and uterine cancers [19,38], and treatment with an mTOR-specific inhibitor suppresses polyph formation in a colon cancer mouse model [38]. In this study, we have shown that activation of beta-catenin and/or AKT increases mTOR activity and that inhibition of that activity with rapamycin suppresses the tumor burden by controlling cell proliferation and death. Everolimus (a rapamycin derivative) treatment has been shown to reduce tumor growth of cisplatin-resistant clear cell ovarian carcinoma cells [56] and both the onset and progression of ovarian cancer in a mouse model expressing SV40 Large T antigen driven by the MIS type II receptor (Amhr2) promoter [2,56]. Presently, mTOR inhibitors are in phase I–III trials for other solid tumors [41]. In light of our studies, their use in OEE-specific trials should also be considered.

**Supporting Information**

**Figure S1** No abnormalities were present in the control (Clamb1<Ex3/>;Pten<+/->) adult ovaries (A & B). In adult mutant (Amhr2-Cre;Clamb1<Ex3/>;Pten<+/->) ovaries, cancerous lesions were present throughout the ovary and in the intrabursal space (arrowheads) (C–E). Beta-catenin staining in adult mutant ovaries (F). Bars represent 50 um. (TIF)

**Figure S2** H&E staining of 10-day old ovaries from control and mutant (Amhr2-Cre;Clamb1<Ex3/>;Pten<+/->) mice (Panel A–C). Representative section of tumor from 8 week-old Amhr2-Cre;Clamb1<Ex3/>;Pten<+/-> mice (Panel D). Bars represent 50 um. (TIF)

**Figure S3** Rapamycin treatment of tumors decreased proliferation and increased cell death. Staining for pH3 (A–F, green), TUNEL (G–L, green), and activated caspase 3 (M–R, red) was performed on three different tumors derived from three different animals. Nuclei were counterstained with DAPI. Bars represent 50 um. (TIF)

**Author Contributions**

Performed the experiments: PST LZ TK-T MDC PR. Analyzed the data: PST DJR JMT. Contributed materials: MMT. Conceived and designed the experiments: PST JMT. Wrote the paper: PST JMT.

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**References**

5. Rosen DG, Yang G, Liu G, Mercado-Uribe I, Chang B, et al. (2009) Ovarian cancer cell lines suppress E-cadherin levels, which correlates with the induction of an EMT-like state and generation of cancer cells with stem cell characteristics [50]. In a previous report, it was observed that combined deletion of APC and PTEN causes formation of ovarian epithelial tumors accompanied by loss of E-cadherin expression and appearance of mesenchymal-like cells suggesting EMT [6].