Introduction

Prostate cancer (CaP) is one of the leading causes of cancer death in men, with the NCI predicting more than 230,000 cases and 29,000 deaths in the US in 2013 (www.cancer.gov/cancerfacts). Prostate cancer is a complex disease, and several signaling pathways are frequently disrupted in human CaP, either by genetic alterations or by changes in expression of key components of the pathway [1]. Recent work with mouse models of prostate cancer (CaP) has shown that inactivation of TGFβ signaling in prostate epithelium can cooperate with deletion of the Pten tumor suppressor to drive locally aggressive cancer and metastatic disease. Here, we show that inactivating the TGFβ pathway by deleting the gene encoding the TGFβ type II receptor (Tgfb2) in combination with a deletion of the Apc tumor suppressor gene specifically in mouse prostate epithelium, results in the rapid onset of invasive CaP. Micro-metastases were observed in the lymph nodes and lungs of a proportion of the double mutant mice, whereas no metastases were observed in Apc single mutant mice. Prostate-specific Apc;Tgfb2 mutants had a lower frequency of metastasis and survived significantly longer than Pten;Tgfb2 double mutants. However, all Apc;Tgfb2 mutants developed invasive cancer by 30 weeks of age, whereas invasive cancer was rarely observed in Apc single mutant animals, even by one year of age. Further comparison of the Pten and Apc models of CaP revealed additional differences, including adenosquamous carcinoma in the Apc;Tgfb2 mutants that was not seen in the Pten model, and a lack of robust induction of the TGFβ pathway in Apc null prostate. In addition to causing high-grade prostate intra-epithelial neoplasia (HGPIN), deletion of either Pten or Apc induced senescence in affected prostate ducts, and this restraint was overcome by loss of Tgfb2. In summary, this work demonstrates that TGFβ signaling restrains the progression of CaP induced by different tumor suppressor mutations, suggesting that TGFβ signaling exerts a general tumor suppressive effect in prostate.

Abstract

Recent work with mouse models of prostate cancer (CaP) has shown that inactivating TGFβ signaling in prostate epithelium can cooperate with deletion of the Pten tumor suppressor to drive locally aggressive cancer and metastatic disease. Here, we show that inactivating the TGFβ pathway by deleting the gene encoding the TGFβ type II receptor (Tgfb2) in combination with a deletion of the Apc tumor suppressor gene specifically in mouse prostate epithelium, results in the rapid onset of invasive CaP. Micro-metastases were observed in the lymph nodes and lungs of a proportion of the double mutant mice, whereas no metastases were observed in Apc single mutant mice. Prostate-specific Apc;Tgfb2 mutants had a lower frequency of metastasis and survived significantly longer than Pten;Tgfb2 double mutants. However, all Apc;Tgfb2 mutants developed invasive cancer by 30 weeks of age, whereas invasive cancer was rarely observed in Apc single mutant animals, even by one year of age. Further comparison of the Pten and Apc models of CaP revealed additional differences, including adenosquamous carcinoma in the Apc;Tgfb2 mutants that was not seen in the Pten model, and a lack of robust induction of the TGFβ pathway in Apc null prostate. In addition to causing high-grade prostate intra-epithelial neoplasia (HGPIN), deletion of either Pten or Apc induced senescence in affected prostate ducts, and this restraint was overcome by loss of Tgfb2. In summary, this work demonstrates that TGFβ signaling restrains the progression of CaP induced by different tumor suppressor mutations, suggesting that TGFβ signaling exerts a general tumor suppressive effect in prostate.
mutations in the \(APC\) gene are rare in human CaP [29]. However, in the majority of cases of advanced human CaP \(\beta\)-catenin is found in the nucleus, suggesting that this pathway is frequently deregulated. Other mechanisms for activation of this pathway have been identified in human CaP, including methylation of the \(APC\) gene [30,31]. Activating mutations in \(\beta\)-catenin itself (encoded by \(CTNNB1\)), that prevent phosphorylation and targeting to the proteasome, have also been identified [32]. While de-regulation of this pathway appears to be quite frequent in human CaP, the importance of nuclear \(\beta\)-catenin in the initiation of human CaP and progression to castration resistant prostate cancer (CRPC) remains to be elucidated.

Recent work with genetically engineered mouse models has begun to shed light on the combinatorial effects of tumor suppressor mutations in prostate cancer progression. Prostate-specific deletions of the \(Tgfbr2\) and \(Snai4\) genes have both been tested in mice. Neither mutation alone is sufficient to initiate tumorigenesis, but when combined with a \(Pten\) deletion, inactivation of the TGF\(\beta\) pathway results in very rapid progression to locally invasive and metastatic disease [33,34]. Mouse models in which a stabilized \(\beta\)-catenin transgene was expressed in the prostate resulted in high-grade prostate intra-epithelial neoplasia (HGPIN) [35,36]. Deletion of the \(Apc\) gene specifically in mouse prostate epithelium also results in HGPIN with high penetrance, but this rarely progresses to invasive cancer, and metastases were not found [37]. Here we show that deletion of both the \(Tgfbr2\) and \(Apc\) genes in mouse prostate epithelium results in rapid progression to invasive cancer, with metastases to lymph nodes and lungs in some cases. Additionally, we show that deletion of either \(Apc\) or \(Pten\) alone induces senescence in prostate ducts with HGPIN and that additional loss of the \(Tgfbr2\) gene overcomes this senescence checkpoint. In summary, this work suggests that TGF\(\beta\) signaling limits progression from HGPIN to invasive prostate cancer, irrespective of the tumor initiating mutation. Thus TGF\(\beta\) signaling plays a key tumor suppressive role in prostate epithelium.

Results and Discussion

Lethal prostate cancer in \(Apc^{null};Tgfbr2^{null}\) mutants

Homozygous deletion of \(Apc\) in mouse prostate epithelium results in HGPIN with squamous differentiation in all prostatic lobes, although it rarely progresses to locally invasive cancer, and metastases have not been detected [37]. TGF\(\beta\) signaling restraining the progression of prostate cancer initiated by loss of the tumor suppressor Pten [33,34]. To test whether TGF\(\beta\) signaling plays a similar role in prostate when \(Apc\) is lost, we used mouse models to combine mutations in the \(Tgfbr2\) and \(Apc\) genes. Conditional, loxP-flanked alleles of both the \(Apc\) and \(Tgfbr2\) genes were combined with the \(PhCre4\) transgene, which is expressed only in prostate epithelium [38]. The recombined alleles, generated by prostate epithelium-specific expression of \(PhCre4\), are referred to hereafter as \(Apc^{null}\) and \(Tgfbr2^{null}\). As a first test of whether combining the \(Apc\) and \(Tgfbr2\) mutations promotes prostate cancer progression, we followed a cohort of \(Apc^{null}\) and \(Apc^{null};Tgfbr2^{null}\) mice to one year of age. Over this time course, none of the \(Apc^{null}\) mice showed signs of distress, whereas all of the double mutants displayed a tumor burden that required euthanasia prior to 30 weeks of age (Figure 1A). For comparison, we also analyzed a number of \(Pten^{null}\) and \(Pten^{null};Tgfbr2^{null}\) mice over the same period. All but one of the \(Pten\) single mutants survived to one year, whereas none of the double mutants survived beyond 16 weeks, consistent with our previous analysis (Figure 1A and [33]). At 18 weeks of age, the prostates of \(Apc\) single null mutants were almost indistinguishable from the wild-type, whereas, prostatic enlargement was readily apparent in \(Pten\) nulls (Figure 1B). By 52 weeks, the \(Apc\) null prostate tumors were clearly evident, but were still much smaller than those in \(Apc^{null};Tgfbr2^{null}\) double null mice at 16 weeks of age (Figure 1B). Thus, deletion of the \(Tgfbr2\) gene in the background of loss of either \(Pten\) or \(Apc\) had a highly significant effect on tumor growth, and reduced the median survival times to 92 days and 140 days respectively (Figure 1A and C). There was also a significant survival difference between the two double mutant combinations that is likely due to the specific pathways affected by loss of \(Pten\) and \(Apc\).

We previously found that a relatively high proportion (~66%) of \(Pten^{null};Tgfbr2^{null}\) mice developed metastases to the lumbar lymph nodes or lung [33]. In the present study, micro-metastases were found in the lumbar lymph nodes of 18% of the \(Apc^{null};Tgfbr2^{null}\) mice, and in the lungs of 12% of the \(Apc^{null};Tgfbr2^{null}\) mice. Thus, metastases in the \(Apc^{null};Tgfbr2^{null}\) mice were significantly less frequent (p<0.01) than in the \(Pten^{null};Tgfbr2^{null}\) mutants examined here, and in our previous analysis (Figure 1C). We never observed metastases in \(Apc^{null}\) single mutant mice, even by one year of age, and only one of fourteen \(Apc^{null}\) mice analyzed at either 36 or 52 weeks of age showed signs of locally invasive cancer. These data clearly show that the combination of mutations in the \(Apc\) and \(Tgfbr2\) genes can accelerate tumor progression to invasive and metastatic disease over that seen with loss of \(Apc\) alone.

Inactivation of TGF\(\beta\) signaling, either by deletion of \(Tgfbr2\) or \(Snai4\), accelerates the progression of \(Pten\) null CaP, and \(Tgfbr2\) deletion has also been shown to cooperate with a constitutively active Akt transgene to drive invasive cancer [33,34]. Expression of a dominant negative TGF\(\beta\) type II receptor transgene in the prostate was able to increase the severity of tumors initiated by prostate specific expression of T antigen, and resulted in increased metastases [39]. In each case, progression to invasive and metastatic disease is accelerated by loss of TGF\(\beta\) signaling, even when the initiating mutation does not on its own result in metastases, as seen here with the \(Apc\) mutants. In summary, the results of these mouse models of CaP together with the data presented here suggest that TGF\(\beta\) signaling plays a major tumor suppressive role in CaP.

Adenosquamous carcinoma in \(Apc^{null};Tgfbr2^{null}\) mutant prostate

Given the differences in survival and in the gross appearance of the tumors from \(Apc^{null};Tgfbr2^{null}\) and \(Pten^{null};Tgfbr2^{null}\) mice we performed a more detailed comparison of these tumors, as well as of prostates from the single null mutants. As previously reported, histological analysis did not reveal any differences between the wild type and \(Tgfbr2\) null prostates (Figure 2 and [33]). Comparison of the \(Pten\) and \(Apc\) single mutants revealed HGPIN in prostates of both genotypes, with squamous differentiation in the \(Apc\) null that was not seen in the \(Pten\) null. Introduction of a \(Tgfbr2\) mutation resulted in progression to poorly differentiated adenosquamous carcinoma (PDA) in all \(Pten;Tgfbr2\) double mutants. When combined with the \(Apc\) mutation, deletion of the \(Tgfbr2\) gene in prostate resulted in progression to invasive cancer, although the majority of \(Apc^{null};Tgfbr2^{null}\) tumors retained the squamous phenotype seen in \(Apc\) single nulls (Figure 2). Note the prominent foci of keratin in the \(Apc\) null HGPIN (arrow, Figure 2) and adenosquamous differentiation indicated in the \(Apc^{null};Tgfbr2^{null}\) tumor shown in Figure 2 (arrowheads). In addition to the histological appearance, increased expression of keratins 1 and 10 has been used as a marker of squamous differentiation in the \(Apc\) null prostate [40]. We, therefore, stained sections of wild type and mutant prostate for keratin 10. As shown in figure 3, strong staining for Krt10 was observed in cells underlying the prominent
foci of keratin present in Apc+/+ HGPIN, with little or no staining evident in wild type, Tgfb2 null or Pten null prostates. In the Apc+/+; Tgfb2−/− tumors large regions of Krt10 positive cells were present throughout the tumor, whereas in the Pten;Tgfb2 double nulls only rare scattered Krt10 positive cells were seen (Figure 3).

To better compare the frequencies of the phenotypes described above we compiled data on the predominant phenotypes in each genotype as scored in a blinded manner based on established mouse prostate phenotypes [41–43]. In Apc−/− mice most prostates analyzed at 8–24 weeks of age had HGPIN with squamous differentiation (adenosquamous HGPIN; Asq-HGPIN) and by 36–52 weeks all had this phenotype (Figure 4A). Only one of the Apc nulls developed regions of locally micro-invasive cancer (at 36 weeks), and this was significantly less frequent (p<0.02) than the incidence of invasive cancer in Pten+/− mice in the 36–52 week age range (Figure 4A). Comparison of the Tgfb2 compound mutants revealed that all Pten+/−;Tgfb2−/− mice had PDA without squamous differentiation, whereas all but one of the Apc−/−;Tgfb2−/− mice euthanized for tumor burden had extensive adenosquamous carcinoma (Figure 4B). We also compared phenotypes in mice with intermediate genotypes, in which one or other mutation was heterozygous (some of the Pten+/−;Tgfb2−/− and Pten−/−;Tgfb2−/− mice included in this phenotype summary were analyzed for survival in ref [33]). For this analysis, mice were analyzed when tumor burden became excessive, or when mice reached at least one year of age. This showed that the frequency of invasive cancer was significantly higher in Pten+/−;Tgfb2−/− than in Apc−/−;Tgfb2−/− mice (p<0.05), and in Pten−/−;Tgfb2−/− than in Apc+/−;Tgfb2−/− Prostates (p<0.001), supporting the idea that loss of Pten results in a more aggressive phenotype than loss of Apc (Figure 4B).

In summary, this analysis suggests that the phenotypes observed are quite specific to each prostate cancer model, and that within each model there is little variation in the type of tumor. For all combinations tested the Pten mutation results in a more aggressive, invasive cancer than Apc deletion. Interestingly, none of the Apc heterozygotes developed tumors, even in the presence of a homozygous deletion of Tgfb2, whereas the majority of Pten heterozygotes developed PDA if they were null for Tgfb2. This is consistent with inactivation of the remaining Pten allele, which has been suggested to be a major route by which Pten heterozygous mouse prostates develop a phenotype [44]. TGFβ signaling appears to restrain the progression of HGPIN to both poorly differentiated adenocarcinoma and to adenosquamous carcinoma, with the difference in phenotype being dependent on the tumor initiating mutation. While nuclear accumulation of β-catenin is found in a high proportion of human CaP, there is no consensus as to how the APC/β-catenin pathway might be disrupted in human CaP. The squamous differentiation induced by loss of Apc is relatively rare in human CaP, although it is more common after androgen deprivation therapy [45]. The presence of adenosquamous carcinoma in Apc;Tgfb2 double nulls suggests that loss of TGFβ signaling does not prevent the squamous differentiation induced by loss of Apc. Similarly, the combination of a Pten mutation with a stabilized β-catenin transgene in prostate results in tumors with squamous differentiation, suggesting that this may be the dominant phenotype of early activation of β-catenin [40]. Perhaps the increased nuclear β-catenin seen in advanced human CaP is a later phenotype, or occurs to a lower level than seen with transgenic manipulation in mice, and thus cannot drive squamous differentiation.
Increased stroma and invasive cancer with Tgfbr2 deletion

Examination of sections stained with H&E revealed evidence of locally invasive cancer, with increased stroma in both Apc<sup>−/−</sup>;Tgfbr2<sup>−/−</sup> and Pten<sup>−/−</sup>;Tgfbr2<sup>−/−</sup> prostates. To visualize expansion of the stroma, we stained sections with Masson’s Trichrome. In wild type and Tgfbr2 null prostates, the duct structure was similar, and there was minimal fibrous tissue between prostatic ducts. In areas of HGPIN in Pten<sup>−/−</sup> animals there were focal areas of increased fibrosis and in the Apc null significant fibrosis was seen between ducts with HGPIN (Figure 5). In contrast, examination of double mutant animals that were euthanized due to tumor burden showed complete breakdown of the basement membrane in areas of invasive cancer (Figure 6). To examine the invasive phenotype, we stained sections for Foxa1, as an epithelial marker, and for Sma to identify the stromal cells. In both the wild type and Tgfbr2 null, prostatic ducts were fully enclosed by Sma positive stromal cells.
Although there was clear evidence of HGPIN in the Apc and Pten null prostates, in most cases the ducts were still surrounded by Sma-positive stroma at ages significantly greater than the median survival times of the double mutants (Figure 7). In contrast, in Apc\(^{-/-}\);Tgfbr2\(^{-/-}\) and Pten\(^{-/-}\);Tgfbr2\(^{-/-}\) animals, the distinct separation between stroma and ducts has clearly broken down, as duct structure is no longer evident upon transition to invasive cancer (Figure 7). Taken together, these analyses suggest that while there are some differences in the histologic type of cancer initiated by loss of Pten or Apc, the additional inactivation of the TGF\(\beta\) pathway causes rapid progression to locally invasive cancer.

To test for evidence of epithelial to mesenchymal transition (EMT) we examined expression of E-cadherin and vimentin. TGF\(\beta\) signaling is a well known driver of EMT and increased TGF\(\beta\) signaling frequently results in increased vimentin expression and a decrease in E-cadherin expression, which can contribute to the breakdown of epithelial cell junctions and an invasive phenotype [46]. As shown in Figure 8, E-cadherin is robustly expressed and present at the cell periphery in the majority of epithelial cells in the Apc and Pten single mutants. E-cadherin expression still appears largely normal in the Pten\(^{-/-}\);Tgfbr2\(^{-/-}\) mice, despite the clear breakdown of duct structure. Some evidence of E-cadherin de-localization from the cell membrane was seen in the Apc\(^{-/-}\);Tgfbr2\(^{-/-}\) prostate, together with a reduction in overall signal compared to the Apc\(^{-/-}\). However, even in the two double mutant prostates, epithelial cell junctions appear to be largely intact with clear E-cadherin staining (Figure 8). Vimentin staining revealed little change in the epithelial cells in any of the mutants, although there was some increased vimentin expression in the stroma of Apc and Pten single null and Apc;Tgfbr2 double null prostates (Figure 8). These data suggest that although the tumors in Pten;Tgfbr2 and Apc;Tgfbr2 double nulls are invasive and can metastasize, this is not accompanied by a large scale EMT phenotype. Given the role of TGF\(\beta\) signaling in driving EMT, it might be expected that the double null tumors would not have E-cadherin and up-regulated vimentin, as they have lost a key component of the TGF\(\beta\) signal transduction pathway. These results do, however, leave open the question of how these tumors become metastatic. One possibility is that rare epithelial cells within the tumor have undergone EMT, presumably driven by a signal other than TGF\(\beta\). Another intriguing possibility is that the double null epithelial cells undergo some form of collective invasion, in which small groups of epithelial cells become invasive and motile, while maintaining their cell junctions.

This type of invasion has been attracting more interest as a potential driver of metastasis [47,48], and it will now be of interest to examine how the tumors examined here become invasive and metastatic.

**Apc deletion does not activate TGF\(\beta\) signaling in prostate**

Deletion of Pten in mouse prostate initiates tumorigenesis and induces the activity of the TGF\(\beta\) pathway [33,34]. Similarly, expression of a constitutively active AKT1 transgene in prostate epithelium increases TGF\(\beta\) signaling [33], suggesting that Akt activation, which occurs downstream of Pten loss, is sufficient to activate this pathway. Given that deletion of the Tgfbr2 gene allowed for progression from HGPIN to invasive cancer in the Apc null prostate, we examined whether the TGF\(\beta\) pathway was induced in this model. Deletion of either Apc or Pten resulted in little change in overall \(\beta\)-catenin levels, whereas phospho-Akt levels were increased specifically in Pten null prostates (Figure 9). To test whether the TGF\(\beta\) pathway was affected by Apc deletion, we first analyzed levels of the TGF\(\beta\) type II receptor and the intracellular mediator, Smad4. While both were significantly increased in the Pten null, there was no significant increase in either Smad4 or Tgfbr2 levels in Apc mutant prostates compared to those from wild type mice (Figure 9). As an indication of pathway activation, we next analyzed levels of phospho-Smad2 phosphorylated at the carboxyl-terminal serines that are a substrate for type I TGF\(\beta\) receptors. Phospho-Smad2 was significantly increased in the Pten null but not in the Apc null prostates, indicating that pathway activation occurs with loss of Pten, but not with loss of Apc. The induction of TGF\(\beta\) signaling by Pten deletion could be driven by signal transduction events downstream of Akt activation, or could

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**Figure 4. Summary of tumor phenotypes in mice with different combinations of Apc, Pten and Tgfbr2 mutations.**

(A) The phenotypes of Apc\(^{-/-}\) and Pten\(^{-/-}\) mice are shown, grouped by two age ranges: 8 to 24 weeks and 36 to 52 weeks. All animals analyzed had some prostate tumor phenotype, which is classified as Focal PIN, HGPIN, or HGPIN with adenosquamous differentiation (Asq-HGPIN). Animals with micro-invasive cancer and Asq-HGPIN or a mixture of HGPIN and PDA are grouped separately. Numbers of animals analyzed are shown above each column, and the distribution of phenotypes is shown as a percentage. The proportion of animals with any signs of invasive cancer at 36–52 weeks is significantly different between the two genotypes (p<0.02). (B) The numbers of mice with each phenotype are shown for animals with different combinations of mutations in both Apc and Tgfbr2 (above), or in both Pten and Tgfbr2 (below). For each genotype the numbers of animals with each phenotype are shown. Normal = no tumor phenotype evident. HGPIN and adenosquamous HGPIN – extensive HGPIN without evidence of invasion. PDA and adenosquamous carcinoma – extensive locally invasive cancer. All mice were euthanized at more than one year of age, unless they had to be sacrificed for tumor burden at a younger age. Significantly more animals with invasive cancer were observed in the Pten\(^{-/-}\);Tgfbr2\(^{-/-}\) and Pten\(^{-/-}\);Tgfbr2\(^{-/-}\) groups than in the Apc\(^{-/-}\);Tgfbr2\(^{-/-}\) and Apc\(^{-/-}\);Tgfbr2\(^{-/-}\) groups (14/14 vs 2/4; p<0.05, and 15/17 vs 0/6; p<0.001). One of the Apc\(^{-/-}\);Tgfbr2\(^{-/-}\) mice (scored as PDA) had only small invasive foci. All others had extensive local invasion if scored as PDA or Asq-carcinoma.

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be a consequence of the type of differentiation in this model – poor glandular differentiation in the Ptén null rather than the squamous differentiation seen with Apc deletion. However, the clear cooperative effects of Apc and Tgfbr2 deletion suggest that even the low level of basal TGFβ signaling present in the Apc mutant tumors is important for restraining cancer progression to locally invasive and metastatic disease.

Loss of TGFβ signaling overcomes senescence

To examine the effects of the Tgfbr2 mutation on proliferation in Ptén and Apc null tumors we examined expression of Cyclin D, increased levels of which correlate with advanced human prostate cancer [34]. As shown in Figure 10A, the number of epithelial cells with high levels of nuclear Cyclin D increased significantly in HGPIN in both the Ptén and Apc single mutant prostates, with further significant increases in invasive cancer in each double mutant compared to the corresponding single mutant. We next examined expression of the CDK inhibitor, p27 (encoded by Cdkn1b) by immunofluorescence microscopy. For this analysis we co-stained for β-catenin to identify cells in which the Apc mutant phenotype was strongest. Expression of p27 increased in the Ptén null and this increase was less pronounced in areas of invasive cancer in the Ptén+/−;Tgfbr2+/− animals (Figure 10B). This observation suggests that the transition to invasive cancer is concomitant with decreased p27 expression, consistent with our previous analysis [33]. In the Apc mutant, we observed a dramatic change in β-catenin from the cell periphery to a more diffuse expression pattern throughout the cell. This change in β-catenin expression was accompanied by a redistribution of p27 from the nucleus, as seen in the wild type prostate, to a diffuse signal throughout the cell (Figure 10B). The change in p27 localization appeared to correlate with altered β-catenin expression, as small foci of cells in which β-catenin was still present at lower levels and only at the cell membrane retained nuclear p27 (Figure 10B arrow). Interestingly, in the Apc+/−;Tgfbr2+/− mutants p27 levels decreased relative to those seen in the Apc single mutant, suggesting that loss of Tgfbr2 has similar effects on p27 in both models (Figure 10B). These data are consistent with loss of TGFβ signaling resulting in increased proliferation in both models of prostate cancer, although the effects of Ptén and Apc deletions appear to differ with respect to p27 expression, and it is possible that the p27 re-localization in the Apc null prostate represents a first step in its inactivation. Phosphorylation of p27 at threonine 157 by AKT1 down-regulates human p27, but this phosphorylation site is not conserved in the mouse protein [49]. Additionally, phosphorylation of p27 on other sites that are conserved between mouse and human, results in redistribution to the cytoplasm and in some cases subsequent proteasomal degradation.

Figure 5. Increased stroma in Apc null prostates. Masson’s Trichrome stained prostate sections are shown, from mice at the following ages. Wild type: 25 weeks, Ptén+/−: 18 weeks, Apc+/−: 36 weeks, Tgfbr2+/−: 18 weeks, Ptén+/−;Tgfbr2+/−: 12 weeks, and Apc+/−;Tgfbr2+/−: 17 weeks.

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Figure 6. Basement membrane breakdown in double null prostates. Indirect immunofluorescence is shown for β-catenin (green) and Collagen IV (red) on sections of prostate from mice of the indicated genotypes. Wild type: 21 weeks, Tgfbr2+/−: 44 weeks, Ptén+/−: 21 weeks, Ptén+/−;Tgfbr2+/−: 11 weeks, Apc+/−: 36 weeks, and Apc+/−;Tgfbr2+/−: 24 weeks old.

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Figure 7. Disruption of stromal integrity in double null prostates. FoxA1 (red) and Sma (green) staining are shown by indirect immunofluorescence on sections of prostate from mice of the indicated genotypes. Ages of the mice are as in Figure 6.

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degradation [50]. However, we do not know if the effect of Apc deletion on p27 localization is due to phosphorylation of p27 itself.

A senescent phenotype can be induced in tumors either by activation of an oncogene, or by inactivation of a tumor suppressor gene [51]. Constitutive Akt activation in prostate epithelium induces cellular features of senescence, including SA-β-Gal (senescence-associated β-galactosidase) activity and increased p27 expression [52]. To test whether deletion of Pten and Apc induced senescence we analyzed the ventral prostates from different genotype mice for SA-β-Gal. Regions of HGPIN in both the Apc and Pten null prostates were positive for SA-β-Gal, whereas no staining was seen in the wild type prostate (Figure 10C). Areas of invasive cancer in the two double mutants were devoid of SA-β-Gal staining, although isolated regions of HGPIN in the double mutants did still retain some SA-β-Gal signal, suggesting that the transition from HGPIN to an invasive phenotype is associated with overcoming senescence (Figure 10C). These data suggest that deletion of either Apc or Pten in prostate epithelium initiates tumorigenesis, and also induces a senescent phenotype that can be overcome by deletion of the Tgfbr2 gene, which allows rapid progression to invasive cancer.

Although Apc deletion in prostate has been shown to generate HGPIN with only rare progression to locally invasive cancer [37], to our knowledge this is the first study to examine the combination of Apc deletion with another mutation in prostate epithelium. Expression of stabilized β-catenin in prostate epithelium was able to cooperate with Pten deletion, or with expression of an activated Ras transgene to accelerate the onset of locally invasive cancer [35,40]. However, either Pten deletion or Ras activation alone are sufficient to cause invasive CaP, albeit with slower kinetics than when combined with stabilized β-catenin. While stabilized β-catenin was able to accelerate the Pten null phenotype, deletion of β-catenin did not slow the progression of Pten null tumors to HGPIN, suggesting that it is not required for the early stages of tumorigenesis in this model [40]. We found that inactivation of TGFβ signaling results in a dramatic acceleration of tumor progression initiated by Apc deletion. Despite the lack of an abnormal phenotype seen with loss of TGFβ signaling in prostate...
epithelium, the combination of inactivating TGFβ pathway mutations with either Pten or Apc deletion results in highly aggressive mouse models of CaP. Thus, TGFβ signaling might restrain a relatively early step in the progression of these tumors, although it does not appear to affect tumor initiation.

It is possible that loss of TGFβ signaling contributes to the transition from androgen-sensitive to CRPC. Androgen deprivation therapy is one of the major treatments for biologically significant human CaP, but tumors virtually always return and in general are more aggressive after becoming castration resistant. Previous work suggests that early castration of prostate-specific Apc null mice slows the progression of tumors in this model [37]. By 32 weeks of age regions of hyperplasia and metaplasia were still evident following castration at six weeks, whereas intact animals at this age display adenosquamous HGPIN. We previously showed that the Pten;Tgfbr2 null tumors were resistant to castration, suggesting that Tgfbr2 deletion speeds the progression to CRPC. However, in this model it is difficult to separate out the effects of Pten deletion from Tgfbr2 deletion, since HGPIN in Pten null mouse prostate has a limited response to castration. Therefore, the Apc model might present an opportunity to test whether inactivation of TGFβ signaling contributes to progression to CRPC.

In summary, we show that loss of TGFβ signaling in mouse prostate epithelium cooperates with loss of either the Apc or Pten tumor suppressor genes to drive invasive CaP, despite clear differences in the pathways activated and the tumor phenotypes. Loss of TGFβ overcomes a restraint on tumor progression resulting in rapid onset of invasive and metastatic disease, further
supporting a major tumor suppressive role for TGFβ signaling in prostate.

**Materials and Methods**

**Ethics statement**
All animal procedures were approved by the Animal Care and Use Committee of the University of Virginia, which is fully accredited by the AAALAC.

**Mice and DNA analysis**
The **loxp** flanked *Pten, Apc** and *Tgfb2* alleles and the *Pb-Cre4* allele have been described previously [30,33–55]. *Tgfb2* and *Apc* mice, and the *Pb-Cre4* transgenics were obtained from the NCI mouse repository. Conditional loxp flanked alleles each contain loxp flanked exons, which when recombined result in null alleles, and are referred to here as ‘r’ for recombinated (null), or ‘f’ for the conditional loxp flanked (equivalent to wild type). All mouse lines were maintained on a mixed C57BL/6J x FVB strain background, and are referred to here as ‘r’ for recombined (null), or ‘f’ for the conditional loxp flanked (equivalent to wild type).

**Histology, IHC and IF**
Prostates were fixed in zinc-formalin, paraffin-embedded and stained with Hematoxylin and Eosin (H&E) or with Masson’s Trichrome by standard techniques. Immunohistochemistry (IHC) staining was performed using Super-Signal West Pico ECL (Pierce). Primary antibodies were against β-catenin (BD Transduction Labs 610153), phospho-Akt (Cell Signaling 3195), vimentin (Abcam Ab20346), Krt10 (Covance PRB-159P), Collagen IV (AbD Serotech 2150-1470), E-cadherin (Cell Signaling 9277), phospho-Smad2 (Millipore AB3849), Smad4 (Millipore 04-1035), Tgfbr2 (Novus NB51-19434) and γ-tubulin (Sigma T6557). Blots were quantified by densitometry using Image J.

**Statistics**
Comparisons of proportions (Figures 1C, 4A and 4B) were performed using an N-1 Chi squared analysis of 2 × 2 contingency tables. Survival data (Figure 1A) was compared by log-rank test (http://bioinf.wehi.edu.au/software/russell/logrank/). Significantly increased protein expression (Figure 9) was tested for using a one-tailed Student’s T test. Differences in Cyclin D expression (Figure 10A) were tested using a two-tailed Student’s T test.

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**Author Contributions**
Conceived and designed the experiments: GAB BMP DW. Performed the experiments: GAB TAM KP HFF. Analyzed the data: GAB TAM KP HFF BMP DW. Wrote the paper: GAB BMP DW.

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