# Elevated levels of IGF-1 receptor convey invasive and metastatic capability in a mouse model of pancreatic islet tumorigenesis

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## Summary

**In a prototypical model of multistage tumorigenesis involving pancreatic islets in RIP1-Tag2 transgenic mice, activation of insulin-like growth factor II (IGF-II) was previously shown to serve as a survival factor that inhibited apoptosis. Now IGF-1R, the receptor tyrosine kinase for IGF-II, has been found to be variably upregulated, first uniformly in dysplastic and angiogenic progenitors and then focally at the margins and in invasive regions of carcinomas. When the levels of IGF-1R were forcibly elevated throughout islet tumorigenesis, progression was accelerated at all stages in the pathway, although apoptosis was not differentially suppressed. Notably, encapsulated tumors were absent; instead, invasive carcinomas with downregulated E-cadherin were prevalent, and the majority of mice had local lymph node metastasis.**

## Introduction

Receptor tyrosine kinases are crucial intermediaries between the extracellular milieu and intracellular signaling pathways that together control cellular functions as diverse as the cell division cycle, apoptosis, gene expression, cytoskeletal architecture, cell adhesion, and cell migration. As the mechanisms controlling cell signaling are better understood, it has become clear that dysregulation of these cellular functions at the level of ligand binding, receptor signaling, or downstream intracellular signaling can have dramatic effects on cellular phenotype. Thus, it is not surprising that activating mutations, gene amplification, or epigenetic increases in gene expression that result in increased receptor kinase activity are associated with a number of different human cancers.

Studies of a transgenic mouse model of cancer (RIP1-Tag2) in which the SV40 T antigen oncoproteins are expressed in the β cells of the pancreatic islets under the control of the rat insulin promoter have proven instructive in delineating the cellular and molecular mechanisms of multistep tumorigenesis (Folkman and Hanahan, 1996; Bergers et al., 1998; Hager and Hanahan, 1999). In particular, insulin-like growth factor-II (IGF-II) has been demonstrated to be a functional determinant of this tumorigenesis pathway, implicating signaling via a receptor tyrosine kinase, the type I insulin-like growth factor receptor (IGF-1R). IGF-II is focally upregulated as oncogene-expressing islet β cells become hyperproliferative, populating hyperplastic and dysplastic pancreatic islets; IGF-II levels increase and become more uniform in the subsequent stages of angiogenic (dysplastic) islets and solid tumors (Christofori et al., 1994; Naik et al., 1994). Knowledge of the role that IGF-II plays in this system came from studies with mice in which IGF-II is genetically ablated. RIP1-Tag2, IGF-II null mice developed markedly smaller tumors whose histopathology was suggestive of a more benign condition—the tumor cells were less prone toward nuclear atypia and had more cytoplasm. Surprisingly, the proliferation index in progenitor islets and solid tumors was comparable to those in wild-type RIP1-Tag2 mice, whereas the frequency of apoptosis was 5-fold higher (Christofori et al., 1994; Naik et al., 1994). Thus, IGF-II was implicated as a survival factor that conveyed resistance to apoptosis in the course of oncogene-induced tumorigenesis, congruent with results from cell culture studies on the *c-myc* oncogene (Harrington et al., 1994).

The expression and activity of IGF-II is subject to multiple levels of regulation. IGF-II is expressed widely during embryonic development in humans and rodents, but then transcription of the Igf-II gene is variably downregulated and in some tissues extinguished after birth (Stewart and Rotwein, 1996). In rodents, Igf-II gene expression is regulated in part by genomic imprinting of the maternal allele (DeChiara et al., 1991; Ferguson-Smith et

### SIGNIFICANCE

**Tyrosine kinases have been widely implicated as causal agents for human cancers, motivating development of pharmacological inhibitors designed to abrogate their functional contributions. While elevated expression of IGF-1R and its ligands has been reported for many human tumors, the IGF signaling system has not been a prominent target for anticancer therapy. This report demonstrates that IGF-1R can induce tumors arising de novo in the pancreas of mice into an invasive and metastatic growth program. Regulatory determinants of invasion and metastasis remain elusive, and yet this is the hallmark of cancer that most often destines lethality. The possibility that IGF-1 receptor signaling conveys invasive and metastatic capability in human tumors deserves broader investigation, and prioritized development of pharmacological inhibitors may be warranted.**

al., 1991; Rappolee et al., 1992). Interestingly, loss of imprinting and concomitant expression of both parental alleles is seen in SV40 T antigen-induced β islet tumors (Christofori et al., 1995). Bioavailability of the IGF-II protein is modulated in certain circumstances by a set of IGF binding proteins (IGF-BP), which can serve to prolong the half-life, sequester the protein within the extracellular matrix, and influence its association with receptors. Release of free IGF-II can be accomplished by proteolytic degradation of the IGF-BPs by proteases that include the kallikrein-like serine proteases, cathepsins, and the matrix metalloproteinases (MMPs), each of which are also extensively regulated on multiple levels (Grimberg and Cohen, 2000). IGF-II binds to two receptors, the IGF-1R, a transmembrane tyrosine kinase, and IGF-2R, also known as the cation-dependent mannose-6 phosphate receptor; IGF-2R/M6P is thought to serve as a membrane bound reservoir or depot for IGF-II, as it appears in general to lack a capability for signal transduction (Braulke, 1999).

Studies in cell culture systems have revealed that IGF-1R can induce a number of "transformed" cellular phenotypes, including hyperproliferation, protection from apoptosis, and tumorigenicity (Werner and LeRoith, 1996; Baserga et al., 1997); more recently, IGF-1R has been causally implicated in invasive and metastatic growth by its ability to convey such capabilities on cultured epithelial cells (Dunn et al., 1998; Long et al., 1998b; Xie et al., 1999; Brodt et al., 2001). IGF-1R can also be activated by the other insulin-like growth factor, IGF-I, as well as by insulin; these ligands have 10 times higher and 100 times lower affinities for IGF-1R than IGF-II, respectively. Furthermore, it has recently been demonstrated that all three ligands can bind to heterodimers composed of IGF-1R and an alternatively spliced isoform of the insulin receptor (IR-A), as well as to IR-A homodimers (Frasca et al., 1999; Pandini et al., 1999; Vella et al., 2002). While IGF-1R is structurally quite similar to the insulin receptor, it has a distinctive region within its cytoplasmic kinase domain (Dupont and LeRoith, 2001; Nakae et al., 2001). Mutational analysis of the cytoplasmic portion of IGF-1R has revealed that its functional activities can be separated with mutations that individually abrogate induction of proliferation, tumorigenicity, and resistance to apoptosis (reviewed by Baserga et al., 1997), and now also invasion and metastasis (Brodt et al., 2001). Thus, IGF ligand signaling emanates from this receptor into multiple intracellular effector circuits, each of which can influence a cellular function deemed important for tumor growth and progression.

In regard to the insulin/IGF signaling system in the RIP-Tag pathway, the current state of knowledge is as follows. (1) The IGF-1 and IGF-2/M6P receptors are both expressed at low but discernable levels in islet tumors and tumor-derived cell lines, with indications that the IGF-2/M6P depot/decoy receptor is more abundant, as shown by ligand binding and RNA analyses (Naik et al., 1994). (2) Expression of the insulin receptor gene is barely detectable in tumors (our unpublished observations). (3) Six of the known IGF-BP are expressed in normal islets and tumors at similar levels (Grewal et al., 1999). (4) Insulin is expressed at high levels (Hanahan, 1985; Efrat et al., 1988), whereas IGF-I is expressed at very low levels (Naik et al., 1994) in islet tumors and tumor cell lines. And (5) as reviewed above, the dormant IGF-II gene is activated and comes to be highly expressed, and its expression is functionally significant, suggesting that IGF-II conveys an important, nonredundant signal in the context of oncogene-induced islet tumorigenesis. Several additional facts further motivated us to investigate the possible involvement of the IGF-1 receptor in the RIP1-Tag2 model. The Igf-1R gene can be transcriptionally repressed by the p53 tumor suppressor protein in cultured cells (Werner et al., 1996), and thus binding and inactivation of p53 by the SV40 large T oncoprotein (Ludlow, 1993) might be predicted to influence IGF-1R expression. Further, studies with transformed fibroblasts have demonstrated a role for IGF-1R in SV40 T antigen-mediated transformation (Sell et al., 1993), revealing a "transforming region" localized in the distinctive cytoplasmic signaling domain (discussed above) not contained in the otherwise structurally similar insulin receptor. Further, a physical association between T antigen and IRS-1, a substrate of activated IGF-1R kinase, is crucial for transformation of fibroblasts in culture (Zhou-Li et al., 1995). In light of these considerations, we first examined in greater depth the endogenous expression of IGF-1R in the discrete stages of tumorigenesis, revealing upregulation in premalignant stages and, provocatively, in the invasive regions of tumors. Then we upregulated expression of IGF-1R by producing transgenic mice in which the Igf-1R gene was under transcriptional control of the rat insulin promoter, and we examined the effect of elevated levels IGF-1R in pancreatic β cells coexpressing the oncoprotein, SV40 large T antigen. The results demonstrate that increased IGF-1R expression can accelerate progression through the stages of islet carcinogenesis, inducing a more invasive and metastatic tumor phenotype.

## Results

### Characterization of IGF-1R expression in the RIP1-Tag2 pathway

In a previous study, semiquantitative RT-PCR analysis of islets representing distinct stages in the RIP-Tag2 progression pathway suggested that levels of Igf-1R mRNA were increased approximately 2-fold upon the initiation of hyperproliferation, persisting similarly in subsequent stages of the pathway (Naik et al., 1994). Cognizant of the range of lesions present at a given time and the potential for variability in expression at the cellular level, we have now performed immunohistochemical analysis of IGF-1R expression after categorizing hematoxylin- and eosin-stained RIP1-Tag2 islet lesions as either normal islets, hyperplastic/dysplastic islets, angiogenic islets, islet tumors or insulinomas (IT) with well-defined margins and frequent fibrous capsules, carcinomas with focal regions of invasion (IC-1), or (rarely) broadly invasive carcinomas (IC-2). These categories, detailed in Table 1, are based on gross morphological and detailed histopathological characteristics, founded upon on extensive experience with this model.

IGF-1R staining was performed on adjacent sections, and the levels and patterns of expression were assessed. Initially, RIP1-Tag2 islets, much as wild-type littermates (normal) (Figure 1A), express a low, uniform level of IGF-1R, while both hyperplastic/dysplastic (Figure 1B) and angiogenic (Figure 1C) islets have cells with a wide range of levels dispersed throughout the lesion in a seemingly random pattern. The benign islet tumors (IT), with well-defined margins and frequent fibrous capsules, evidence little or no detectable IGF-1R expression (Figure 1D). By contrast, islet carcinomas (IC-1 and IC-2) exhibit distinctive IGF-1R expression at the tumor margins and invasive fronts (Figure 1E) and comparatively reduced levels in the interior (Figure 1F). Notably, the immunohistochemical analysis, although consistent with mRNA expression levels obtained by semiquan-

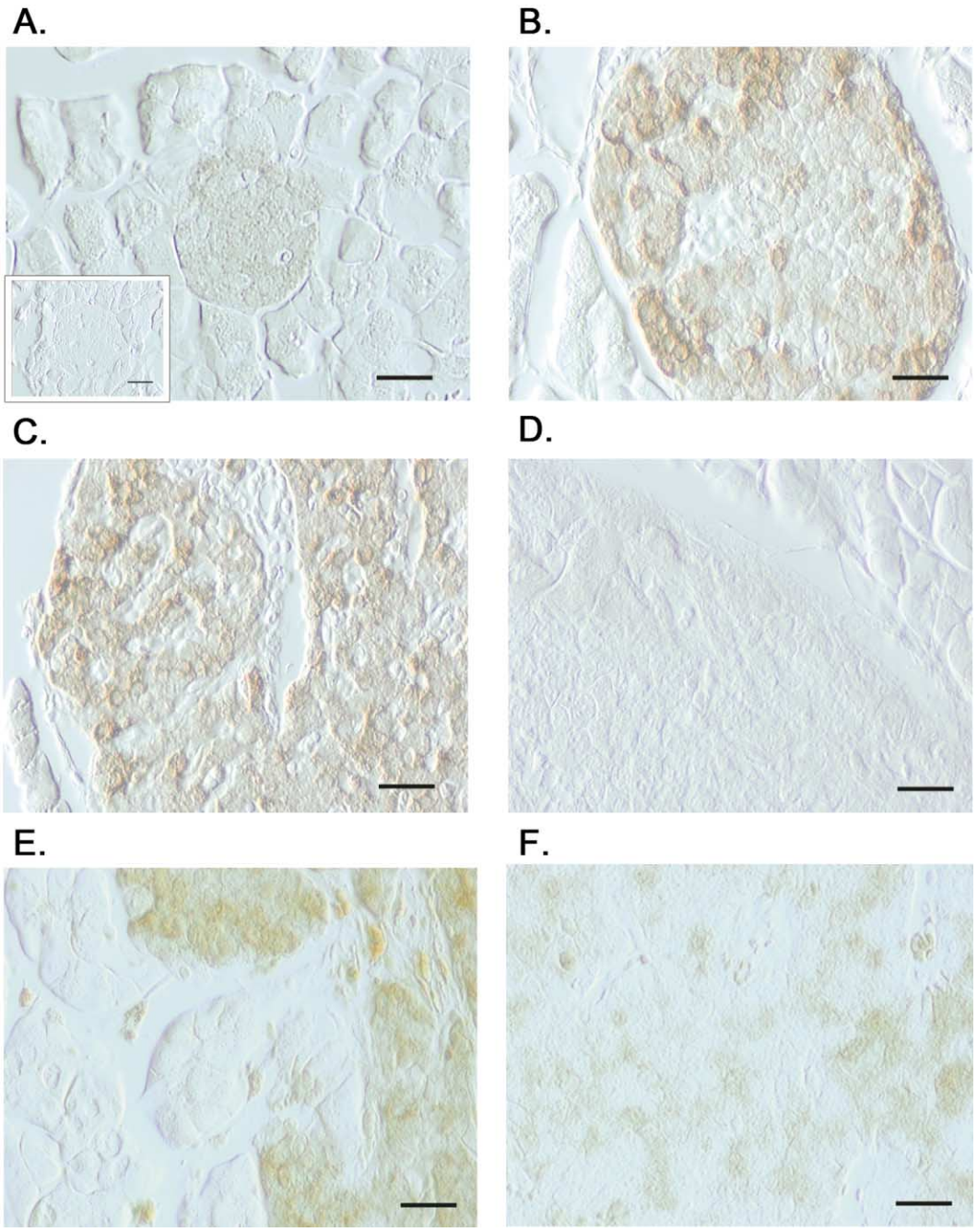


**Figure 1.** IGF-1 receptor is variably upregulated during islet tumorigenesis in RIP1-Tag2 mice

Immunostaining of tissue sections was used to visualize expression of the endogenous mouse IGF-1 receptor. **A** shows a normal islet in a nontransgenic littermate; inset shows the normal rabbit immunoglobulin-stained control. IGF-1R is upregulated in hyperplastic/dysplastic islets (**B**) and angiogenic islets (**C**). There is little expression evident in islet tumors with clear margins lacking invasion (**D**). By contrast, expression of IGF-1R is elevated at the margins and in invasive regions of islet carcinomas (**E**); interestingly, levels are reduced in the interior of carcinomas (**F**), much like for the benign islet tumors. The scale bars represent 20 μm.

titative RT-PCR, has revealed a striking variability between the interior and periphery of invasive tumors and an apparent downregulation in the noninvasive islet tumors compared to the progenitor lesions. The presence of high levels of IGF-1R in cells at the margins and within frankly invasive regions suggests a role for the IGF signaling in the acquisition and/or maintenance of invasive growth program.

### Genetic upregulation of Igf-1R during RIP1-Tag2 carcinogenesis

We sought to test the hypothesis that induction of IGF-II and increased expression of its receptor IGF-1R together play a role in islet carcinogenesis by perturbing expression of the receptor using transgenic mice. Previous studies had revealed that constitutive upregulation of IGF-II in transgenic mice by expression

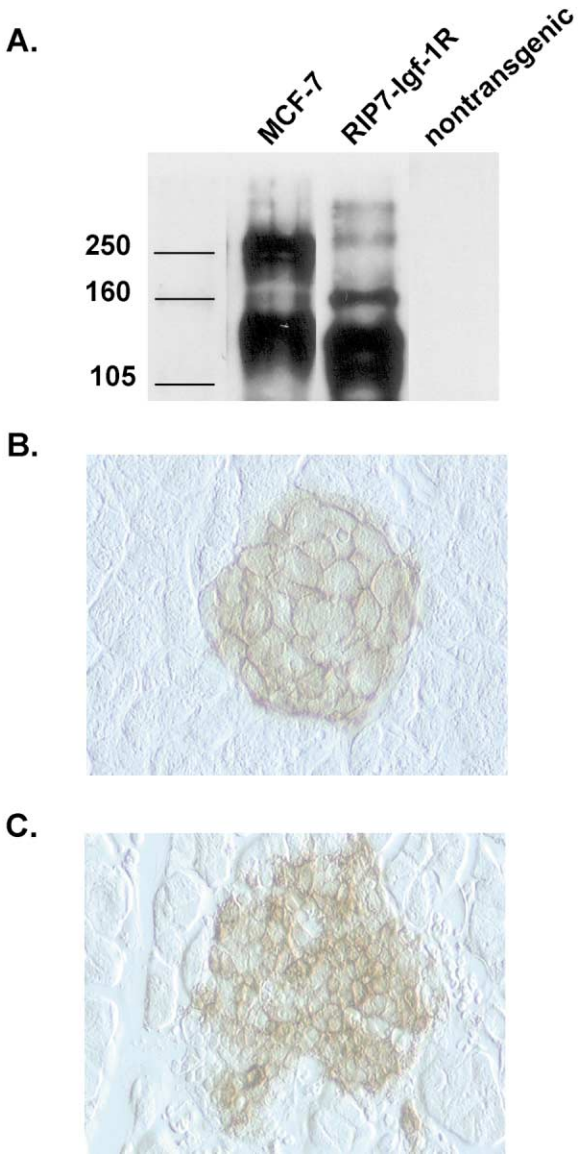


**Figure 2.** Expression of human IGF-1 receptor in the islets of RIP7-Igf-1R transgenic mice

Among several RIP7-Igf-1R transgenic lines produced, one (designated line #1 or RIP7-Igf1R$^{L1}$) was chosen for this study after hu-IGF-1R protein was found to be expressed in islets at a similar level to that in a human breast cancer-derived cell line, MCF-7 (**A**). Pancreatic islets were isolated, and cell lysates were subjected to immunoprecipitation of intact IGF-1R followed by Western blotting to detect the 130–135 kDa IGF-1R $\alpha$ chain. The apparent molecular weight of hu-IGF-1R was consistently found to be slightly lower in this and several other transgenic mouse lines than in MCF-7 cells (data not shown). Immunostaining with an antibody specific for IGF-1R revealed uniform expression in the islets of single-transgenic mice, with clear hypertrophy of the islet $\beta$ cells (**B**). When crossed to RIP1-Tag2, the islets in double-transgenic mice also expressed IGF-1R at elevated levels, albeit without evident $\beta$ cell hypertrophy, as illustrated for a dysplastic islet (**C**). The scale bars are 20 $\mu$m.

of the Igf-II cDNA under control of the rat insulin promoter did not enhance tumorigenesis (Naik, 1996), suggesting that the ligand levels achieved by activation and progressive upregulation of the endogenous Igf-II gene were not limiting. In light of the complex patterns of expression of the Igf-1R gene in the stages of this pathway and its association with cell transformation, we sought to increase its levels throughout the stages of islet carcinogenesis. Several lines of RIP7-Igf-1R transgenic mice were generated, wherein a human Igf-1R cDNA was controlled by a version of the rat insulin gene promoter that consists of 9.5 kb of 5′ flanking regulatory region and the 5′ untranslated region (see Experimental Procedures). The mature IGF-1 receptor exists as a disulfide-linked heterotetramer composed of two $\alpha$ and two $\beta$ chains. Expression levels of the transgene-encoded receptor were determined by immunoprecipitation of the intact receptor and Western blot analysis of the 130–135 kDa $\alpha$ chain of the IGF-1R. One line, RIP7-Igf-1R$^{L1}$, expressed IGF-1R in the pancreatic islets at a level comparable to that of the endogenous Igf-1R gene in a human breast carcinoma line, MCF-7, that has been used to study receptor signaling (Figure 2A); this transgenic mouse line was selected for further characterization. Immunohistochemical staining for IGF-1R expression in paraffin-embedded pancreas sections from RIP7-Igf-1R single-transgenic mice demonstrated that IGF-1R was uniformly expressed on the plasma membrane of cells throughout the islets (Figure 2B). Expression of IGF-1R in the islet $\beta$ cells did not alter life expectancy or glucose homeostasis as determined by fasting glucose tolerance (data not shown). Consistent with the described role of IGF-1R in controlling cell size (Stewart and Rotwein, 1996) through the phosphoinositide 3-kinase-dependent serine/threonine protein kinase Akt (Tuttle et al., 2001), transgenic $\beta$ cells exhibited an increased cytoplasmic to nuclear ratio (Figure 2B), indicating that transgenic expression of human Igf-1R gene resulted in sufficient levels of a functional receptor to activate intracellular signaling of a previously ascribed phenotype. There was no gross or histological evidence for a transformed phenotype in the RIP7-Igf-1R single-transgenic mice.

Next, the RIP7-Igf-1R transgenic mice were crossed with RIP1-Tag2 mice, and islets in young double-transgenic mice were examined by immunohistochemistry to confirm that IGF-1R was upregulated in the context of oncogene expression. Figure 2C shows that islet cells from 5-week-old double-transgenic RIP7-Igf-1R, RIP1-Tag2 mice have increased levels of IGF-1R, and receptor expression was more uniform throughout the islet than in the RIP1-Tag2 single-transgenic mice (Figure 1). Notably, even at 5 weeks of age, histological examination (Figure 2C) revealed marked morphological differences in the islets compared to the hyperplastic and early dysplastic stages (Figure 1B) in age-matched littermate RIP1-Tag2 mice. The boundary between some islets and adjacent exocrine pancreatic tissue was poorly defined, and cells could be seen extending into the exocrine pancreas. We went on to assess the impact of constitutive upregulation of IGF-1R on islet carcinogenesis.

### Increased Igf-1R expression accelerates islet carcinogenesis

A cohort of RIP1-Tag2, RIP7-Igf-1R mice along with littermate RIP1-Tag2 controls were aged and examined for life span and for tumor phenotype at regular intervals. Survival time of the RIP7-Igf-1R, RIP1-Tag2 mice was significantly decreased in comparison to the RIP1-Tag2 mice. The double-transgenic mice ($n = 12$) lived on average 9.9 weeks in comparison to the 12.6 weeks typical for RIP1-Tag2 single-transgenic mice, in particular for littermate siblings of the cross that generated the compound mice. (The difference in life span is statistically significant, with $p = 0.0007$ using the two-sample, two-tailed t test assuming

**Table 1.** Staging criteria for H&E-stained pancreatic sections in mouse models of islet carcinogenesis

| Stage {Symbol} | Nucleus/cytoplasm ratio | Lesional size (compared to normal wt islets) | Vascular morphology | Margins with exocrine tissue |
|---|---|---|---|---|
| "Normal" Tag+ {N*} | Similar to wt β cells | Similar (<0.2 mm diameter) | Similar to wt islet capillaries | Well-defined, similar to wt islets |
| Hyperplastic/dysplastic islet {H/Dl} | Increased relative to wt β cells | Variably larger (0.2–0.5 mm diameter) | Similar to wt islet capillaries | Well-defined, similar to wt islets |
| Angiogenic islet {Al} | Increased relative to wt β cells | Variably larger (<1 mm diameter) | Abnormal vessels appearing dilated and/or torturous; blood islands often evident | Well-defined similar to wt islets |
| Islet tumor or insulinoma {Tum} | Increased relative to wt β cells | Much larger (1–10 mm diameter) | Abnormal vessels appearing dilated and/or torturous; blood islands may be evident | Well-defined with no invasive regions; may or may not have a fibrous capsule and/or enveloped acinar islands or single cells |
| Invasive carcinoma type 1 {IC-1} | Increased relative to wt β cells | Much larger (1–10 mm diameter) | Abnormal vessels appearing dilated and/or torturous; blood islands may be evident | Focal regions of invasion with adjacent margins; tumor cells intercalated into exocrine tissue, and/or present as fronds projecting into normal tissue |
| Invasive carcinoma type 2 {IC-2} | Increased relative to wt β cells | Variable, from similar to normal islets up to large tumors, as above | Variable, from normal in small lesions to dilated and torturous in larger lesions | Widespread invasion with no evidence of margins: extensive intercalation of tumor cells into exocrine pancreas |

The basis for these criteria came from exhaustive analysis here and elsewhere of RIP1-Tag2 transgenic mice.
N*, histologically normal, but expressing the Tag oncogene.

unequal variance.) All double-transgenic mice were dead by 11 weeks, while single-transgenic littermates lived up to 16 weeks. Remarkably, tumors were detectable in double-transgenic mice at 7 weeks of age, significantly earlier than seen in the RIP1-Tag2 single-transgenic mice. Tumor burden rapidly increased over 40-fold in the ensuing 2 week period (Table 2), whereas the single-transgenic RIP1-Tag2 mice had not yet developed macroscopic tumors by this age.

We next evaluated the temporal appearance and frequency of tumor formation and of progenitor lesions in comparison to the well-established parameters of the RIP1-Tag2 pathway. Islet lesions from cohorts of RIP1-Tag2 controls and RIP7-Igf-1R, RIP1-Tag2 mice were analyzed for the frequency of distinct stages of RIP1-Tag2 tumorigenesis using the criteria detailed in Table 1. As described in earlier studies, the single RIP1-Tag2 mice exhibit a temporal pattern of lesional progression that begins with normal, oncogene-positive islets, followed by the appearance of hyperplastic/dysplastic and angiogenic islet lesions, and finally solid tumors begin forming at 10–12 weeks. The histological characteristics of the prototypical stages are described in Table 1 and illustrated in Figure 3A; the abundance of the various stages at ages 5–15 weeks is shown in Figure 4A. In the course of the extensive histological comparison performed here (Table 2), we also detected uniformly invasive tumors that lacked any vestige of the well-defined margins and frequent encapsulation of the typical solid tumors seen in the RIP1-Tag model. These small, uniformly invasive tumors (called invasive carcinoma type 2, or IC-2; see Table 1 and Figure 3A) were evident as early as 7 weeks of age, well before the more benign tumor types were detectable. It is presently unclear whether this rare class of tumor was overlooked in the previous analyses of RIP1-Tag2 mice, or if the continuous backcrossing into the C57Bl/6 background (currently >N40) has enabled it. Comparison of the frequency of islet lesions in the double-transgenic mice revealed marked differences (Figure 4B). At 5 weeks, the earliest time examined, only 60% of double-transgenic islet lesions were identifiable as normal or hyperplastic/dysplastic in comparison to almost 90% of islets in the single-transgenic mice. Moreover, at this time point, 30% of the lesions in the double transgenic exhibit the tortuous, dilated vessels characteristic of an angiogenic islet, which is significantly greater than the 12% seen in the single-transgenic RIP1-Tag2 mice. Remarkably, the more benign islet tumors characterized by the presence of a well-demarcating tumor-exocrine boundary were completely absent from the double-transgenic pancreases at all time points. Instead, small invasive carcinomas were detected in RIP7-Igf-1R, RIP1-Tag2 pancreases as early as 5 weeks of age (Figure 3B), and the frequency of the IC-2 lesions at this young age was higher than at any time point in the RIP1-Tag2 pathway (Figure 4B). Further, these invasive carcinomas came to constitute nearly 40% of all lesions detected in the pancreas of late-stage (9- to 10-week-old) double-transgenic mice.

### IGF-1R enhances both proliferation and apoptosis in premalignant stages

We have previously implicated both increased cell proliferation and varying rates of apoptosis in the progression of tumorigenesis in the RIP1-Tag2 pathway, and in particular have suggested that, as in many systems (Evan and Littlewood, 1998), the balance between the rates of cell proliferation and cell death govern tumor growth (Naik et al., 1996; Bergers et al., 1998; Hager and

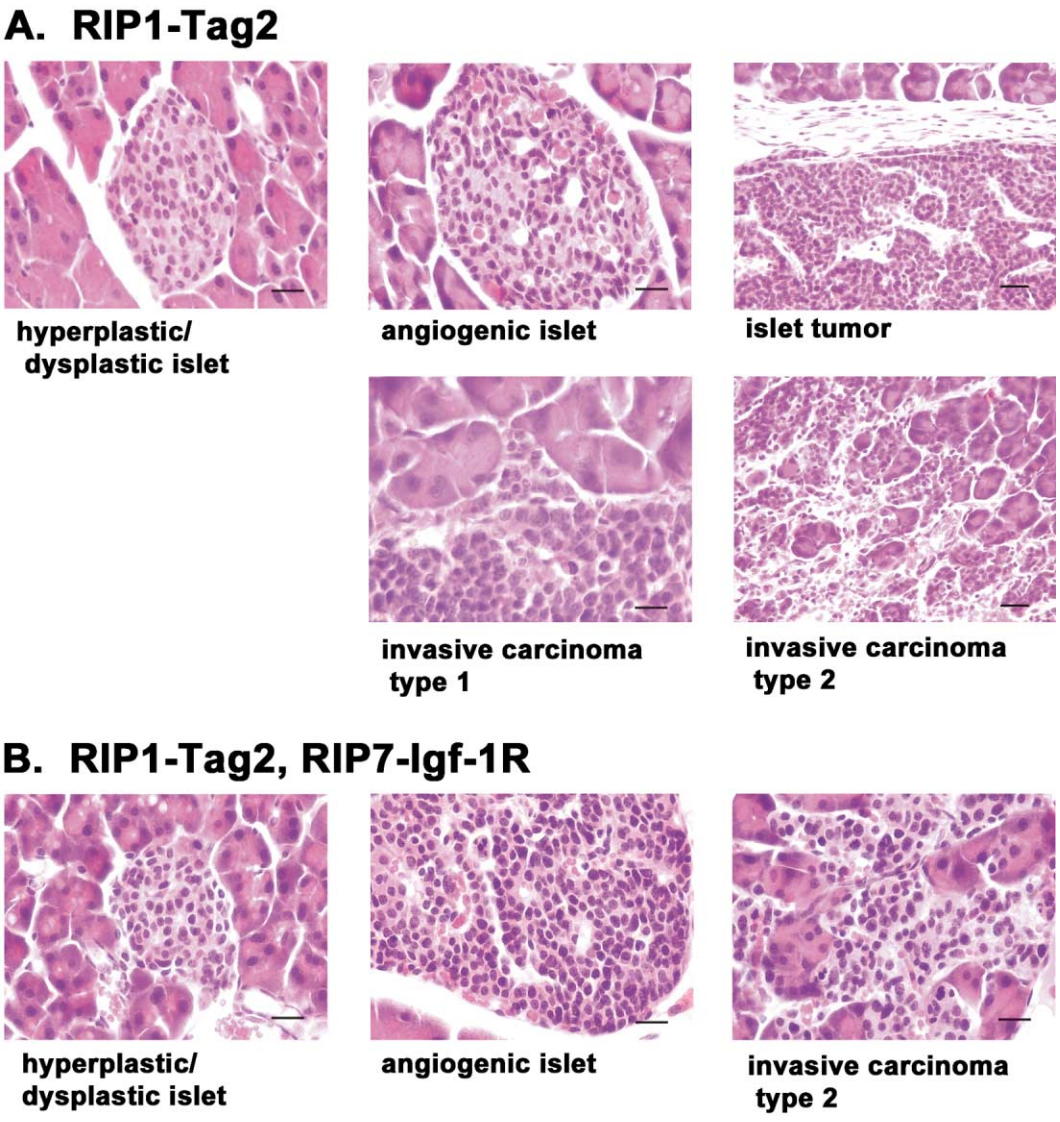


**Figure 3.** Morphology of the distinctive stages in islet carcinogenesis

Typical examples of the stages evident in the pathways to islet carcinogenesis are shown for standard RIP1-Tag2 mice (**A**) and for the predominant pathway resulting from upregulation of IGF-1R in RIP1-Tag2, RIP7-Igf-1R double-transgenic mice (**B**). These morphologies are described in Table 1 and form the basis for the quantitative comparison of the temporal appearance and statistical abundance of the stages seen during progression to invasive carcinoma of the pancreatic islets. Note that the double-transgenic mice also develop IC-1 lesions, which are similar to that shown in **A** for RIP1-Tag2. Scale bars represent 20 μm on all micrographs except the islet tumor and invasive carcinoma type 2 in **A**, where the scale is 40 μm.

Hanahan, 1999). We assessed, therefore, both parameters in the pancreases of double-transgenic mice at defined points during their lifetime. To assess proliferation frequencies, cells incorporating BrdU were quantitated in the histologically distinct populations of islets and neoplastic lesions from nontransgenic controls, from RIP7-Igf-1R, from RIP1-TAg2, and from double-transgenic RIP7-Igf-1R, RIP1-TAg2 littermates (Figure 5A). Islets from the RIP7-Igf-1R single-transgenic mice possess a slightly elevated level of BrdU incorporation (6.4%) when compared to the nontransgenic littermate control (2.5%). The increase in proliferation rate in the Igf-1R single-transgenic mice is consistent with both the slight increase in islet size and the presence of a functional receptor that may be activated either in a ligand-independent manner or by endogenous serum levels of IGF-I and/or IGF-II. In the double-transgenic mice, a significant increase in the frequencies of cells in cycle are seen in the hyperplastic/dysplastic and angiogenic lesions as compared with lesions from the single RIP1-Tag2 transgenic littermate. By contrast, the average proliferation rate seen in the tumors of the double-transgenic mice was not enhanced in comparison to the tumors in the RIP1-Tag2 single-transgenic mice.

To assess possible changes in the apoptotic rate as a consequence of increasing the levels of IGF-1R, the TUNEL assay was performed on islet lesions from RIP1-Tag2 single- and RIP7-Igf-1R double-transgenic littermates. As seen in Figure 5B, the frequency of TUNEL positive apoptotic cells is increased in normal, hyperplastic/dysplastic, and angiogenic lesions, but remains unaltered in the more advanced lesions in the double

**Table 2.** Comparison of tumor burden and summary of lesional stages analyzed in wild-type versus Igf-1R-upregulated RIP1-Tag2 mice

| | Mice analyzed | | Lesions evaluated (total) | | Tumor burden ($mm^3$) | |
|---|---|---|---|---|---|---|
| Age | RT2 | DTg | RT2 | DTg | RT2 | DTg |
| 5 weeks | 4 | 4 | 380 | 272 | <0.5 | <0.5 |
| 7 weeks | 4 | 3 | 488 | 220 | <0.5 | 1.21 ± 0.45[a] |
| 9 weeks | 6 | 5 | 314 | 100 | <0.5 | 55.34 ± 20.41[b] |
| 12 weeks | 6 | ND | 197 | ND | 37.7 ± 20.26 | ND |
| 15 weeks | 5 | ND | 76 | ND | 57.9 ± 14.21 | ND |

Abbreviations: RT2, RIP1-Tag2; DTg, RIP7-Igf1R, RIP1-Tag2 double transgenic mice; ND, not available, as DTg mice are dead by 11 weeks.
[a] $p = 0.035$
[b] $p = 0.0079$

transgenics in comparison to the RIP1-Tag2 single-transgenic mice. This result was unexpected, given the prediction that IGF-1R would signal resistance to apoptosis in response to activation of IGF-II. Thus, elevated IGF-1R differentially impacts the growth phenotype, as reflected by the proliferation and apoptosis rates: it initially enhances apoptosis and less so proliferation in the earliest stages (compared to RIP1-Tag2 islets) where IGF-II is off or focally low (Christofori et al., 1994); then, it comparatively upregulates proliferation in the angiogenic stage when IGF-II levels are elevated; and finally, it has no impact on either parameter in tumors.

**IGF-1R downregulates E-cadherin**

Cell-cell adhesion is an important component of the normal architecture of the islets, and loss of those intercellular contacts is postulated to be important for the development of malignancy. Prior studies have demonstrated that loss of the gap-junction protein E-cadherin is functionally involved in the transition from well-differentiated adenoma to invasive carcinoma in the RIP1-Tag2 pathway (Perl et al., 1998). Furthermore, downregulation of E-cadherin is associated with the malignant phenotype in many human cancers (Christofori and Semb, 1999; Cavallaro and Christofori, 2001). Given the prevalence of highly invasive tumors in the double-transgenic mice, we sought to determine whether E-cadherin expression was altered in the stages of islet carcinogenesis. Islets from single- and double-transgenic mice were stained for both E-cadherin and large T antigen, with the oncoprotein serving to visualize the neoplastic cells. Figures 6B and 6D show a clear loss of E-cadherin expression in oncogene-expressing cells in a relatively small double-transgenic islet lesion. A comparable lesion in the RIP1-Tag2 single-transgenic mice shows no loss of E-cadherin expression (Figures 6A and 6C). Visual inspection revealed downregulation of E-cadherin to be prevalent but not absolute in the numerous islet lesions analyzed in the double-transgenic mice. To substantiate that conclusion, we sought to quantitatively assess the apparent downregulation of E-cadherin by performing image analysis of the fluorescence intensity of E-cadherin immunostaining in islet lesions compared to adjacent normal exocrine pancreas. Evaluation of relative fluorescence intensity of E-cadherin in the different stages of the generic tumorigenesis pathway confirmed downregulation in both invasive carcinoma stages but not in progenitor islets or encapsulated islet tumors (Figure 6E). In contrast, E-cadherin expression in RIP1-Tag2, RIP7-Igf-1R mice was decreased throughout tumorigenesis, in premalignant lesions as well as in invasive carcinomas (Figure 6E). Notably, we did not detect downregulation of E-cadherin in the islets of RIP7-Igf1R single-transgenic mice (not shown), which indicates that additional factors are necessary to effect its suppression; amongst the candidate cofactors in RIP1-Tag2 mice are the induced IGF-II ligand, the expressed Tag oncoproteins, and the proliferative condition the oncogene elicits.

**Elevated IGF-1R increases pancreatic islet metastasis**

Suppression of E-cadherin has also been associated with increased but still relatively low-frequency metastasis in the RIP1-Tag2 model (Perl et al., 1998), motivating assessment of the metastatic phenotype in the Igf-1R double-transgenic mice. Indeed, the intrapancreatic lymph node (PLN) contained metastatic nodules in 77% of the double-transgenic mice analyzed; by comparison, 8.3% of single-transgenic RIP1-Tag2 mice had metastatic lesions (Table 3). More distant metastases were observed only occasionally in RIP1-Tag2, RIP7-Igf1R mice; we suspect the brief 10 week life span of the double-transgenic mice limited the opportunity for distant metastases to develop macroscopically. The intrapancreatic lymph node is the initial draining node for the pancreas (Cook, 1983; see also Mandriota et al., 2001, and references therein). This local lymph node has proved to be the predominant site for β cell metastasis in RIP-Tag2 mice, most notably in response to additional genetic perturbations. Thus, PLN metastasis was observed in the context of dominant-negative suppression of E-cadherin (Perl et al., 1998), ablation of NCAM expression (Perl et al., 1999), or coexpression of the lymphangiogenic growth factor VEGF-C in the oncogene-expressing β cells (Mandriota et al., 2001). The patterns and relatively low frequencies of metastasis observed in these studies led to the proposition that multiple factors govern the capability for islet metastasis (Cavallaro and Christofori, 2001). The comparatively high frequency of metastasis produced by elevated levels of IGF-1R suggests that IGF-1R signaling affects more of the necessary conditions for the metastatic growth program in addition to its clear association with downregulation of E-cadherin (Figure 6).

## Discussion

Motivated by the correlation of IGF-II gene activation with the onset of neoplastic proliferation in the RIP1-Tag2 tumorigenesis pathway and by its functional association with resistance to apoptosis, we sought to investigate the role played by one of its receptors, IGF-1R. The insulin-like growth factor receptor type 1 is a transmembrane tyrosine kinase with well-established

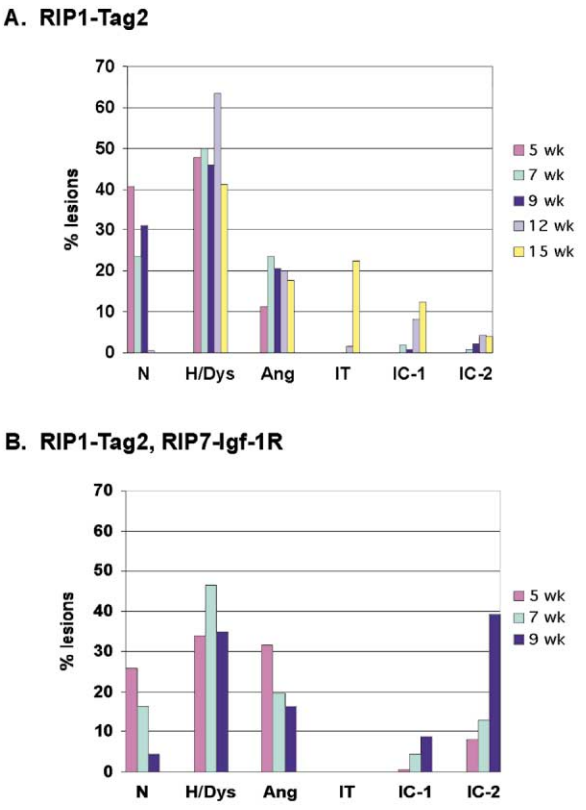


**Figure 4.** The temporal dynamics of multistage progression is accelerated and biased by elevated IGF-1R

Using staging criteria summarized in Table 1, lesions were classified at various time points in the life spans of RIP1-Tag2 (**A**) and RIP7-Igf-1R, RIP1-Tag2 double-transgenic (**B**) mice. Substantial numbers of lesions were evaluated at the noted ages, as summarized in Table 2. Comparison of the islet lesions in the cohorts of RIP1-Tag2 and RIP7-Igf-1R, RIP1-Tag2 mice at 5, 7, and 9 weeks of age demonstrated a statistically significant shift toward invasive carcinomas (5 weeks, $p = 0.0304$; 7 weeks, $p = 0.0518$; 9 weeks, $p = 0.0051$ by the Wilcoxon score for variable grade).

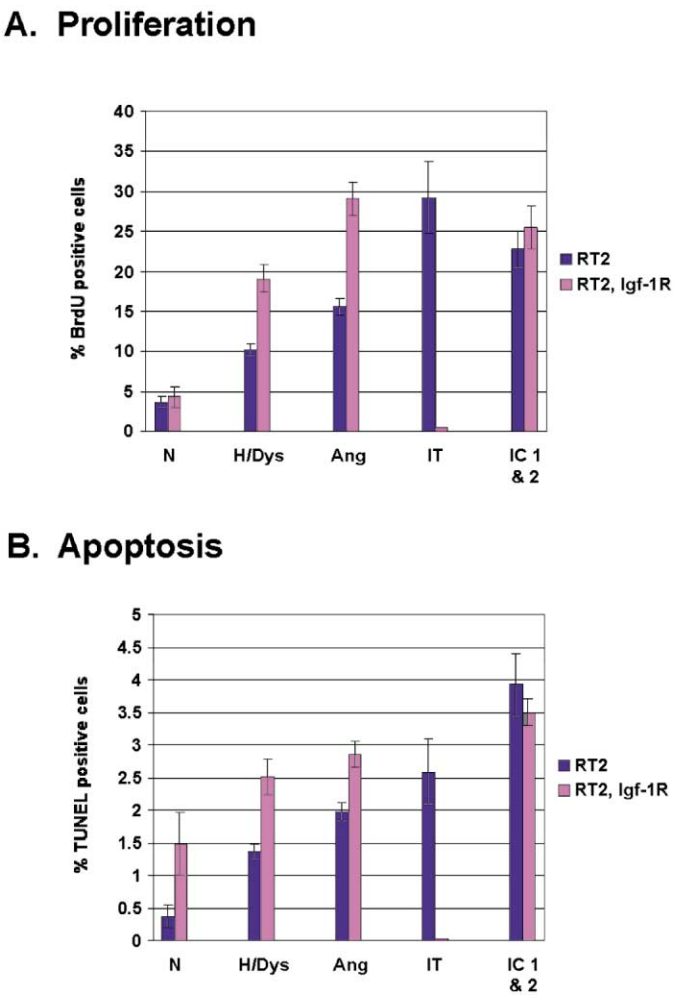


**Figure 5.** Elevated levels of IGF-1R selectively increase proliferation and apoptosis in premalignat stages of islet carcinogenesis

The proliferation index (**A**) and the apoptotic index (**B**) in staged lesions (Table 1) was determined and compared between RIP1-Tag2 single-transgenic and RIP1-Tag2, RIP7-Igf-1R double-transgenic mice. Differences between the two genotypes at a particular stage of carcinogenesis were analyzed by the unpaired, two-tailed t test. For the proliferation index (**A**), the significance values were: "normal" islets, $p = 0.6312$; hyperplastic/dysplastic islets, $p < 0.0001$; angiogenic islets, $p < 0.0001$; and invasive carcinoma types 1 and 2 (combined), $p = 0.4647$. The incidences of apoptosis (**B**) were similarly compared at each stage, and the significance values were: normal islets, $p = 0.1213$, hyperplastic/dysplastic islets, $p < 0.0001$; angiogenic islets, $p = 0.0005$; and invasive carcinoma types 1 and 2 (combined), $p = 0.1493$. Notably, increased levels of IGF-1R did not reduce apoptosis, but rather increased both proliferation and apoptosis in the premalignant stages while having no impact on the cancer stages. (The double-transgenic mice do not develop encapsulated islet tumors (IT) lacking invasive regions.)

intracellular signaling capabilities, and a knowledge base of correlative and functional evidence implicates it in cancer phenotypes. In generic RIP1-Tag2 single-transgenic mice, the endogenous IGF-1R protein was found to be upregulated from a nearly undetectable level in normal pancreatic islets, coincident with the transition from "normal" (oncogene-expressing) islets to hyperplastic/dysplastic islets, and concomitant with the heterogeneous induction of its previously dormant ligand, IGF-II. Receptor levels continued to be elevated in angiogenic islets, but then shifted from the uniform expression pattern seen in these premalignant lesions to one where increased expression was localized to cells at the periphery and invasive fronts of established tumors, consistent with a role in promoting invasion. Little or no expression was detectable in islet tumors with well-defined margins and fibrous capsules, and expression was patchy in the core of invasive carcinomas. We currently have no clues into the dynamic regulation of endogenous IGF-1R levels in the stages of islet carcinogenesis. We suspect that the sporadic activation and heterogeneous expression of the dormant (and imprinted) IGF-II gene (Christofori et al., 1994, 1995) in β cells expressing the T antigen oncogene may be part but probably not all of the regulatory equation. Seeking to probe the role of IGF-1R in this prototypical carcinogenesis pathway, transgenic mice were generated that constitutively expressed IGF-1R at elevated levels in the islet β cells of the pancreas. There was little effect on the islets of single-transgenic mice. By contrast, in the context of tumorigenesis, IGF-1R clearly accelerated neoplastic progression, most dramatically by promoting the capability for invasion and metastasis.

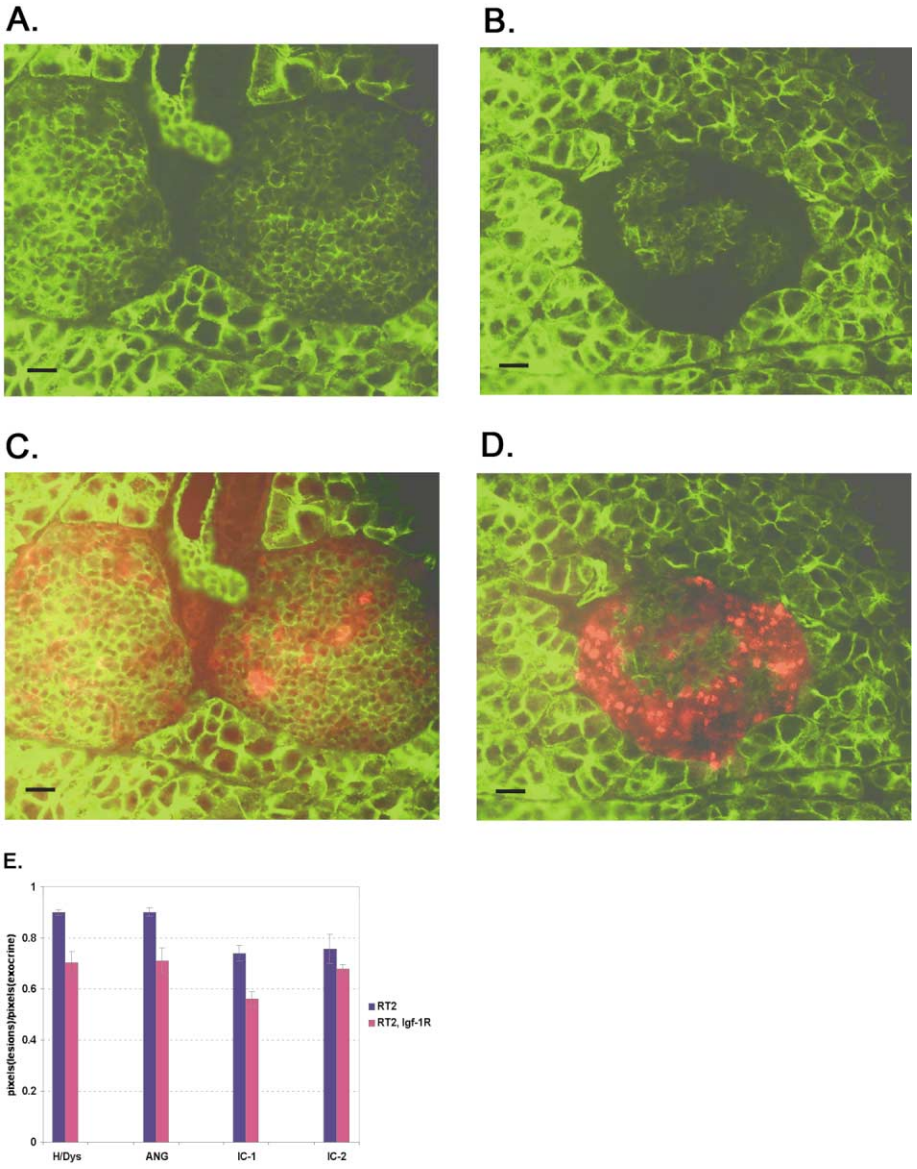


**Figure 6.** E-cadherin is downregulated early during tumorigenesis in RIP-7-Igf-1R, RIP1-Tag mice

**A** and **B** show immunostaining (green) for E-cadherin in hyperplastic/dysplastic islets of 5-week-old (**A**) single- and (**B**) double-transgenic mice, revealing marked suppression of E-cadherin in the latter. The regions devoid of E-cadherin in **B** are tumor cells expressing the large T oncoprotein, as revealed by coimmunostaining for Tag-expressing cells (labeled red); **C** and **D** show the same islets visualized for both E-cadherin and the oncogene. Downregulation of E-cadherin is prevalent (but heterogeneous) in all subsequent stages of the double-transgenic mice. Notably, the invasive regions of islet carcinomas in both single- and double-transgenic mice had reduced levels of E-cadherin (not shown), consistent with the results of Perl et al. (1998). Scale bars are 20 μm. **E:** Islet lesions from five RIP1-Tag2 (n = 121 total lesions) and four RIP1-Tag2, RIP7-Igf-1R (n = 76 total lesions) were categorized according to the criteria outlined in Table 1, and the ratio of mean fluorescence intensity of the islet lesion/adjacent exocrine pancreas was determined. Lesions were pooled from all mice of the same genotype for statistical analysis. Immunofluorescent staining revealed that the level of E-cadherin is decreased in the double transgenic in comparison to the single transgenic in the hyperplastic/dysplastic ($p = 0.002$), angiogenic ($p = 0.018$), and invasive carcinoma type 1 ($p = 0.008$) stages and equivalent in the invasive carcinoma type 2 ($p = 0.325$) by the two-tailed t test assuming unequal variances.

In designing this study, we predicted that elevating the levels of IGF-1R throughout tumorigenesis would cause a generalized decrease in apoptosis at all stages, much as we observed when the antiapoptotic factor Bcl-XL was similarly upregulated (Naik et al., 1996). The basis for this prediction came not only from the analysis of the phenotype in RIP1-Tag2, IGF-II null mice, but also from analysis of mice that had IGF-II constitutively upregulated. A RIP7-IGF-II transgenic line was produced and shown to express high levels of IGF-II in normal islet β cells without consequence; when crossbred to RIP1-Tag2, the double-transgenic mice had unchanged proliferative and apoptotic profiles during tumorigenesis (Naik, 1996). From this, we concluded that the levels of IGF-II resulting from upregulation of the endogenous IGF-II gene were sufficient to manifest its contributions to tumorigenesis, and therefore we postulated that the low levels of its receptor might be limiting. Indeed, the results of elevating this IGF receptor suggest that is the case, but in addition they have revealed an intriguing complexity to IGF-1R signaling. Evaluation of proliferation and apoptosis frequencies in the different lesional stages demonstrated that both proliferation and apoptosis rates were significantly increased in the premalignant stages, contrary to expectations. The discrepancy between the protection from apoptosis afforded by IGF-II upregulation in the standard RIP1-Tag2 pathway and the increased apoptosis in progenitor lesions in the RIP1-Tag2, RIP7-Igf-1R transgenic mice may have a basis in the dynamics of signal transduction. Ligand binding to IGF-1R has been shown to induce at least three intracellular signaling pathways: MAP kinase, phospholipase Cγ, and phosphoinositol 3-kinase. Mutational analyses of key residues in the cytoplasmic domain of the IGF-1R β chain have demonstrated that it is possible to functionally separate the survival, proliferation, transformation, and invasive/metastatic responses (Baserga et al., 1997; Brodt et al., 2001). Moreover, analysis of the MAP kinase pathway in other cell culture models has shown that decisions between cell survival and apoptosis as well as between proliferation and cell cycle

**Table 3.** Frequency of metastasis to the local pancreatic lymph nodes

| Transgenic genotype | Number of mice analyzed | Age (weeks) | Mice with PLN metastasis | Frequency of metastasis |
|---|---|---|---|---|
| RIP1-Tag2 (colony) | 12 | 13–14 | 1 | 8.3% |
| RIP1-Tag2 (littermates) | 12 | 11–16 | 1 | 8.3% |
| RIP1-Tag2, RIP7-Igf-1R$^{L1}$ | 9 | 8–10 | 7 | 77% |

In this analysis, RIP1-Tag2 and RIP1-Tag2, RIP7-Igf-1R pancreases were collected, fixed, and serially sectioned, and tissue sections every 100 μm were evaluated to reveal the pancreatic lymph nodes (PLN), which are the inital collecting point for the pancreatic lymphatic vasculature. The frequency of metastasis reported in generic RIP1-Tag mice has varied historically (Grant et al., 1991, Perl et al., 1998), perhaps as a function of genetic background, backcross generation, and age. To compare RIP1-Tag2 mice having the closest possible genetic background to the double transgenic mice at a similar stage of disease progression, a cohort of single transgenic RIP1-Tag2 littermates were sacrificed somewhat later than when their double transgenic siblings were at endstage and both were analyzed. Separately, endstage RIP1-Tag mice from the standard breeding colony were evaluated. Histological analyses by H&E, confirmed by insulin immunostaining, established the presence of metastatic nodules inside the lymph nodes. The difference in metastatic frequency is statistically significant compared to both RIP1-Tag2 cohorts ($p = 0.0022$ for the double transgenic mice versus RIP1-Tag littermates, and $p = 0.0022$ versus RIP1-Tag mice from the colony, as determined using the Fisher's Exact test).

arrest can be effected through alternative signaling thresholds determined by both the strength and duration of the activating signal (Woods et al., 1997; Cook et al., 1999). Based on these considerations, we envision that the modest upregulation of IGF-1R in premalignant lesions of RIP1-Tag2 single-transgenic mice establishes the receptor at a level that primarily signals antiapoptosis. Then, when the receptor level is further elevated via the transgene, a threshold is crossed such that the predominant signaling output is biased to the proliferative and invasive/metastatic effector circuits instead of antiapoptosis. We suspect that the invasive effector signal is also activated (but perhaps to a lesser extent) by the focally elevated IGF-1R seen in the invasive regions of carcinomas of RIP1-Tag2 single-transgenic mice. Additional considerations involve the heterogeneous expression of IGF-II, the potential of IGF binding proteins to modulate its bioavailability, and the possible involvement of the other IGF-1R ligands, IGF-I, and insulin. Moreover, the insulin receptor is now known to have an alternative isoform with higher IGF affinity (Frasca et al., 1999; Vella et al., 2002) and the potential to make functional heterodimers with IGF-1R (Pandini et al., 1999). It will be of interest to assess the possible contributions of these members of the IGF axis to islet carcinogenesis.

### E-cadherin and other effectors of IGF-1R invasive and metastatic signaling

IGF-1R signaling has long been associated with cellular transformation and more recently with invasion and metastasis (Dunn et al., 1998; Long et al., 1998b; Xie et al., 1999; Brodt et al., 2001). So too has suppression of E-cadherin, a cell adhesion molecule causally associated with invasion in this model and in a variety of human cancers (Christofori and Semb, 1999; Cavallaro and Christofori, 2001). Two previous reports have suggested a possible connection between E-cadherin and IGF-1R signaling: Andre et al. (1999) and Playford et al. (2000) each reported that IGF-I ligand increased tyrosine phosphorylation of β-catenin in colon carcinoma cell lines, resulting in dissociation of E-cadherin from the plasma membrane, thereby suppressing its cell adhesion function. Here, by elevating IGF-1R levels in the RIP-Tag2 pathway, we have shown that E-cadherin is downregulated early and far more prevalently that in the unperturbed pathway, where IGF-1R levels are more variable. Notably, the data presented above show that IGF-1R is expressed at the highest levels in single RIP1-Tag2 tumors at the boundaries and in invasive regions, where E-cadherin is known to be downregulated (Perl et al., 1998). Our interpretation is that signals from IGF-1R and from the Tag oncogene collaborate, likely with additional agents (perhaps IGF-II), to effect E-cadherin downregulation, a possibility that deserves future investigation.

It is pertinent to consider other candidate effectors of invasion and metastasis elicited by IGF-1R signaling, as there is reason to suspect that suppression of E-cadherin is necessary but not sufficient. One rationale involves the comparatively low frequency of metastasis seen when E-cadherin was suppressed (Perl et al., 1998) relative to when IGF-1R levels were elevated (this study), suggesting that IGF-1R signaling regulates other effectors of invasion. A second is that invasive carcinomas arise much earlier and at higher frequency in the case of IGF-1R upregulation than in that involving forcible E-cadherin downregulation. Several previous studies have identified candidate effectors of invasive and metastatic capabilities regulated by IGF-1R in cultured cells: Dunn et al. (2001) found that urokinase was upregulated by IGF-1R in the MDA-MB-213 human breast cancer line, and Long et al. (1998a) reported IGF-1R regulation of MMP-2 in a variant Lewis lung carcinoma line, whereas Mira et al. (1999) found that MMP-9 was induced by IGF-I in MCF-7 human breast cancer cells. Extracellular proteases have long been implicated in invasion and metastasis, and as such these are attractive candidates that bear investigation in the RIP1-Tag2, RIP7-Igf-1R tumorigenesis pathway. A counterpoint is that neither urokinase, MMP-2, nor MMP-9 appear to be individually important for induction of the invasive phenotype in islet carcinomas arising in the wild-type RIP1-Tag2 model. The evidence comes from gene knockout mice lacking each of these proteinases and from pharmacological inhibition studies with MMP inhibitors that target MMP-2 and MMP-9. When gene knockouts for urokinase (uPA), MMP-2, or MMP-9 were crossbred into the RIP-Tag2, and the protease null mice were analyzed, the surprising result was that mice lacking any one of these three proteases still developed highly invasive islet carcinomas (Bergers et al., 2000); MMP-2 and MMP-9 were, however, demonstrably important for tumor growth. Furthermore, treatment of RIP1-Tag2 mice with MMP inhibitors also impaired tumor growth but did not affect progression to invasive carcinomas (Bergers et al., 1999, 2000). Finally, MMP-9 was not expressed by the islet tumor cells coexpressing the Tag oncogene, IGF-II, and IGF-1R; rather, it is expressed in perivascular inflammatory cells (Bergers et al., 2000, and unpublished observations of the Hanahan laboratory). While these data argue against

their involvement in local invasion in the pancreas, we cannot exclude that one or another of these proteinases will prove to be induced in metastatic islet tumor cells and functionally involved therein; the possibility deserves future investigation. Similarly, the suggestions that IGF-1R-induced invasiveness is dependent on upregulation of integrins (Mira et al., 1999) or phosphorylation of β-catenin and IRS-1 (Playford et al., 2000) should be considered. While these candidates may or may not prove to be the implicit cofactors or the functional effectors of E-cadherin suppression that are necessary for signaling of invasion and metastasis by IGF-1R, we expect the critical genes will be identified as new candidates presented from other systems or by comparative analysis in the various RIP-Tag models, who are then confirmed via genetic manipulation of their functionality during islet carcinogenesis.

### IGF-1R directs tumorigenesis into an invasive growth program

In the course of analyzing the effects of elevating the levels of the IGF-1R throughout the well-established RIP1-Tag2 tumorigenesis pathway, the complete spectrum of lesions was thoroughly examined at three different time points (5, 7, and 9 weeks of age) in both the RIP1-Tag2 single- and RIP1-Tag2, RIP7-Igf-1R double-transgenic mice. The analysis of the single-transgenic mice confirmed the multistep paradigm proposed in earlier studies in the RIP1-Tag2 model. Although expression of the oncoprotein is known to initiate in the embryonic pancreas (Alpert et al., 1988), the islets were found to be histologically "normal" until 3–4 weeks after birth, when a subset activated diffuse β cell hyperproliferation and concomitant apoptosis, developing the histological characteristics of hyperplasia and dysplasia. Of these lesions, a subset switched on angiogenesis, as evidenced by the dilated, tortuous vasculature and microhemorrhaging characteristic of the angiogenic islet stage. At 5 weeks, all of these lesions are evident (Figures 3 and 4). Islet tumors with well-defined margins and often capsules (IT) begin appearing at about 10 weeks, which then progress into tumors with focal invasive regions (invasive carcinoma type 1, or IC-1), representing at endstage approximately 5% of the 400 pancreatic islets "at risk." In addition, our analysis of 7- and 9-week-old RIP1-Tag2 mice has revealed the presence of rare, highly invasive lesions that appear to have developed in the absence of progenitor tumors (IT) having well-defined margins. We now propose this class of tumor arises via a heretofore unappreciated minor pathway leading directly from angiogenic islets to widely invasive carcinomas that lack both an identifiable capsule and a clear margin with adjacent exocrine pancreas (IC-2). In Figure 7A, we have diagrammed this modification of RIP1-Tag2 pathway, in which a branch point at the hyperplastic/dysplastic or angiogenic islet stage shunts a subset of progenitor lesions away from the path to encapsulated islet tumors, directing them instead to form highly invasive carcinomas.

The existence of a branch in the tumorigenesis pathway that leads directly to invasive carcinoma is corroborated by our analysis of tumorigenesis in the double-transgenic RIP1-Tag2, RIP7-Igf-1R mice. Elevated levels of IGF-1R accelerated the temporal appearance and abundance of hyperplastic/dysplastic and angiogenic islet lesions and of highly invasive carcinomas (IC-2). There was, remarkably, a complete absence of benign islet tumors (IT). At 9 weeks, ~40% of the islets in the double-transgenic mice had already progressed to highly invasive carcinomas, with a spectrum of sizes ranging from microscopic (similar to islets) to macroscopic. By contrast, 9-week-old RIP1-Tag single-transgenic mice lacked macroscopic tumors of any class (IT, IC-1, or IC-2) and had at most 1–2 microscopic IC-2 lesions. These comparisons make an important point, namely that the highly invasive carcinomas induced by IGF-1R do not reflect a novel phenotype peculiar to the RIP1-Tag2, RIP7-Igf-1R mice overexpressing IGF-1R. Rather, the IGF-1R transgene biases carcinogenesis in favor of a newly appreciated branch of the pathway that is also evident in single-transgenic RIP1-Tag2 mice. Our revised view of the islet carcinogenesis pathway that unfolds in RIP1-Tag mice diagrammed in Figure 7, both for the RIP1-Tag2 single-transgenic case (Figure 7A) and for the

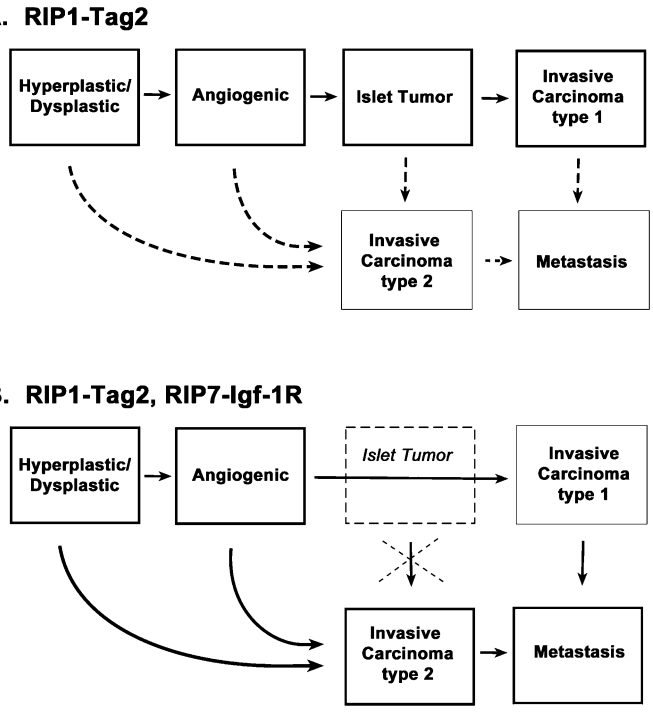


**Figure 7.** A modified pathway of multistage tumorigenesis of the pancreatic islets in RIP1-Tag2 mice

Extensive analysis of the temporal appearance and histological characteristics of lesional stages that appear in the pancreas of RIP1-Tag2 mice suggests a branched pathway to invasive carcinoma, as shown in **A**; the stages are described in Table 1 and exemplified in Figure 3A. The prevailing evidence from this study and those of Christofori and colleagues implicates a major pathway wherein angiogenic dysplasias ("angiogenic islets") in RIP1-Tag2 mice progress predominantly to islet tumors with well-defined margins that lack invasion. Invasive regions subsequently appear, focally projecting out of such well-defined islet tumors, producing the invasive carcinoma type 1 (IC-1), which is relatively frequent (8%–10% of islets at risk at 15 weeks). An exhaustive histopathological analysis (Table 2) of RIP1-Tag single-transgenic mice has revealed a less-frequent carcinoma that lacks evidence for a margin or capsule (invasive carcinoma type 2, IC-2). Both carcinoma types may produce the infrequent metastases observed. Elevated expression of IGF-1R biases the branched pathway in favor of the otherwise minor IC-2 branch (**B**), in part by suppressing expression of E-cadherin, producing a high incidence of broadly invasive carcinomas (40% of the focal islet lesions at 9 weeks) and frequent local metastasis. These results, considered in the context of independent studies in other experimental systems, suggest that elevated levels of IGF-1R may be a causal factor in neoplastic progression to highly invasive and metastatic phenotypes in settings of natural carcinogenesis.

illustrative perturbation whereby the levels of IGF-1R were forcibly elevated (Figure 7B). Thus, while we have historically presented islet carcinogenesis as a simple linear pathway, we now consider it as branched, wherein the invasive growth program can be induced at early or late stages, with a likely determinant being variable dynamics of upregulating IGF-1R. Interestingly, we have previously shown that genesis of intestinal carcinoids, arising from a related neuroendocrine cell type in the gut in a variant genetic background of RIP1-Tag2, also evidences early invasiveness in a pathway that similarly lacks an encapsulated tumor stage (Grant et al., 1991).

## A perspective on IGF signaling for the capabilities of carcinomas

The results of this investigation support a proposition that elevated expression of IGF-1R in the presence of both a ligand, IGF-II, and an oncoprotein, in this case the SV40 large T antigen that abrogates the pRb and p53 tumor suppressors, can enhance malignant progression, in particular evoking the capability for invasion and metastasis, thought to be crucial for most cancers (Hanahan and Weinberg, 2000). Elucidation of the mechanisms regulating the invasive and metastatic growth program remains a major agenda for cancer biology. One well-characterized invasive growth regulator is another receptor tyrosine kinase, c-met, which is capable of both ligand-dependent and ligand-independent signaling (Furge et al., 2000; Comoglio et al., 1999; Wang et al., 2001). Notably, c-met is able in some circumstances to downregulate E-cadherin (Hiscox and Jiang, 1999; Noe et al., 1999), perhaps the best-characterized effector of the invasive phenotype. Herein we show that a second receptor, IGF-1R, can elicit invasive and metastatic capabilities in the context of oncogenesis and that these phenotypes are also associated with downregulation of E-cadherin. Thus, IGF-1R may well serve as another key regulator of the invasive growth program. It is pertinent to recall, in this context, that a considerable body of research has documented elevated expression in human tumors of IGF-1R and its primary ligands, IGF-I and IGF-II (e.g., Macaulay, 1992; Quinn et al., 1996; Werner and LeRoith, 1996; Grothey et al., 1999; Xie et al., 1999; Scharf et al., 2001). This correlative data supports the conclusions of the functional studies performed here in a mouse model of carcinogenesis and by others in a variety of cell-based assay systems. Collectively, the evidence encourages the prediction that IGF-1R will prove to make profound functional contributions to the development of human cancer, conveying in some organs (and perhaps in early stages of tumorigenesis) resistance toward apoptosis, and in other organs (or later stages of progression) capability for invasion and metastasis. As such, it is reasonable to suggest that broader efforts are warranted to evaluate the expression of IGF-1R and its ligands in human cancers and to develop pharmacological inhibitors of IGF-1R signaling.

### Experimental procedures

#### Transgenic mice

The RIP7-IGF-1R transgene utilized a full-length cDNA clone of the human insulin-like growth factor type 1 (Igf-1R) gene (Ullrich et al., 1986) that had been shown to be biologically active when transfected in an expression vector into cultured mammalian cells (Yamasaki et al., 1991). The Igf-1R cDNA was inserted as a 4.4. kb fragment into the RIP7 vector (Naik et al., 1996), which consists of ~10 kb of the 5′ flanking region of the rat insulin II gene carried in a Bluescript-derived plasmid vector; the insulin gene regulatory sequences included the proximal promoter-enhancer region and most of the 5′ untranslated region of the transcribed portion of the gene with its associated small intron. Plasmid DNA was isolated using standard methods and purified by cesium density gradient for microinjection. The RIP7-Igf-1R mice were produced by DNA microinjection into zygotes of a C3B6F2 genotype using standard techniques. Mice were initially identified by Southern blot. Genetic screening to identify the transgene was performed by PCR using the following primers: 5′-GATGTTGTAGGTGTCTGCGGC and 5′-GACGGCACCATCGACATTGAGG. The identified transgenic founder mice (including the line #1 described here) were backcrossed to C57Bl/6 for 2–5 generations and then crossed to RIP1-Tag2 mice (effectively inbred in C57Bl/6, >N40) to generate double-transgenic mice. Unless noted, littermate RIP1-Tag2 single-transgenic mice were used in comparisons to the double-transgenic mice. In some cases, late-stage RIP1-Tag2 single-transgenic mice from the core colony (>N40 in C57Bl/6) were separately analyzed. The RIP7-Igf-1R$^{L1}$ lineage was maintained by backcrossing transgenic males and females with C57Bl/6 females and males (Jackson Laboratory), whereas only RIP1-Tag2 males were used for breeding.

#### Tissue preparation

Pancreases were removed and either fixed overnight in 4% paraformaldehyde in 1× PBS or immediately placed in OCT (Tissue-Tek) and frozen in a $CO_2$/ethanol bath. Fixed tissue was dehydrated through 30%, 50%, 70%, 80%, 95%, and 100% ethanol and 100% xylene before embedding in paraffin (Paraplast). Fixed and frozen tissues were cut into 5 μm and 10 μm sections, respectively.

#### Histology and immunohistochemistry

Frozen sections were air dried prior to acetone fixation, while paraffin-embedded sections were rehydrated through 100% xylene, 100%, 95%, 70%, and 50% ethanol before immersion in 1× PBS. For hematoxylin and eosin staining, sections were stained with hematoxylin Gill #3 and eosin Y (Sigma) according to standard protocols. For immunohistochemistry, frozen sections were routinely blocked for nonspecific biotin binding with a blocking kit (Vector Laboratories) diluted in 1% bovine serum albumin (Fraction V, Sigma) in 1× PBS. All tissue sections were blocked in 1% bovine serum albumin in 1× PBS prior to addition of the primary antibody. The following immunoglobins were used as negative controls for the species-matched primary antibody: rat IgG (ChromoPure Rat IgG whole-molecule Jackson ImmunoResearch), mouse IgG (MOPC 21, clarified ascites, Sigma), and rabbit IgG (Vector Laboratories). The following antibodies were used for specific staining: monoclonal rat anti-mouse E-cadherin (ECCD-2; 1:800; Zymed Laboratories), rabbit polyclonal anti-IGF-1R (SC-712; 1:250; Santa Cruz Laboratories), and rabbit polyclonal anti-large T antigen (1:250; Hanahan laboratory preparation). The following secondary antibodies were used at a 1:200 dilution: biotinylated goat anti-rabbit IgG (Vector), biotinylated goat anti-rat IgG (Vector), and Cy3-conjugated AffiniPure F(ab′2) goat anti-mouse IgG + IgM (Jackson ImmunoResearch). Biotinylated antibodies were developed with either FITC-conjugated streptavidin (1:200; BD Pharmingen) or Vectastain Elite ABC kit (Vector Laboratories) with Fast DAB (3′3′ Diaminobenzidine tetrahydrachloride; Sigma). Fluorescently labeled tissues were mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories). OCT-embedded samples were counterstained with 1% methyl green and dehydrated in isobutanol and xylene before mounting in Cytoseal #60 (Fisher). Paraffin-embedded samples were counterstained with hematoxylin followed with a brief incubation in dilute ammonium hydroxide solution before sequential dehydration in 70%, 95%, and 100% ethanol and 100% xylene, and then mounting as above.

#### Bromodeoxyuridine labeling

Mice were injected intraperitoneally with 5-bromo-2′-deoxyuridine (Sigma) to give a final dose of 100 μg BrdU/g body weight and euthanized 2 hr later. Tissues were collected and prepared as described above for paraffin embedding. The 5-Bromo-2′-Deoxy-Uridine Labeling and Detection Kit II (Roche) was used with the following modifications. After deparaffinization and rehydration, antigen retrieval (Citra Solution, Biogenex) was performed according to the manufacturer's protocol. Anti-BrdU was detected with biotinylated goat anti-mouse IgG (1:200; Vector Laboratories) and then processed for biotinylated antibodies as described above.

**TUNEL staining**

TdT-mediated dUTP-digoxigenin nick-end labeling was done on paraformaldehyde-fixed, paraffin-embedded tissues. Sections were prepared as described above and preincubated with Proteinase K solution (undiluted, Dako) for 3 min followed by an incubation with 0.15 M NaCl, 0.5% bovine serum albumin in TdT buffer (Invitrogen). Sections were incubated with terminal deoxynucleotidyl transferase (200 U/ml; Invitrogen) and alkali-stable digoxigenin-conjugated dUTP (2 μM; Boehringer-Mannheim) diluted in TdT buffer. Finally, sections were incubated with horseradish peroxidase-conjugated Fab fragments of sheep anti-digoxigenin-POD (5 U/ml; Roche). The horseradish peroxidase reaction, counterstaining, and mounting were carried out as above.

**Immunoprecipitation and Western blot**

Islets from nontransgenic and RIP7-Igf-1R mice were isolated by hand picking individual islets from collagenase Type XI (Sigma)-digested, Ficoll gradient-sedimented pancreases. Islets and MCF-7 cells were lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% Na deoxycholate, 0.02% Na azide, and complete EDTA-free protease inhibitor cocktail (Roche Applied Science). Protein concentration was determined by protein assay (BioRad), and equal amounts were precleared with protein G agarose (GIBCO-BRL/Invitrogen) before incubating with the mouse monoclonal MOPC 21 (myeloma IgG; Sigma) or αIR3 (specific for human, mouse, and rat IGF-1R; Oncogene Science) and protein G agarose. Immunoprecipitated protein was boiled in loading buffer with β-2 mercaptoethanol and run on a 8% SDS-polyacrylamide gel. Proteins were blotted on Immobilon-P membrane (Millipore) and probed with the rabbit polyclonal anti-IGF-1R (human, mouse, and rat IGF-1R α chain; Santa Cruz Biotechnology) in 5% milk powder in 20 mM Tris, 137 mM NaCl, 0.1% Tween-20. The blot was developed with horseradish peroxidase-conjugated goat anti-rabbit IgG (BioRad) and ECL (Amersham).

**Determination of E-cadherin levels**

Mouse pancreas sections stained with the antibody combinations anti-large T antigen/Cy3 and anti-E-cadherin/FITC were examined with a Zeiss Axioskop-$2^+$ microscope, and regions of interest were defined by large T antigen reactivity. E-cadherin expression levels within the region of interest were measured using the Openlab software (Improvision) as mean fluorescence intensity (pixels). To permit comparison between different areas of the same slide and different slides, an adjacent region-of-interest was defined in the exocrine tissue for each islet lesion, and the ratio of fluorescence intensity of islet lesion to adjacent exocrine pancreas was determined.

**Acknowledgments**

We thank Molly Daniels for construction of the RIP7-Igf-1R transgene and Shlomo Melmed for providing the human Igf-1R cDNA clone; Anne Neill, Eva Soliven, Raquel Santos, and Jose Imperio for technical support; Jeff Hager and Zena Werb for comments on the manuscript; and Mae Moredo for assistance with the manuscript. This work was supported by grants from the National Cancer Institute.

Received: March 21, 2002
Revised: May 1, 2002

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