Characterization of a Novel Primary Mammary Tumor Cell Line Reveals that Cyclin D1 Is Regulated by the Type I Insulin-Like Growth Factor Receptor

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Abstract

The importance of type I insulin-like growth factor receptor (IGF-IR) overexpression in mammary tumorigenesis was recently shown in two separate transgenic models. One of these models, the MTB-IGFIR transgenics, was generated in our lab to overexpress IGF-IR in mammary epithelial cells in a doxycycline (Dox)-inducible manner. To complement this transgenic model, primary cells that retained Dox-inducible expression of IGF-IR were isolated from a transgenic mammary tumor. This cell line, RM11A, expressed high levels of IGF-IR, phosphorylated Akt, and phosphorylated extracellular signal–regulated kinase 1/2 in the presence of Dox. IGF-IR overexpression provided the primary tumor cells with a survival advantage in serum-free media and seemed to induce ligand-independent activation of the IGF-IR because RM11A cells cultured in the presence of Dox were largely nonresponsive to exogenous IGFs. IGF-IR overexpression also augmented the growth of RM11A cells *in vivo* because injection of these cells into mammary glands of wild-type mice produced palpable tumors in 15.8 ± 3.4 days when the mice were administered Dox, compared with 57.8 ± 6.3 days in the absence of Dox. DNA microarray analysis revealed a number of genes regulated by IGF-IR, one of which was *cyclin D1*. Suppression of IGF-IR expression *in vitro* or *in vivo* was associated with a decrease in cyclin D1 protein, suggesting that at least some of the proliferative actions of IGF-IR are mediated through cyclin D1. Therefore, this article characterizes the first primary murine mammary tumor cell line with inducible IGF-IR expression. These cells provide a powerful *in vitro/in vivo* model to examine the function of IGF-IR in mammary tumorigenesis.
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Introduction

The type I insulin-like growth factor receptor (IGF-IR) plays an important role in a number of human cancers including those of the lung, breast, and prostate (1-5). The physiologic effects of IGF-IR are mediated through a number of signaling pathways including phosphatidylinositol 3-kinase and mitogen-activated protein kinase. Activation of the tyrosine kinase domain following binding of IGF-I or IGF-II permits the association of a number of intracellular docking proteins including Shc and insulin receptor substrate 1. Induction of the Shc pathway leads to extracellular signal–regulated kinase (Erk)-1/Erk2 phosphorylation and activation of downstream transcription factors that regulate cell cycle progression (1, 3, 4, 6-8). Phosphorylation of insulin receptor substrate 1 leads to sequential activation of phosphatidylinositol 3-kinase and Akt (9-11). Once activated, Akt can promote cell proliferation by enhancing p70 S6 kinase activity and inhibiting glycogen synthase kinase-3–mediated degradation of cyclin D1 (12-14). Akt can also suppress apoptosis by regulating Bad and caspase-9 activation (15-17). Other signaling pathways activated by IGF-IR include p38 mitogen-activated protein kinase and c-jun NH_2-terminal kinase/stress-activated protein kinase (18).

The function of IGF-IR in breast tumorigenesis has extensively been investigated *in vitro*. Mouse embryo fibroblasts null for the IGF-IR are resistant to transformation by a number of chemical, viral, and genetic factors (19). Reintroduction of IGF-IR into these cells restored their sensitivity to transforming agents. Moreover, it has been shown that overexpression of IGF-IR itself is sufficient for cell transformation provided a sufficient number of IGF-IR molecules are expressed (20-22). The IGF-IR is expressed on the surface of breast cancer cell lines, and activation of IGF-IR induces proliferation, inhibits apoptosis, improves three-dimensional growth, and enhances drug and radiation resistance in these cells (4, 5, 8, 23). Further, IGF-IR suppression through a variety of techniques, including dominant negative and antisense RNA constructs, inhibits breast cancer cell proliferation and promotes apoptosis (24-28).

In human breast cancer, IGF-IR is expressed on the surface of malignant epithelial cells and levels have been found to be elevated as high as 14-fold (8, 29-32). Moreover, hyperphosphorylation of IGF-IR has been observed in primary breast tumors compared with normal mammary epithelial cells (8).

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A recent article has shown that patients carrying *BRCA1* mutations have elevated IGF-IR expression (33). Although elevated IGF-IR levels and activity have been found in breast cancer, high breast tumor IGF-IR levels have been associated with both enhanced and reduced disease-free survival (3, 8, 29, 31, 34). Therefore, the exact function of IGF-IR in human breast cancer remains unclear. Despite the incomplete understanding of IGF-IR function in mammary physiology/tumorigenesis *in vivo*, this receptor is being touted as a potential therapeutic target for breast cancer (24, 35).

The recent development of two transgenic models where the IGF-IR is overexpressed in mammary epithelial cells has provided insight into the *in vivo* functions of IGF-IR in mammary development and tumorigenesis. Mammary-specific IGF-IR transgenic mice developed by Carboni et al. (36), known as CD8-IGF-IR, express a fusion protein containing the cytoplasmic portion of the human IGF-IR and the extracellular and transmembrane portions of the human T-cell antigen CD8α. This fusion protein results in constitutive activation of the IGF-IR and is driven by the mouse mammary tumor virus promoter, which permits constitutive expression of the CD8-IGF-IR fusion protein in mammary epithelial cells. The mammary-specific IGF-IR transgenic mice developed in our lab, known as MTB-IGFIR, overexpress the full-length human IGF-IR cDNA in a doxycycline (Dox)-inducible manner. Thus, the MTB-IGFIR transgenic mice only overexpress IGF-IR when the animals are administered Dox in either their water or food. Both IGF-IR transgenic models displayed similar phenotypes. Overexpression of IGF-IR during mammary ductal development inhibited elongation of the mammary ducts (36, 37). In addition, constitutive overexpression of the IGF-IR fusion protein in the CD8-IGF-IR transgenics or overexpression of IGF-IR in the MTB-IGFIR transgenics beginning at 21 days of age resulted in the formation of palpable mammary tumors by ~10 weeks of age. Thus, the *in vivo* data indicate that IGF-IR overexpression is sufficient to initiate mammary tumor development.

To complement our IGF-IR transgenic animals, we have generated a primary mammary tumor cell line from one of the mammary tumors that developed in an MTB-IGFIR transgenic mouse. This cell line, called RM11A, retains the ability to overexpress IGF-IR in the presence of Dox and induces the activation of similar signaling pathways as observed in the mammary tumors of the MTB-IGFIR mice. Using these cells, we have shown that IGF-IR overexpression provides RM11A cells with a survival advantage over primary mammary tumor cells expressing basal levels of IGF-IR, particularly in serum-free medium. To gain a broader perspective of the alterations induced by IGF-IR overexpression, DNA microarray analysis was done on these RM11A cells expressing high or basal levels of IGF-IR. IGF-IR overexpression resulted in the up-regulation of 114 and the down-regulation of 164 genes (at least 2-fold; $P < 0.005$). One of the genes induced by IGF-IR overexpression was the key cell cycle regulator *cyclin D1*. Given that *cyclin D1* is one of the most commonly overexpressed oncogenes in breast cancer, we proceeded to examine this molecule further. Our findings indicate that IGF-IR overexpression regulates *cyclin D1* at both the mRNA and protein levels *in vitro*. Furthermore, using the inducible nature of the IGF-IR transgene in our MTB-IGFIR mice and RM11A-injected tumors, we provide evidence that overexpression of IGF-IR regulates *cyclin D1* in breast tumor cells *in vivo*. The ability of IGF-IR to regulate *cyclin D1* was, at least in part, mediated by activation of Akt signaling.

Results

Isolation of Primary Murine Mammary Tumor Cells That Retain Dox-Inducible Expression of IGF-IR

We have previously shown that transgenic overexpression of IGF-IR in the mammary epithelium in a Dox-dependent manner results in the rapid development of mammary tumors (37). To further investigate the role of IGF-IR in mammary tumorigenesis, we isolated several cell lines from an MTB-IGFIR mammary tumor. One of these cell lines, termed RM11A, was identified as containing IGF-IR levels that could be induced by Dox (Fig. 1A). The levels of both phosphorylated IGF-IR and phosphorylated Akt were also elevated in RM11A cells treated with Dox compared with those cultured in the absence of Dox, as were the levels of phosphorylated Erk1/Erk2 (Fig. 1A).

Repression of IGF-IR transgene expression (through removal of Dox from the culture media) was associated with a morphologic change from spindle-shaped cells (high IGF-IR) to cells with a more cuboidal appearance (low IGF-IR) within 2 days after Dox removal (Fig. 1B and C). The shape change was not associated with altered cytokeratin expression because RM11A cells cultured in the presence or absence of Dox expressed high levels of cytokeratins 5 and 14 (Fig. 1D-G) and little or no cytokeratins 8 and 18 (data not shown). This altered cell morphology was maintained in long-term Dox-free cultures, and these cells, termed KR, remain viable and proliferate in the absence of Dox. Interestingly, this morphologic change seems to be irreversible because readministration of Dox to the culture media did not induce IGF-IR transgene expression, nor did it induce KR cells to revert back to the spindle-like morphology.

IGF-IR Overexpression Enhances Ligand-Independent Tumor Cell Proliferation

To determine whether the level of IGF-IR expression affected cell proliferation or survival of RM11A cells, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. When cells were cultured in serum-free media, RM11A cells cultured in the presence of Dox (high IGF-IR expression) displayed a 2.7-fold increase in cell number compared with RM11A cells cultured in the absence of Dox (low IGF-IR expression; Fig. 2A). When the cells were grown in fully supplemented media, the difference in growth rate was not significantly different in RM11A cells with elevated or basal IGF-IR expression (Fig. 2A). It is interesting to note that the addition of serum to RM11A cells expressing low levels of IGF-IR resulted in a significant increase in proliferation, whereas serum did not significantly alter the proliferation rates of RM11A cells expressing high levels of IGF-IR (Fig. 2A).

Consistent with the finding that the addition of serum to RM11A cells expressing high levels of IGF-IR did not significantly increase growth rate, the addition of IGF-I or IGF-II

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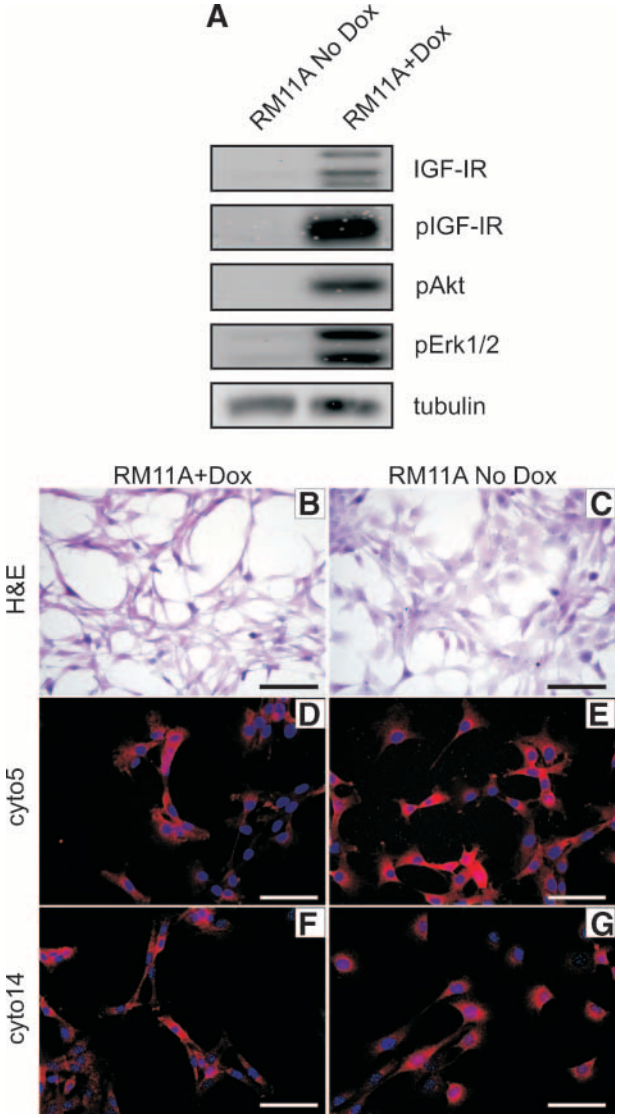

FIGURE 1. RM11A cells retain Dox-inducible expression of IGF-IR. **A.** Western blot analysis of protein levels of IGF-IR, phospho-IGF-IR (Tyr^{1131}), phospho-Akt (Ser^{473}), and phospho-Erk1/Erk2 (Thr^{202}/Tyr^{204}) in RM11A cells cultured in the presence of Dox and following withdrawal of Dox for 48 h. Tubulin served as a protein loading control. RM11A cells undergo a morphologic change when cultured in the absence of Dox. H&E-stained RM11A cells cultured with (**B**) and without (**C**) Dox supplementation to the culture medium. Immunofluorescence staining of RM11A cells cultured in the presence and absence of Dox for basal cell markers cytokeratin 5 (**D** and **E**) and cytokeratin 14 (**F** and **G**). Bar, 100 μm.

to the media did not significantly increase the proliferation rate of RM11A cells expressing high levels of IGF-IR (Fig. 2B and C). In contrast, RM11A cells expressing low levels of IGF-IR that were cultured in serum-free media displayed a significant increase in proliferation in response to IGF-I or IGF-II treatment (Fig. 2B and C). Because it is possible that the high IGF-IR–expressing RM11A cells are making their own IGFs, a recombinant IGF binding protein 1 was added to the cells grown in serum-free media. In preliminary trials, the addition of IGF binding protein 1 did not significantly reduce cell proliferation (data not shown). Therefore, the RM11A cells expressing high levels of IGF-IR have enhanced proliferative capacity that seems to be independent of exogenous growth factors.

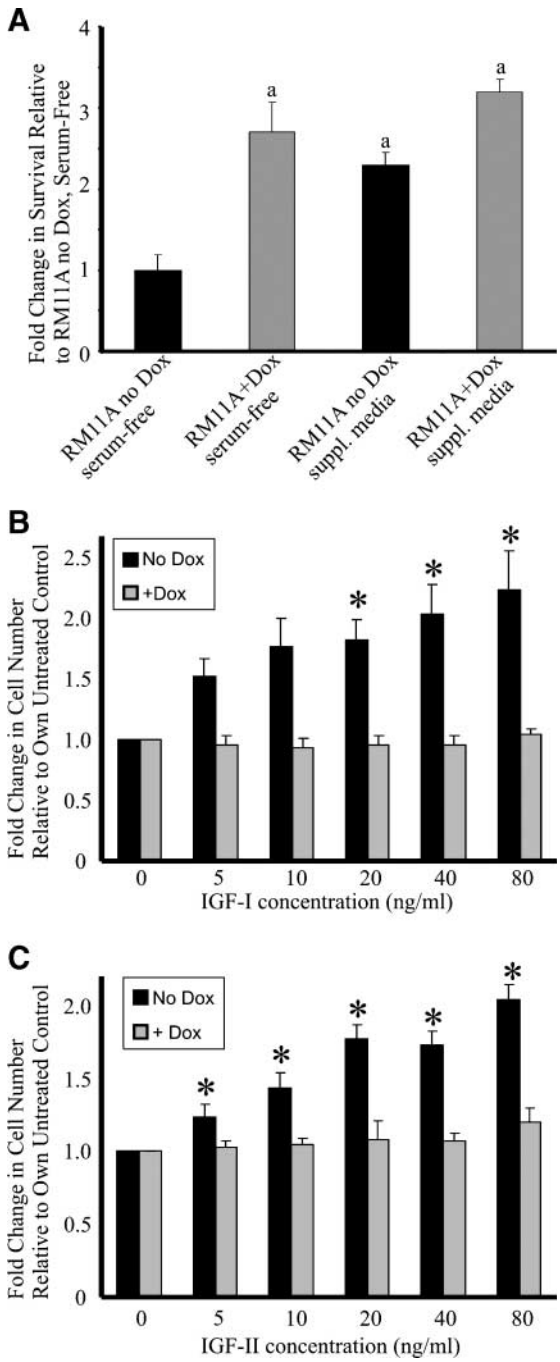

FIGURE 2. RM11A cells overexpressing IGF-IR have enhanced proliferation/survival *in vitro*. **A.** MTT assay of RM11A cells cultured in serum-free media or fully supplemented media in the presence and absence of Dox and RM11A cells grown in serum-free media, in the presence and absence of Dox, supplemented with various concentrations of human recombinant (**B**) IGF-I or IGF-II (**C**). [a], $P < 0.05$, these three groups are significantly different from the RM11A no Dox, serum-free group but are not significantly different from each other (Tukey's test). *, $P < 0.05$, a significant increase in proliferation relative to control (Dunnett's test).

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Overexpression of IGF-IR in RM11A Cells Accelerates Tumor Formation In vivo

The tumorigenic potential of RM11A cells was determined by injecting $\sim 5 \times 10^5$ cells into the mammary fat pad of wild-type, FVB (syngeneic) mice. The wild-type mice were then provided drinking water that contained 2 mg/mL Dox to induce IGF-IR expression. Palpable mammary tumors were evident ~2 to 3 weeks following injection of RM11A cells (Fig. 3A). To determine whether the level of IGF-IR expression altered mammary tumor formation, two experiments were done: (*a*) RM11A cells were injected into wild-type FVB mice that were provided normal drinking water and (*b*) KR cells were injected into wild-type FVB mice that were provided normal drinking water. RM11A cells still produced palpable mammary tumors when injected into mice not treated with Dox; however, tumor onset was delayed to ~8 weeks following injection. KR cells were also capable of forming tumors *in vivo* with a latency of ~8 weeks.

Histologically, tumors that arose in the presence or absence of Dox had similar appearances in that they were relatively homogeneous and were primarily composed of solid sheets of tumor cells with sparse stroma (Fig. 3B and C). IGF-IR immunohistochemistry was done to determine whether the IGF-IR protein was elevated independent of Dox administration. RM11A tumors in Dox-treated mice had higher levels of IGF-IR than RM11A cells in mice not treated with Dox (Fig. 3D and E) or KR cells in mice not treated with Dox (data not shown). Mammary tumors that developed in the presence or absence of Dox stained positive for cytokeratin 5 whereas stainings for cytokeratins 8, 14, and 18 were very weak or negative (data not shown). Immunohistochemistry for estrogen and progesterone receptors in RM11A-injected tumors revealed no positive staining (data not shown). This finding is consistent with our observations in the MTB-IGFIR transgenic mice in that large mammary tumors were estrogen and progesterone receptor negative (37).

Gene Expression Alterations Associated with IGF-IR Overexpression

Given that IGF-IR overexpression in RM11A cells enhances cell proliferation/survival *in vitro* and accelerates tumor formation *in vivo*, we next conducted microarray analysis to explore the target genes that are associated with elevated IGF-IR signaling. Using a cutoff of at least a 2-fold change in gene expression and a significance level of $P < 0.005$, IGF-IR overexpression in RM11A cells was associated with the

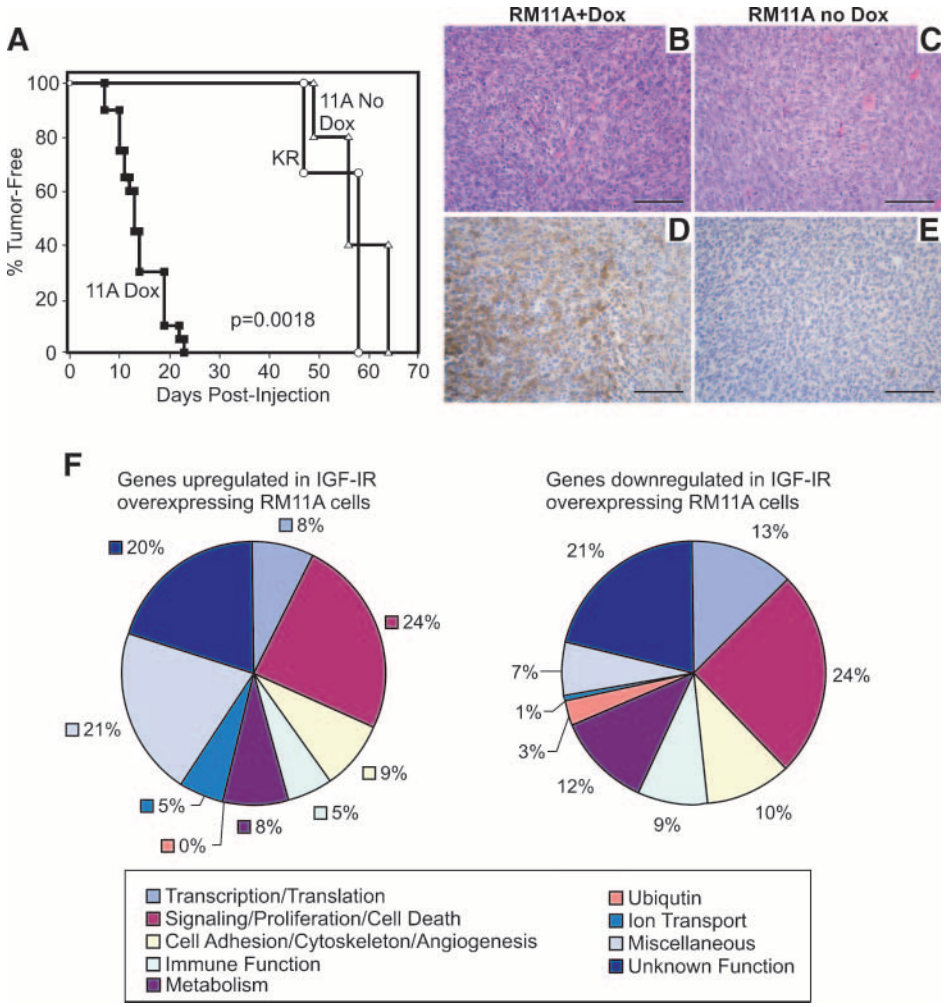

FIGURE 3. **A.** Kaplan-Meier plot of tumor onset in wild-type mice injected with RM11A cells and fed Dox-supplemented food (■), injected with RM11A cells and fed unsupplemented food (△), or injected with KR cells (low IGF-IR expression) and fed unsupplemented food (○). **B** and **C.** H&E-stained sections of tumors arising in mice injected with RM11A cells and treated with Dox (**B**) or without Dox (**C**). **D** and **E.** Immunohistochemistry for IGF-IR. Bar, 100 μm. **F.** Summary of DNA microarray data. The pie charts summarize the function of the genes significantly (at least 2-fold change and $P < 0.005$) up-regulated or down-regulated in RM11A cells with elevated IGF-IR expression compared with RM11A cells with basal IGF-IR expression.

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up-regulation of 114 genes and the down-regulation of 164 genes. These genes were separated into the following classifications: (*a*) transcription/translation, (*b*) signaling/proliferation/cell death, (*c*) cell adhesion/cytoskeleton/angiogenesis, (*d*) immune function, (*e*) metabolism, (*f*) ubiquination, (*g*) ion transport, (*h*) miscellaneous functions, and (*i*) unknown function. The pie charts illustrate the relative distribution of the genes into the aforementioned functional classes (Fig. 3F). A complete gene list is provided in Supplementary Table S1.

A number of genes of interest were selected from the microarray data and their expression was confirmed by real-time PCR or Western blot (Table 1). Genes up-regulated in response to IGF-IR overexpression and subsequently confirmed by quantitative real-time PCR included the transcription factors *ETV-1* and *ETV-5*, which belong to the PEA3 subfamily of Ets transcription factors, and the transcriptional regulator *Notch 4*. Genes confirmed to be down-regulated in response to overexpression of IGF-IR included the Wnt signaling mediators *WISP1* and *WISP2*.

IGF-IR Overexpression Induces Cyclin D1 mRNA and Protein Expression In vitro

Microarray analysis also indicated that IGF-IR overexpression induced expression of the *cyclin D1* gene. Because cyclin D1 is commonly overexpressed in breast cancer and has been implicated in mediating IGF-induced cell cycle progression, we proceeded to examine this protein further. To determine whether IGF-IR regulated cyclin D1 at the protein level, Western blotting was done on RM11A cells cultured in the presence and absence of Dox. As illustrated in Fig. 4A, IGF-IR overexpression induced an increase in cyclin D1 protein levels. Immunofluorescence for cyclin D1 protein also revealed that overexpression of IGF-IR results in higher levels of cyclin D1 protein, and most of this protein was localized to the nucleus (Fig. 4B-E).

To ensure that the increase in cyclin D1 levels was due to IGF-IR overexpression and not a result of Dox being present in the culture media, IGF-IR expression was suppressed using RNA interference. Knockdown of IGF-IR using two different oligonucleotides specific for the human *IGF-IR* gene suppressed IGF-IR protein levels by ~60% and 45% and was associated with a corresponding decrease in cyclin D1 levels (Fig. 4F).

IGF-IR Regulates Cyclin D1 in Mammary Tumors In vivo

To investigate whether IGF-IR regulated cyclin D1 levels in mammary tumors *in vivo*, Western blotting was done on tumor-bearing MTB-IGFIR transgenic mice and age-matched, Dox-treated wild-type mice. Indeed, IGF-IR–induced mammary tumors contained higher levels of cyclin D1 protein compared with wild-type mammary glands (Fig. 5A). Immunohistochemistry confirmed that the mammary tumors induced by IGF-IR overexpression (Fig. 5B) contained high levels of cyclin D1 (Fig. 5D) that was localized to the nucleus. Even the lung metastases (which occur in ~33% of the mice) express high levels of IGF-IR (Fig. 5C) and cyclin D1 protein (Fig. 5E).

The IGF-IR transgene in the MTB-IGFIR mice can be regulated by the addition and withdrawal of Dox from the animal's drinking water. Thus, to further establish whether overexpression of IGF-IR regulates cyclin D1 *in vivo*, Dox was removed from tumor-bearing MTB-IGFIR mice for 24, 48, and 72 hours. Dox withdrawal resulted in rapid down-regulation of IGF-IR expression, which was also associated with a corresponding decrease in phosphorylated IGF-IR, cyclin D1, and phosphorylated Akt levels (Fig. 5F). In addition, withdrawal of Dox for 48 hours from mice bearing tumors induced by the injection of RM11A cells also resulted in a decrease in both IGF-IR and cyclin D1 protein levels (Fig. 5G). In both the MTB-IGFIR– and RM11A-injected mammary tumors, Dox withdrawal was associated with rapid tumor regression (data not shown).

TABLE 1. Confirmation of Genes Regulated in RM11A Cells Overexpressing IGF-IR

Unigene	Gene	Fold Change	qRT-PCR	Western blot
Genes up-regulated				
Mm.4866	*Etv-1*	2.3	↑	—
Mm.155708	*Etv-5*	5.3	↑	—
Mm.173813	*Notch 4*	2.9	↑	—
Mm.273049	*Cyclin D1*	2.4	—	↑
Genes down-regulated				
Mm.10222	*WISP1*	2.1	↓	—
Mm.13828	*WISP2*	4.0	↓	—

NOTE: The table indicates genes that were significantly increased or decreased as determined by DNA microarray analysis in RM11A cells overexpressing IGF-IR and confirmed by quantitative real-time PCR (qRT-PCR) or Western blotting.

Discussion

As molecules targeting the IGF-IR are entering clinical trials, it is essential to understand (*a*) how the IGF-IR promotes mammary tumorigenesis; (*b*) which signaling pathways are used by the IGF-IR during tumor progression; and (*c*) whether a molecule downstream of the IGF-IR represents a more selective and effective therapeutic target. To address these questions, MTB-IGFIR transgenic mice were created. These mice overexpress the human IGF-IR in a Dox-inducible manner (37). Although incredibly valuable, the MTB-IGFIR transgenics suffer the same limitations as the other transgenic models. First, propagation and characterization of transgenic mice is time-consuming and expensive. Second, unlike cultured cells where additional genetic alterations can easily be introduced using expression plasmids or RNA interference, incorporation of additional genetic alterations into transgenic mice is considerably more complicated. Finally, the use of transgenic animals requires a particular level of expertise and appropriate housing facilities that may not be available to all researchers. Thus, to complement our IGF-IR transgenic mice, a primary tumor cell line, derived from a mammary tumor that developed in an MTB-IGFIR transgenic, was created.

A number of different cultures were generated following disaggregation of the MTB-IGFIR mammary tumor; however, one culture, named RM11A, was selected based on its ability to overexpress the IGF-IR following the addition of Dox to the culture media. Overexpression of IGF-IR in RM11A cells resulted in IGF-IR phosphorylation as well as enhanced phosphorylation of Akt and Erk1/Erk2.

MTT cell survival assays of RM11A cells suggest that overexpression of IGF-IR provides cells with an enhanced

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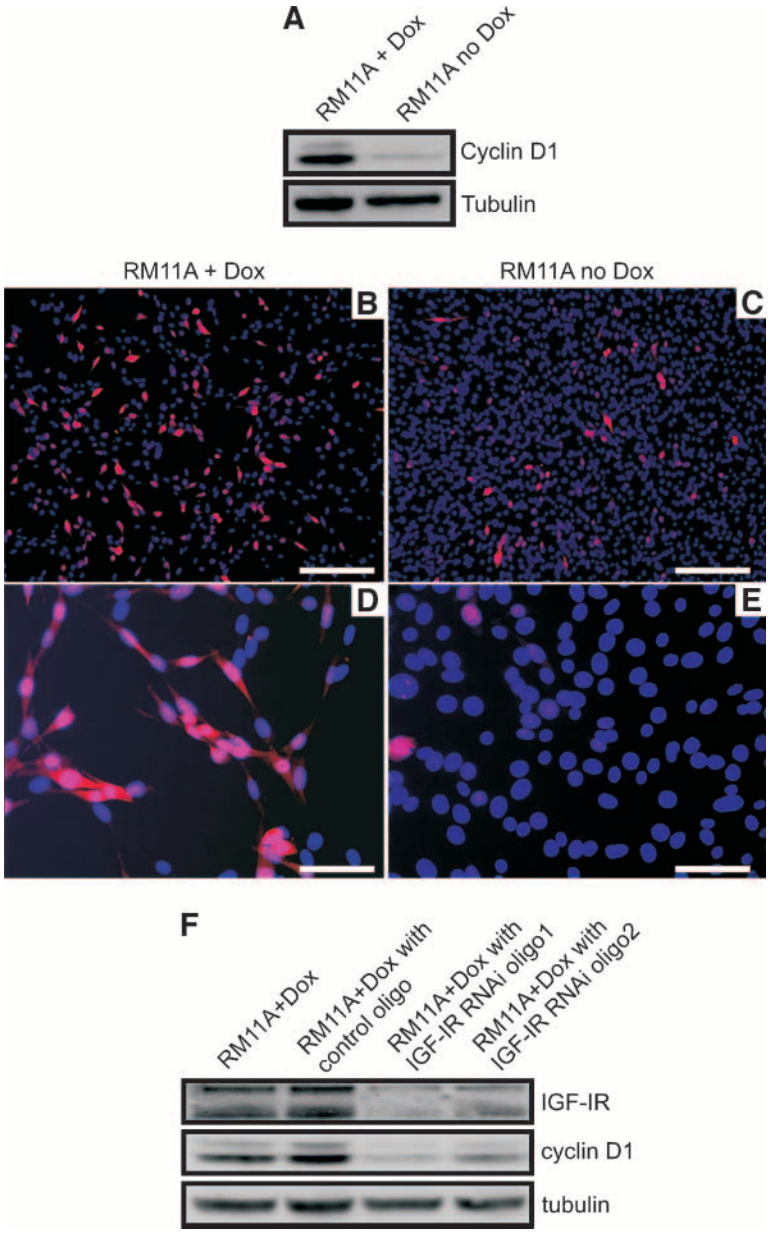

FIGURE 4. IGF-IR overexpression increases cyclin D1 protein levels. **A.** Western blot analysis of cyclin D1 protein levels in RM11A cells cultured in the presence of Dox and following Dox withdrawal for 48 h. Immunofluorescence staining of RM11A cells cultured in the presence (**B** and **D**) and absence (**C** and **E**) of Dox for cyclin D1 showing that more cells in the +Dox treatment express cyclin D1 and most of this protein is localized to the nucleus. Bar, 200 μm (**B** and **C**); 50 μm (**C** and **E**). **F.** Knockdown of IGF-IR using RNA interference results in a decrease in cyclin D1 protein as determined by Western blot.

proliferative/survival advantage in serum-deprived conditions compared with RM11A cells with low IGF-IR levels. Treatment of RM11A cells containing high levels of IGF-IR with exogenous IGF-I, IGF-II, or serum did not enhance the proliferative/survival advantage. The ability of RM11A cells expressing low levels of IGF-IR to respond to IGF stimulation is most likely due to activation of the endogenous murine IGF-IR. It is possible that IGF-IR overexpression results in constitutive activation of the IGF-IR independent of the presence of ligands. Autoactivation of IGF-IR, when expressed at sufficient levels, has previously been shown in IGF-IR null cells that have been transfected with different levels of IGF-IR (38, 39). Ligand-independent activation of IGF-IR would have an important effect on the design of therapeutic molecules targeting the IGF-IR. For instance, inactivation of IGF-IR signaling with the use of small-molecule inhibitors that target the catalytic sites of IGF-IR may prove more effective than interfering with the ligand-receptor interaction. Alternatively, the increase in cell proliferation or survival observed in RM11A cells overexpressing IGF-IR under serum-deprived conditions may be due to autocrine production of IGF-I and/or IGF-II leading to IGF-IR activation. Initial experiments involving treatment of RM11A cells with IGF binding protein 1 in the presence of Dox did not support the presence of an IGF/IGF-IR autocrine loop.

Following isolation of RM11A cells, it was observed that removal of Dox induced a morphologic change in RM11A cells. In the presence of Dox, RM11A cells maintain a spindle-like morphology similar to fibroblasts. Culturing RM11A cells in the absence of Dox for more than 48 hours induced a transition to a more cuboidal shape commonly associated with

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epithelial cells. Interestingly, reversion of the cuboidal morphology could not be achieved through the readdition of Dox. Moreover, Dox no longer induced IGF-IR expression in cells cultured in Dox-free media for at least 48 hours. Because the removal of Dox induced irreversible changes in cell morphology and IGF-IR expression, it was possible that RM11A cells actually contained two populations of cells: one that was dependent on high IGF-IR for survival and one population that was IGF-IR independent. If two different populations of cells existed, removal of Dox would result in the death of the IGF-IR–dependent cells, leaving a homogenous population of IGF-IR–independent cells behind. Removal of Dox from the culture media of RM11A cells did not produce a visible decrease in cell number or an increase in the number of dead cells in the culture media. Moreover, there was no increase in the number of apoptotic cells following Dox removal as determined by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling analysis (data not shown). Alternatively, overexpression of IGF-IR may induce a mesenchymal-like phenotype. A recent report has shown that overexpression of a constitutively active IGF-IR in immortalized human mammary epithelial cells and in mammary epithelium of transgenic mice resulted in cellular transformation that was associated with an epithelial-to-mesenchymal transition (40). However, down-regulation of IGF-IR in RM11A cells following Dox withdrawal failed to induce protein expression of a number of epithelial cell markers such as E-cadherin, cytokeratin 8, or cytokeratin 18. Therefore, the decrease in IGF-IR expression does not convert RM11A cells from mesenchymal cells to epithelial cells but rather may alter cytoskeleton reorganization, a characteristic that is currently under investigation.

Another possible explanation for the nonreversible change in IGF-IR transgene expression and alterations in cell shape may be due to epigenetic alterations. DNA methylation, which involves the methylation of cytosines, regulates gene expression, maintains chromosomal integrity, and is essential for

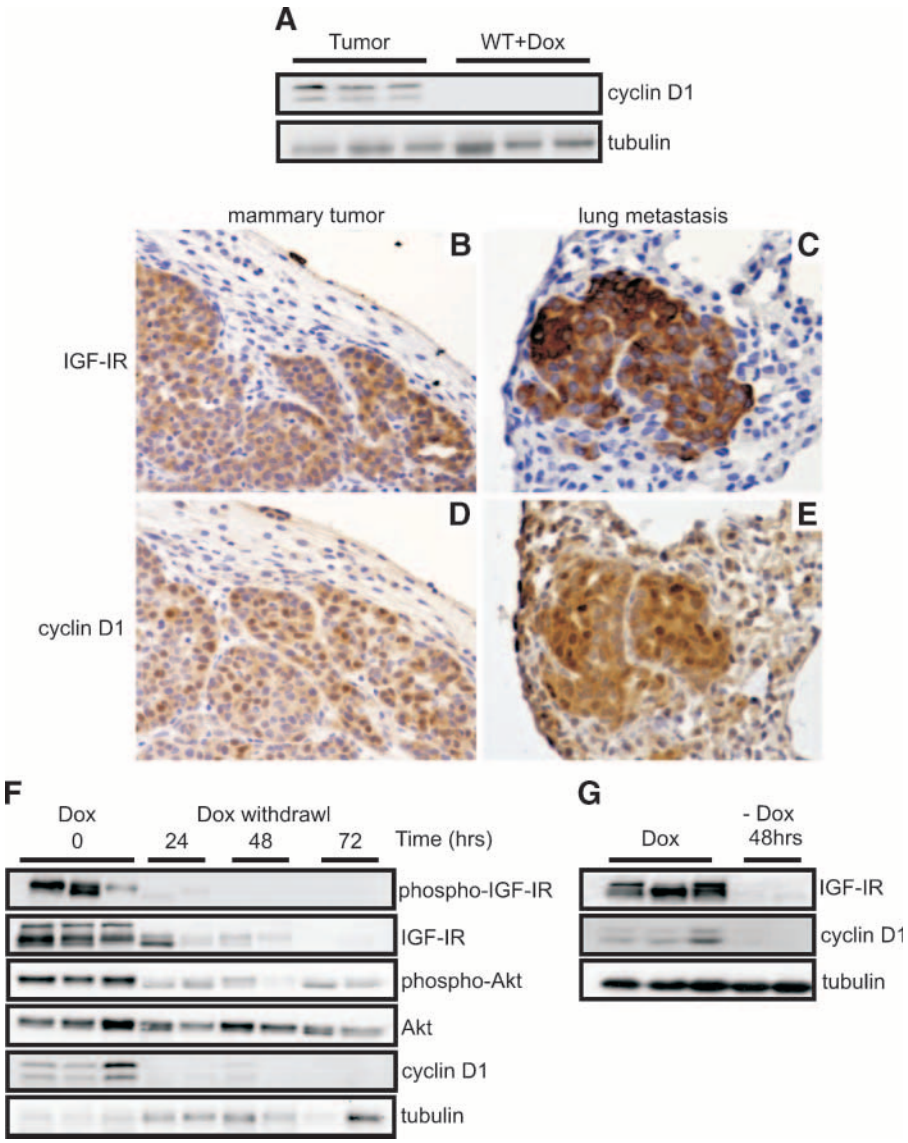

FIGURE 5. Overexpression of IGF-IR regulates cyclin D1 *in vivo*. **A.** Western blot analyses of cyclin D1 protein in MTB-IGFIR primary mammary tumors and age-matched Dox-treated wild-type mammary glands. Immunohistochemistry for IGF-IR (**B** and **C**) and cyclin D1 (**D** and **E**) in an MTB-IGFIR primary mammary tumor (**B** and **D**) and a lung metastasis (**C** and **E**). **F.** Western blot analyses of phosphorylated IGF-IR (Tyr^{1131}), IGF-IR, phosphorylated Akt (Ser^{473}), total Akt, and cyclin D1 in MTB-IGFIR mammary tumors obtained from mice treated with Dox (*lanes 1-3*) or removed from Dox for 24, 48, and 72 h (*lanes 4-12*). **G.** Western blot analyses of IGF-IR and cyclin D1 in RM11A tumors obtained from mice treated with Dox (*lanes 1-3*) or 48 h after Dox withdrawal (*lanes 4-5*). Tubulin served as a loading control for total protein.

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normal development (reviewed in ref. 41). Therefore, loss of IGF-IR transgene expression may alter the DNA methylation pattern of promoter regions, including the promoter of the IGF-IR transgene, resulting in differential gene expression and silencing of the IGF-IR transgene.

Tumorigenecity of RM11A cells was determined through the injections of the cells into the mammary glands of wild-type mice. Three conditions were examined: (*a*) RM11A cells injected into mice that received Dox supplementation; (*b*) RM11A cells injected into mice that did not receive Dox supplementation; and (*c*) KR cells (RM11A cells cultured in the absence of Dox) injected into mice that did not receive Dox supplementation. These conditions were established to determine whether IGF-IR overexpression in RM11A cells was necessary for tumor growth. Although continual overexpression of IGF-IR (RM11A cells injected into mice receiving Dox supplementation) produced tumors significantly faster than either the RM11A cells or KR cells injected into mice that did not receive Dox supplementation (basal IGF-IR expression), the cells with basal IGF-IR expression still eventually produced mammary tumors. A possible explanation for mammary tumor formation in the absence of Dox supplementation is that RM11A cells were isolated from an established mammary tumor. During the development of this mammary tumor, IGF-IR overexpression presumably increased cell proliferation and decreased apoptosis. Loss of cell cycle control and apoptosis would provide a genetically unstable environment with an increased mutation rate within the cells. A small subset of cells may have acquired mutations that allowed them to become independent of IGF-IR signaling. Thus, a subset of cells within the population of either RM11A cells or KR cells may have acquired IGF-IR independence and can thus form a tumor in the absence of Dox supplementation. The delayed tumor onset of the KR cells would also be consistent with this theory as the tumor would originate from a smaller population of cells and thus require additional population doublings before becoming palpable. Also consistent with this theory is the finding that on Dox withdrawal, most of the mammary tumors that develop in MTB-IGFIR transgenic mice or following injection of RM11A cells initially regress; however, some of the tumors recur in the absence of Dox or elevated IGF-IR expression.[1] Therefore, the genetically unstable environment of the tumor is conducive to the acquisition of mutations, some of which may render cells independent of IGF-IR signaling.

To identify genes regulated by IGF-IR that may contribute to the enhanced growth and survival of tumor cells that we observed *in vitro* and *in vivo*, microarray analysis was done on RM11A cells cultured in the presence or absence of Dox. This analysis identified 278 differentially expressed genes that were altered at least 2-fold at a significance level of $P < 0.005$. From the 114 up-regulated and 164 down-regulated genes, 6 genes of interest were chosen; 4 were up-regulated genes (*cyclin D1, Etv-1, Etv-5*, and *Notch 4*) and 2 were down-regulated genes (*WISP1* and *WISP2*), which were confirmed to be differentially expressed by quantitative real-time PCR or Western blotting.

[1] In preparation.

Because cyclin D1 is frequently overexpressed in human breast cancer (42-45), can by itself promote mammary tumor formation in mice (46), and is necessary for mammary tumor induction in ErbB2 transgenic mice (47), further studies were done to examine the regulation of cyclin D1 by IGF-IR. Cyclin D1 promotes proliferation by binding to cyclin-dependent kinase 4 or 6 and translocating to the nucleus where it phosphorylates retinoblastoma, resulting in the release of E2F transcription factors, which activate genes essential for cell cycle progression. Overexpression of IGF-IR seems to regulate cyclin D1 at the transcriptional level (increased gene expression on DNA microarray). Interestingly, transcriptional regulation of cyclin D1 following IGF-IR activation was recently found to be associated with nuclear translocation of the major IGF-IR docking protein insulin receptor substrate 1, followed by activation of the cyclin D1 promoter (48). Thus, IGF-IR may regulate cyclin D1 levels through insulin receptor substrate-1 activation.

Overexpression of IGF-IR may also regulate cyclin D1 at the posttranscriptional level through enhanced activation of Akt. Akt can promote cyclin D1 nuclear accumulation and protein stability by inhibiting glycogen synthase kinase-3β (12-14). To evaluate the physiologic relevance of cyclin D1 regulation by IGF-IR, cyclin D1 levels were examined following manipulation of IGF-IR expression *in vivo*. Mammary tumors that arose in the MTB-IGFIR transgenic mice due to elevated IGF-IR expression had higher levels of cyclin D1 compared with normal mammary glands (basal levels of IGF-IR).

Using the fact that the IGF-IR transgene is dependent on the presence of Dox, cyclin D1 levels were evaluated in MTB-IGFIR mammary tumors following Dox withdrawal. Removal of Dox resulted in a time-dependent decrease in IGF-IR and cyclin D1 levels. Moreover, Akt phosphorylation was also suppressed following Dox withdrawal in MTB-IGFIR mammary tumors. A decrease in IGF-IR and cyclin D1 levels was also observed in RM11A-injected tumors following Dox withdrawal. Thus, the *in vivo* findings support those obtained *in vitro* that cyclin D1 levels are regulated by IGF-IR and this effect is potentially mediated in part through the Akt signaling pathway.

In summary, this article describes the characterization of a primary mammary tumor cell line where IGF-IR expression can be regulated in a Dox-inducible manner. These cells provide a relatively rapid *in vitro/in vivo* system to examine the function of IGF-IR in mammary tumorigenesis. Further characterization of these primary mammary tumor cells should enhance our understanding of the function of IGF-IR in mammary tumorigenesis and provide insight into the usefulness of IGF-IR, or a downstream molecule, as a therapeutic target for breast cancer.

Materials and Methods

Generation of Primary Murine Mammary Tumor Cells

A piece of a mammary tumor was removed from an MTB-IGFIR1 transgenic mouse (37) and was placed in DMEM (Life Technologies, Inc.) containing 10% tetracycline-free fetal bovine serum (Clontech), 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, 4 mmol/L glutamine, 2 mmol/L hydrocortisone, 5 μg/mL estrogen, 5 μg/mL prolactin, 10 μg/mL

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epidermal growth factor, 10 μg/mL insulin, and 10 μg/mL Dox (Sigma) and manually disaggregated with sterile scalpel blades. The pieces were then dispersed in a six-well plate and maintained in fully supplemented media containing Dox. Cell colonies displaying different morphologies were removed with a rubber policeman and placed individually into new six-well plates in fully supplemented media containing Dox. Cells were expanded and either used for subsequent experiments or frozen and stored in liquid nitrogen.

Histology, Immunohistochemistry, and Immunofluorescence

For H&E staining, cells were grown on sterile glass coverslips placed in wells of a six-well plate for ~48 h. Cells were then fixed for 1 h at room temperature in 10% buffered formalin and then washed with PBS. Hematoxylin was added directly to the well for 1 min and then washed in water. Eosin was then added to the well for 30 s and washed with 95% ethanol. The coverslips were then incubated in 100% ethanol followed by xylene before mounting the coverslips on glass slides using Permount.

Immunohistochemistry and immunofluorescence were done as previously described (49). Primary antibodies were used at a dilution of 1:100 in 5% bovine serum albumin in PBS containing 0.1% Triton X-100. The antibodies used were anti–cytokeratin 5 and anti–cytokeratin 14 (Abcam); anti–cytokeratin 8 (Fitzgerald Industries International, Inc.); anti–cytokeratin 18 (Research Diagnostics, Inc.); anti–IGF-IR (R&D Systems, Inc.); and anti–phospho-IGFIR (Tyr^{1131}/Tyr^{1146}), anti–phospho-Akt (Ser^{473}), and cyclin D1 (Cell Signaling Technology).

Western Blotting

Western blotting was done as previously described (50). Primary antibodies for IGF-IR (R&D Systems), phospho-IGFIR (Tyr^{1131}/Tyr^{1146}), phospho-Akt (Ser^{473}), phospho-Erk1/Erk2 (Thr^{202}/Tyr^{204}), cyclin D1 (Cell Signaling Technology), and tubulin (Santa Cruz Technologies) were used at a dilution of 1:1,000 in 5% bovine serum albumin in TBST. Protein levels were detected with a 1:2,000 dilution of the appropriate secondary antibody (Cell Signaling Technology) and Western Lightning Chemiluminescence substrate (Perkin-Elmer). Images were captured and quantified on a FluorChem 9900 Gel Documentation Systems (Alpha Innotech) imaging system.

MTT Survival Assay

RM11A cells were seeded in growth media containing 10% fetal bovine serum at a density of 5,000 per well in a 96-well plate in the presence or absence of 10 μg/mL Dox. To assess cell survival in the absence of exogenous growth factors, cells were seeded in media containing 10% fetal bovine serum to allow for cell attachment and were then switched to serumfree media with or without Dox supplementation. After 72 h, 5 mg/mL MTT was added to each well for 1 h, cells were then lysed, and absorbance was read at 570 nm. For IGF-II treatments, RM11A cells were serum starved for 24 h in the presence and absence of Dox and were then treated in a dose-dependent manner with IGF-II (5-80 ng/mL) for a 3-d period. All experiments were done in triplicate.

Mammary Gland Injection

Wild-type FVB mice were anesthetized with isofluorane, and a small T-shaped incision was made in the abdomen to expose the 4th inguinal mammary glands. Approximately 5×10^5 cells in 10 μL of sterile PBS were then injected into the mammary fat pad and the incisions were closed with surgical staples. Mice were then given drinking water supplemented with 2 mg/mL Dox and 5% sucrose to induce IGF-IR expression or given normal drinking water without Dox supplementation.

RNA Extraction and Affymetrix DNA Microarrays

Subconfluent RM11A cells were cultured in the presence of 10 μg/mL Dox or in the absence of Dox for 48 h. RNA was extracted using the ArrayGrade Total RNA Isolation Kit following the manufacturer's instructions (SuperArray). Total RNA from four independent sets of RM11A cells (presence and absence of Dox) was sent to the Microarray Centre at the University Health Network, Toronto, Ontario, Canada. RNA quality was confirmed by an Agilent BioAnalyzer before cRNA generation and hybridization using the Affymetrix GeneChip Mouse Genome 430 2.0. Data were analyzed using a PLIER algorithm and transformed to a log scale using ArrayAssist software (Stratagene). An unpaired t test was used to identify genes differentially expressed.

Real-time PCR

First-strand cDNA was synthesized from 1 μg of total RNA using oligo-$(dT)_{12\text{-}18}$ primers and SuperScript II Reverse Transcriptase following the manufacturer's protocols (Invitrogen). Real-time PCR reactions were done in a 20-μL reaction mixture consisting of 1 μL of cDNA, 1× Platinum Quantitative PCR Supermix-UDG with bovine serum albumin (Invitrogen), and 0.5 μmol/L of each primer. Quantification was done using LightCycler technology and Relative Quantification software (Roche Diagnostics). Primers specific for mouse *ETV-1, ETV-5, Notch 4, WISP1, WISP2*, and *β-actin* were obtained from SuperArray.

Statistics

All values are presented as mean ± SE. Statistical significance was determined with Student's t test for the comparison of two values, ANOVA followed by Dunnett's test to compare multiple treatments to an individual control, or Tukey's test to compare all samples within an experiment. The log-rank test was used to determine whether the tumor-free survival curves differed significantly.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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