

# Transgenic overexpression of IGF-II induces spontaneous lung tumors: a model for human lung adenocarcinoma

Roger A Moorehead<sup>1,2,3</sup>, Otto H Sanchez<sup>1,3</sup>, R Mitchell Baldwin<sup>1,2</sup> and Rama Khokha<sup>1</sup>

<sup>1</sup>Ontario Cancer Institute, University of Toronto, Toronto, Ontario, Canada, M5G 2M9

Elevated levels of insulin-like growth factor (IGF)-II are associated with a poor prognosis in human pulmonary adenocarcinoma; however, a causal role for IGF-II in pulmonary adenocarcinoma has not been demonstrated. Here, we show that transgenic overexpression of IGF-II in lung epithelium induces lung tumors in 69% of mice older than 18 months of age. These tumors displayed morphological characteristics of human pulmonary adenocarcinoma such as their epithelial origin, tubulo-acinar architecture and expression of TTF-1, SP-B and proSP-C. Examination of signaling molecules downstream of the IGF-IR showed the activation of either the Erk1/Erk2 or p38 MAPK pathways, but not both, within the lung tumors. Notably, all lung tumors contained high levels of phosphorylated CREB, suggesting that both the Erk1/Erk2 and p38 MAPK pathways converged on this transcription factor. Moreover, IGF-II induced proliferation and CREB phosphorylation in human lung cancer cell lines, suggesting that IGF-II and CREB also contribute to the growth of human lung tumors. Thus, IGF-II is an important genetic factor in the development of lung tumorigenesis, in which activation of CREB is a ubiquitous event. The MMTV-IGF-II transgenic mice provide a critical model for elucidating the role of IGF-II in this fatal human disease.

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The insulin-like growth factor (IGF) system has been implicated in human lung cancer. Many studies have shown that IGFs and the type I IGF receptor (IGF-IR) are present in human lung tumor tissue (Minuto *et al.*, 1986, 1988; Jaques *et al.*, 1988; Nakanishi *et al.*, 1988; Rotsch *et al.*, 1992; Kaiser *et al.*, 1993; Schardt *et al.*,

1993). A case-control analysis of plasma IGF levels from lung cancer patients revealed that elevated plasma IGF-I was associated with an increased risk of lung cancer (Yu *et al.*, 1999). An increased risk of lung cancer is also associated with reduced IGF binding protein (IGFBP)-3 levels (Yu *et al.*, 1999), which are generally thought to regulate the interaction of IGFs with their receptors and thus moderate the physiologic effects of IGFs (Wetterau *et al.*, 1999; Grimberg and Cohen, 2000; Schneider *et al.*, 2000). Prospective studies, however, have failed to demonstrate a correlation between plasma IGF-I and lung cancer risk (Lukanova *et al.*, 2001; London *et al.*, 2002). IGF-II was not associated with increased lung cancer risk in the above case-control study (Yu *et al.*, 1999). This mitogen appears to act in an autocrine and paracrine manner rather than in an endocrine manner in tumorigenesis (Toretzky and Helman, 1996), thus suggesting that tissue-specific levels of IGF-II are more relevant than plasma IGF-II levels. Consistent with this theory, high tumor IGF-II immunoreactivity has been significantly correlated with decreased survival in patients with pulmonary adenocarcinoma (Takanami *et al.*, 1996). Overall, the exact role of the IGF system in human lung cancer is yet to be established.

Extensive literature from cell culture systems convincingly demonstrates that IGFs are vital mitogens for lung tumor cells. Administration of IGF-I or IGF-II induces proliferation in human small cell and nonsmall cell lung cancer cell lines (Rotsch *et al.*, 1992; Zia *et al.*, 1996; Nakanishi *et al.*, 1988) and the proliferative effects of the IGFs were inhibited by the anti-IGF-IR antibody,  $\alpha$ -IR3 (Zia *et al.*, 1996). Alpha-IR3 was also capable of inhibiting the growth of human lung tumors transplanted into nude mice (Zia *et al.*, 1996). In addition, antisense-mediated reduction of IGF-IR expression in lung cancer cell lines inhibited clonogenic capacity and growth of these cell lines in nude mice (Lee *et al.*, 1996). Thus, the IGF system clearly promotes lung tumor cell proliferation *in vitro*.

To help resolve the apparent disparity between the clinical- and culture-based studies and determine the importance of IGF-II *in vivo*, an animal model is required. Here we demonstrate that overexpression of IGF-II is sufficient to induce lung cancer *in vivo* and provide the initial characterization of a valuable model of IGF-II-induced pulmonary adenocarcinoma.

\*Correspondence: R Khokha, Department of Medical Biophysics, Ontario Cancer Institute, 610 University Avenue, Toronto, Ontario, Canada, M5G 2M9; E-mail: rkhokha@oci.utoronto.ca

<sup>2</sup>Current address: Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

<sup>3</sup>These authors contributed equally to this manuscript  
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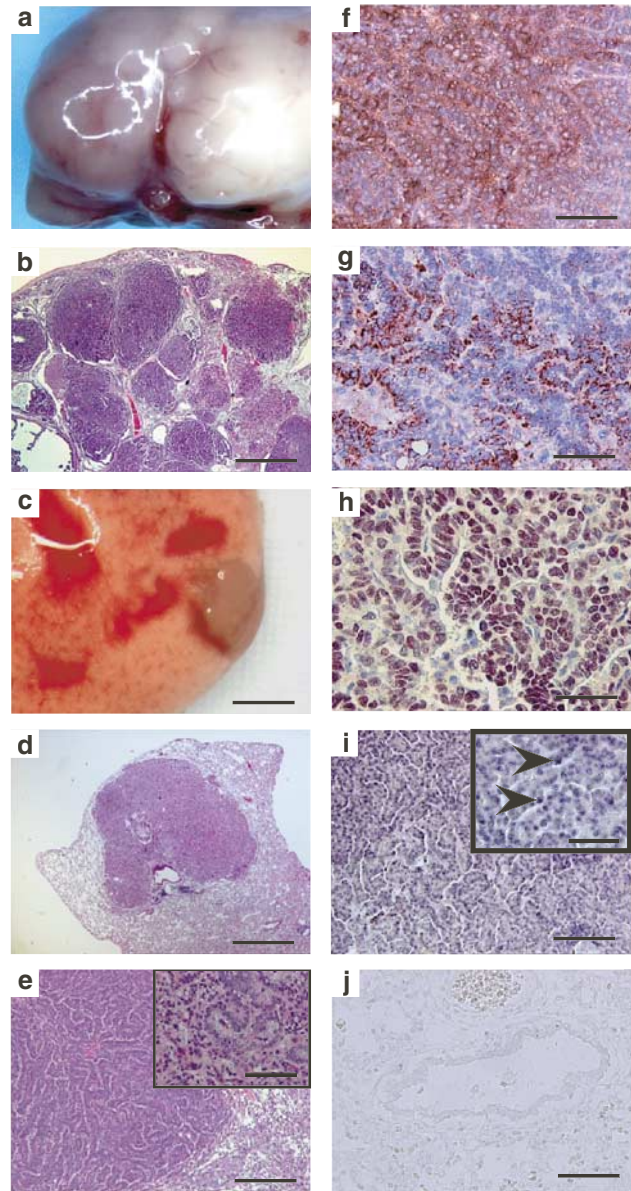
**Table 1** Lung tumor incidence in MMTV-IGF-II transgenic mice

	Number of tumors in mice > 6 months (%)	Number of tumors in mice > 18 months (%)
Male	7/33 (21)	5/8 (62)
Female	10/44 (23)	6/8 (75)
Total	17/77 (22)	11/16 (69)

Examination of MMTV-IGF-II transgenic mice (Moorehead *et al.*, 2001) revealed that lung tumors frequently developed in these mice. Lung tumors were found in MMTV-IGF-II transgenic mice as early as 6 months of age, and in mice older than 18 months, the tumor incidence reached 69% (Table 1). The tumors observed in these transgenic mice displayed morphological characteristics similar to those observed in human pulmonary adenocarcinoma. Specifically, compromised lungs showed one or multiple subpleural well-circumscribed nodules that histologically were solid epithelial tumors, not encapsulated, tubulo-acinar in nature, without preservation of alveolar architecture. Cytologically, tumors were composed of cuboidal or columnar cells, often showing multi-vacuolated cytoplasm (Figure 1a–e). There was no evidence of metastatic lesions. According to Rehm *et al.* (1994) these tumors can be histogenetically classified as solid bronchiolo-alveolar adenomas or adenocarcinomas, a classification supported by their peripheral subpleural location and histological and cytological features. Other tumors also developed in these mice including lymphomas, uterine/cervix and ovarian, harderian, and bulbourethral, and rarely mammary tumors. Wild-type controls never developed tumors.

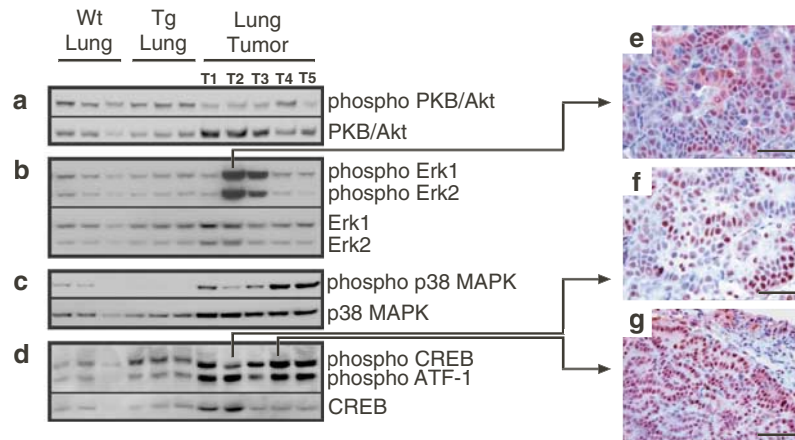
Tumor cells expressed high levels of prosurfactant protein C (proSP-C) (Figure 1f), surfactant protein B (SP-B) (Figure 1g) and thyroid transcription factor (TTF)-1 (Figure 1h), while immunoreactivity for these proteins was considerably lower in normal lung epithelium (data not shown). ProSP-C, SP-B and TTF-1 are normally expressed at high levels in fetal lung epithelium, but declined in adult lung epithelium (Mendelson, 2000). These proteins become re-expressed in pulmonary epithelial tumors (Di Loreto *et al.*, 1997; Kaufmann and Dietel, 2000; Ordonez, 2000). SP-B is not expressed in squamous cell carcinoma, large cell carcinomas of the lung or nonpulmonary adenocarcinomas (Chailley-Heu *et al.*, 2001). The expression of pro-SPC and TTF-1 is also restricted primarily to type II alveolar cells and Clara cells. Therefore, detection of these three markers indicated that the tumor cells arose from Clara cells or type II epithelial cells (Mendelson, 2000; Clark *et al.*, 2001).

To explore the mechanism of IGF-II-induced lung tumorigenesis, signaling pathways downstream of the IGF-IR were investigated. We first examined the PKB/Akt pathway since we had previously shown that the phosphorylation status of this molecule is altered in the mammary tissue of the MMTV-IGF-II transgenic mice (Moorehead *et al.*, 2001). Further, activation of PKB/



**Figure 1** Representative macroscopic (a, c) and histologic sections (b, d, e) of lung tumors found in MMTV-IGF-II transgenic mice. Immunohistochemical detection of proSP-C (f), SP-B (g), and TTF-1 proteins (h). Histology and immunohistochemistry were performed on formalin-fixed tissue as previously described (Fata *et al.*, 1999; Moorehead *et al.*, 2001) using antibodies for proSP-C and SP-B (Research Diagnostics Inc., Flanders, NJ, USA) at a dilution of 1:500 and 1:100, respectively. The TTF-1 antibody (Dako Diagnostics, Mississauga, Canada) was used at a dilution of 1:100. *In situ* hybridization for IGF-II mRNA in (i) lung tumor and (j) wild-type lung tissue. DIG *in situ* hybridization was performed as previously described (Harvey *et al.*, 1995; Inderdeo *et al.*, 1996; Fata *et al.*, 1999) with the following modifications. Specifically, tissue was fixed in 4% (w/v) buffered formalin overnight, tissue sections were treated with 20 µg/ml of Proteinase K for 20 min at room temperature and sections were washed three times in 4 × SSC for 15 min each at room temperature. IGF-II positive cells stain a purplish blue. Scale bars (a, c) 625 µm, (b, d) 200 µm, (e, i, j) 50 µm and inset in e, i, 20 µm. Scale bars for images 200 µm, inset, 50 µm

Akt has been observed in a number of tumor types as this protein promotes proliferation and inhibits apoptosis (Datta *et al.*, 1999; Brazil and Hemmings, 2001;



**Figure 2** Western analysis of Akt and phosphorylated Akt (Ser473) (a), Erk1/Erk2 and phosphorylated Erk1/Erk2 (Thr202/Tyr204) (b), p38 MAPK and phosphorylated p38 MAPK (Thr180/Tyr182) (c), and, CREB and phosphorylated CREB (Ser133) (d) in wild-type lung, transgenic non tumor-bearing lung and MMTV-IGF-II lung tumors. Protein isolation and Western blotting was performed as described (Moorehead *et al.*, 2001). All primary antibodies (Cell Signaling Technologies, Beverly, MA, USA) were used at a dilution of 1:1000 in 5% skim milk in Tris-buffered saline containing 1% Tween 20 and membranes were incubated with the appropriate primary antibody overnight at 4°C. Sequential probing of membranes was performed after stripping in 62.5 mM Tris pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS for 30 min at 50°C. Immunohistochemistry of phosphorylated Erk1/Erk2 (e) and phosphorylated CREB (f, g) in lung tumor T2 (e, f) and T4 (g). Immunohistochemistry was performed as described in Figure 1 using a 1:100 dilution of the primary antibodies

Lawlor and Alessi, 2001; Scheid and Woodgett, 2001). The levels of phosphorylated PKB/Akt were significantly reduced in the tumor tissue compared to wild-type tissue, whereas there was no significant difference between wild-type tissue and nontumor-bearing transgenic lung tissue (Figure 2a). This suggested that enhanced PKB/Akt activation is not a critical element in the development of MMTV-IGF-II lung adenocarcinoma.

Next, we investigated other proliferative pathways downstream of the IGF-IR, namely Erk1/Erk2, p38 MAPK and JNK/SAPK. Two of the five lung tumors (T2 and T3) had elevated levels of phosphorylated Erk1/Erk2 (Figure 2b). Interestingly, the other three tumors (T1, T4 and T5) had elevated levels of phosphorylated p38 MAPK (Figure 2c). The levels of phosphorylated JNK/SAPK were barely detectable and were not different between lung tumor tissue and wild-type lungs (data not shown). We then examined two downstream targets common to these signaling proteins, namely Elk-1 and CREB. Phosphorylated Elk-1 was undetectable in these samples. The levels of phosphorylated CREB were notably elevated in all five of the lung tumor samples compared to wild-type tissue (Figure 2d). The antiphospho-CREB antibody also recognizes phosphorylated ATF-1, which was also elevated in these samples (Figure 2d). The level of phosphorylated ATF-2, a transcription factor downstream of p38 MAPK and JNK/SAPK, was not increased in the tumor tissue (data not shown).

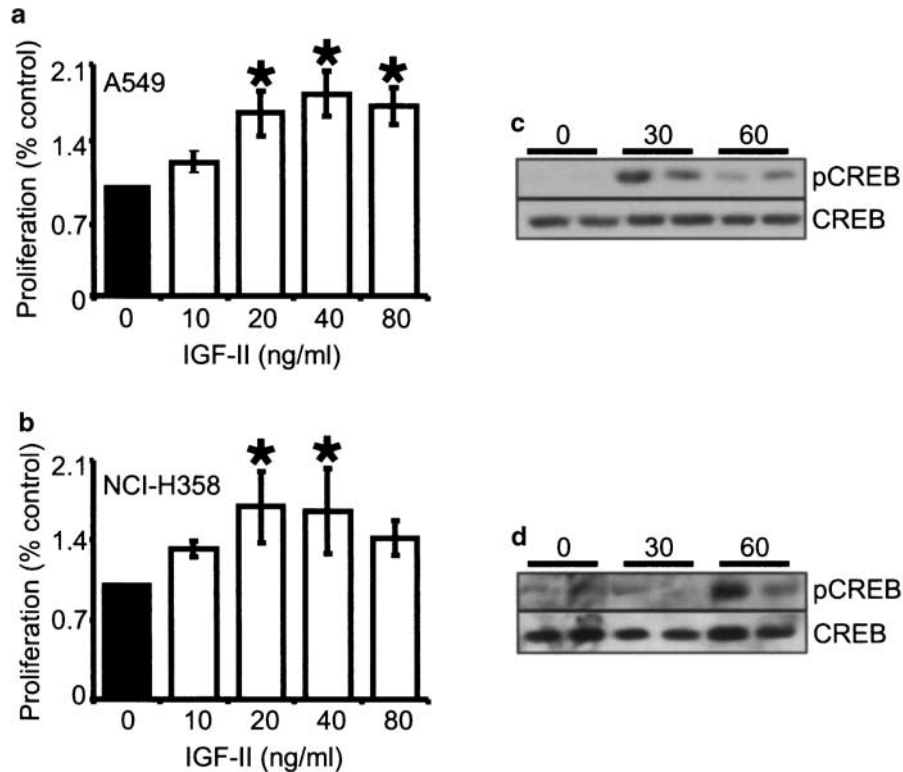
Immunohistochemistry was performed to identify the cellular localization of signaling molecules involved. Lung tumors that had high levels of phosphorylated Erk1/Erk2 by Western analysis (T2 and T3) showed heterogeneous nuclear immunostaining in tumor cells that was most predominant at the tumor periphery (Figure 2e). Phosphorylated CREB was also detected in

tumor cells and displayed a heterogeneous nuclear staining with high levels at the tumor periphery (Figure 2f, g). As expected from Western analysis, phosphorylated CREB was evident in all lung tumors (T1–T5). Altogether, these data indicate that although different signaling pathways (Erks and p38 MAPK) may be utilized by IGF-II in individual tumors, convergence on particular transcription factors (CREB, ATF-1) may represent a more important event.

Next, we investigated whether CREB could be activated in human lung cancer cell lines in response to IGF-II administration. Using human lung tumor cell lines A549 and NCI-H358, we first tested the proliferative response of these cells to IGF-II. Recombinant IGF-II induced a dose-dependent increase in proliferation in both cell lines (Figure 3a, b). This increase in proliferation was solely because of the IGF-II treatment, as all experiments were performed in serum-free media. Western blot analysis demonstrated that IGF-II administration stimulated CREB phosphorylation in both cell lines within 60 min (Figure 3c, d). Thus, IGF-II stimulated human lung tumor cell proliferation, concomitant with CREB activation. Whether CREB plays a causal role in lung cancer remains open and needs further investigation.

The activation of CREB is consistent with our findings of TTF-1, SP-B and proSP-C overexpression in the tumor cells. CREB is a transcription factor that has been implicated in regulating TTF-1 expression as well as the expression of SP-B (Yan and Whitsett, 1997; Nguyen *et al.*, 2000). Since TTF-1 induces the expression of the surfactant proteins (Yan and Whitsett, 1997; Mendelson, 2000), one would expect high levels of TTF-1 and surfactant protein in cells containing activated CREB.

This is the first study to demonstrate the importance of CREB in lung tumorigenesis. Although CREB is



**Figure 3** Proliferation induced by recombinant IGF-II in (a) A549 and (b) NCI-H358 human lung tumor cells. Cells were seeded in serum-free media at a density of 4000 cells per well in 96-well plates and were allowed to adhere for 4 h. After the attachment period, cells were treated with recombinant IGF-II at the indicated concentration. IGF-II administration was repeated daily for 4 days. A volume of 25  $\mu$ l of MTT (5 mg/ml) was then added to each well. Following a 3 h incubation period, cells were lysed (20% SDS, 40% dimethylformamide) and the amount of MTT converted to a purple precipitate was quantified at 570 nm using a microplate reader. Western analysis of phosphorylated CREB in (c) A549 and (d) NCI-H358 cells following treatment with 20 ng/ml of IGF-II in serum-free media. Western analysis was performed as described in Figure 2. \* $P < 0.01$  as determined by Dunnett's test

activated in lungs in response to insults such as hemorrhage (Shenkar and Abraham, 1999; Abraham *et al.*, 2001; Shenkar *et al.*, 2001), endotoxemia (Shenkar and Abraham, 1999; Abraham *et al.*, 2001; Shenkar *et al.*, 2001), hyperoxia (George *et al.*, 1999) and metal exposure (Samet *et al.*, 1998) and is important for normal lung development (Rudolph *et al.*, 1998), its role in lung tumorigenesis has not been reported.

In summary, our study demonstrates that IGF-II overexpression is sufficient to cause lung tumorigenesis *in vivo*. Lung tumor development in the MMTV-IGF-II appears dependent on the constitutive activation of the

transcription factor CREB and upstream signaling molecules Erk1/Erk2 and p38 MAPK. Concentrations of IGF-II that promote proliferation in human lung tumor cell lines and also induce CREB phosphorylation. The MMTV-IGF-II transgenic mice provide a valuable model of pulmonary adenocarcinoma and for elucidating the role of IGF-II in this disease. Further, our data suggest that IGF-II expression and/or activation of CREB represent important biological markers of both human and murine pulmonary adenocarcinoma and thus, potential therapeutic targets for the disease.

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