Integrin α11 regulates IGF2 expression in fibroblasts to enhance tumorigenicity of human non-small-cell lung cancer cells


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April 2, 2007

Integrin α11 (ITGA11/α11) is localized to stromal fibroblasts and commonly overexpressed in non-small-cell lung carcinoma (NSCLC). We hypothesized that stromal α11 could be important for the tumorigenicity of NSCLC cells. SV40 immortalized mouse embryonic fibroblasts established from wild-type (WT) and Itga11-deficient (KO) mice were tested for their tumorigenicity in immune-deficient mice when implanted alone or coimplanted with the A549 human lung adenocarcinoma cells. A549 coimplanted with the fibroblasts showed a markedly enhanced tumor growth rate compared with A549, WT, or KO, which alone formed only small tumors. Importantly, the growth was significantly greater for A549 + WT compared with A549 + KO tumors. Reexpression of human α11 cDNA in KO cells rescued a tumor growth rate that is comparable with the A549 + WT tumors. These findings were validated in two other NSCLC cell lines, NCI-H460 and NCI-H520. Gene expression profiling indicated that IGF2 mRNA expression level was >200 times lower in A549 + KO compared with A549 + WT tumors. Stable short-hairpin RNA (shRNA) down-regulation of IGF2 in WT (WTshIFG2) fibroblasts resulted in a decreased growth rate of A549+WTshIFG2, compared with A549 + WT tumors. The results indicate that α11 is an important stromal factor in NSCLC and propose a paradigm for carcinoma–stromal interaction indirectly through interaction between the matrix collagen and stromal fibroblasts to stimulate cancer cell growth.

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here is abundant evidence that tumor–stromal cell interaction plays a critical role in tumor growth, invasion, metastases, angiogenesis, and chemoresistance (1–5). Factors derived from the carcinoma-associated fibroblast (CAF) or activated fibroblast contribute to the transformation of immortalized epithelial cells (6, 7) and enhance the tumorigenicity of cancer cells (8). Irradiated fibroblasts increase the incidence of breast cancer compared with the nonirradiated fibroblasts when they are cotransplanted with untransformed mammary epithelia into the mammary fat pad (9). These studies demonstrated that changes in stromal fibroblasts might contribute to epithelial transformation and tumorigenicity possibly through paracrine secretion of growth factors. However, the precise mechanisms underlying communication among stromal fibroblasts, tumor cells, and the environment have not been completely defined despite implication of some growth factor loops such as hepatocyte growth factor/Met (10, 11), TGF-β1 (2), IL-8 (12), and stromal cell-derived factor-1 (13).

Integrins are a large family of transmembrane receptors that mediate physical interactions between cells and extracellular matrix protein collagens. There are at least 24 different integrins that may be formed as heterodimers of 18α and 8β subunits; each integrin heterodimer demonstrates distinct ligand binding and signaling properties (14). Integrins play crucial roles in diverse cellular and developmental processes, including cell growth, differentiation, and survival, as well as carcinogenesis, cancer cell invasion, and metastases (15). The α11 integrin subunit was first identified as a major integrin in cultured skeletal muscle cells (16, 17). It contains a transmembrane domain and a 24-aa cytoplasmic tail. α11 dimerizes with β1 to form one of the four collagen receptors (14). In human embryos, α11 expression is localized to the mesenchyme. α11-deficient mice are viable and fertile, but display dwarfism and increased mortality (18). We have previously used a representational difference analysis method to identify α11 as a commonly overexpressed gene in primary non-small-cell lung carcinoma (NSCLC) (19). We report here that α11 plays an important role in the ability of fibroblasts to promote the growth of NSCLC cells in vivo, and such activity is partially mediated by its regulation of IGF2 expression.

Results

α11 Protein Is Overexpressed Mainly in the Stroma of Human NSCLC. The α11 mRNA was overexpressed in both lung adenocarcinoma and squamous cell carcinoma (19). This differential expression was confirmed at the protein level by Western blot on three of four unselected paired primary NSCLC and corresponding nonneoplastic lung tissue (Fig. 1A). Laser-capture microdissection demonstrated that α11 mRNA expression was two to eight times higher in the stromal tissue compared with the tumor cells (Fig. 1B), despite low expression in the epithelial tumor cells. Immunofluorescence microscopy showed that the α11 protein was mainly localized in the stroma (Fig. 1D) and was scanty present in the control normal lung sections (Fig. 1E).

α11 Overexpression in CAF. To demonstrate further that α11 was differentially overexpressed in lung cancer stromal fibroblasts,
we compared α11 expression in fibroblasts isolated from five pairs of primary NSCLC and their corresponding nonneoplastic lung tissue. α11 was differentially overexpressed 2-fold or higher in four CAFs, compared with their counterpart fibroblasts (Fig. 1C), suggesting that increased stromal α11 mRNA expression is associated with tumor formation.

**α11 Expression in Fibroblasts Enhances Tumorigenicity of NSCLC Cells.** We hypothesized that the expression of α11 in tumor stroma fibroblasts plays an important role in the tumorigenicity of lung cancer cells. To test this hypothesis, we evaluated the tumorigenicity of A549 lung adenocarcinoma cells when coimplanted with immortalized wild-type (WT) or α11-deficient [knockout (KO)] mouse embryonic fibroblasts (MEFs) in a 1:1 ratio in the s.c. tissue of immune-incompetent SCID mice (Fig. 2A). Individual cell lines were also tested as controls. The latter cell lines formed small tumors. Although A549 cells formed poorly differentiated adenocarcinoma, the WT and KO MEFs formed fibrosarcomas. In contrast, A549 cells coimplanted with either WT or KO MEFs demonstrated enhanced tumor growth, compared with the individual cell line alone (Fig. 2A). The effect appeared synergistic rather than additive. More important, the A549+WT group showed markedly greater tumor growth rate than the A549+KO group (P = 0.024; Fig. 2A). Histological examination revealed that the tumors were mainly (60–80%) composed of carcinoma cells. There was no significant difference in the relative cellularity of the spindle fibroblasts between the A549+WT and A549+KO tumors (Fig. 2B and C). Consistent with this evaluation, there was no significant difference in the mRNA expression levels of the mouse mesenchymal cell marker vimentin between these tumors (Fig. 2E).

Both WT and KO cells expressed α1, α2, and β1 integrin subunits [supporting information (SI) Fig. 6]. To confirm that the reduced tumorigenicity was because of the loss of α11 expression in KO fibroblasts, we reexpressed the human α11 gene into the KO cells (knockin, KI) (Fig. 2D and SI Fig. 6). The coimplanted A549+KI cells formed tumors at a rate similar to the A549+WT and at a rate significantly greater than the A549+KO (P = 0.028; Fig. 2A). None of the experimental groups demonstrated metastasis. Quantitative RT-PCR (qRT-PCR) analysis of the tumors formed confirmed the high expression of α11 in groups with WT fibroblasts, low expression in those with KO fibroblasts, and high expression of human α11 in those with KI fibroblasts (Fig. 2D).

To validate our findings, we repeated the coimplantation studies with two other NSCLC cell lines, NCI-H460 large-cell carcinoma and NCI-H520 squamous cell carcinoma lines (Fig. 3 A and B). Tumor growths were significantly greater when these cells were coimplanted with WT than with KO MEFs (Fig. 3A and B; H460, *P* < 0.001; H520, *P* = 0.039). q-RT-PCR confirmed the significantly lower α11 levels in tumors formed by NSCLC+KO compared with NSCLC+WT cells (SI Fig. 7). These results provide strong evidence that the role of α11 observed previously with the A549 cells was not cell line-specific. The tumors formed by the coimplanted H460 or H520 with WT or KO MEFs also showed no significant differences in the cellularity of carcinoma cells, compared with spindle fibrosarcoma cells (SI Fig. 7).

The overexpression of integrins (20), including some of the collagen receptor integrins such as α1β1 and α2β1 in tumor cells (21, 22), has been reported to play a role in angiogenesis. To explore whether angiogenesis was involved in the tumor-
α11 Regulates the Expression of IGF2 in Fibroblasts. Microarray studies were conducted to obtain insights into the genes that could potentially mediate α11’s effect on enhanced tumorigenicity in A549 + WT compared with A549 + KO tumors. With the mouse chip that would detect mainly gene expression of host-derived mouse cells, α11 expression or lack of it was associated with the differential expression of ≥2-fold in 1,630 probe sets. The highest differentially expressed gene was IGF2, which was 250 times higher in α11 WT compared with KO tumors. This result was confirmed by q-RT-PCR (Fig. 4A), which showed α11-deficient MEFs (KO), and KI MEFs that reexpressed human α11 (KI). (B and C) H&E staining of xenografts formed by A549 coimplanted with WT (B) and KO (C) MEFs. No apparent difference in the cellularity of carcinoma cells, compared with spindle fibrosarcoma cells, was noted. (D) Mouse α11 (filled bar) and human α11 (open bar) mRNA expression in tumors formed by WT, KO, and KI cells alone or when coimplanted with A549 cells. When WT was coimplanted with A549, there was an ∼10-fold increase in mouse α11 expression level compared with WT tumors. KI tumors only expressed human α11. The low level of mouse α11 detected in KO tumors was putatively from the host stromal cells. Note the lack of detection of human α11 in WT and KO tumors and the negligible level of mouse α11 in KO and A549 + KI tumors, confirming the specificity of primers used. All expression levels were arbitrarily referenced to the mean of A549 + KO tumors. (E) Vimentin mRNA expression in tumors. No significant difference was noted between A549 + WT versus A549 + KO, H460 + WT versus H460 + KO, and H520 + WT versus H520 + KO. All expression levels were relative to that of A549 + KO. (F) CD31 mRNA expression levels in xenograft tumors specified in E. Error bar represents SE. The number in brackets indicates the number of mice in each group. The numbers of samples used for q-RT-PCR analysis were: WT, 7; KO, 7; KI, 4; A549 + WT, 7; A549 + KO, 8; A549 + KI, 4.

Promoting effect of stromal α11, the mouse CD31 mRNA expression level of the A549 xenografts was quantified by q-RT-PCR. No significant differences were found (Fig. 2F), indicating that the stromal α11-mediated increase in tumorigenesis was unlikely because of enhanced angiogenesis.

Relative Importance of Integrin α11-Subunit Collagen Receptors. Compensatory effects within gene families are common in KO studies. We next asked whether the expression of other collagen receptor integrin subunit genes were altered in KO tumors (SI Fig. 8). q-RT-PCR of xenografts showed a clear compensatory elevation of α2 expression in A549 + KO (P = 0.007 compared with A549 + WT; P = 0.014 compared with A549 + KI). Although α10 mRNA expression was increased 2-fold in KO, compared with WT (P = 0.006), the difference between A549 + KO and A549 + WT was insignificant. In addition, the levels of α1 and β1 were inconsistently altered. These findings further support the unique importance of fibroblast α11 in regulating the tumorigenicity of NSCLC cells.

IGF2 Partially Mediates the Fibroblast α11 Effect on Tumorigenicity of A549 Cells. To investigate further the role of IGF2 in α11-mediated enhanced tumorigenicity of A549 cells by fibroblasts, we stably down-regulated the IGF2 expression in WT fibroblasts using short-hairpin RNA (shRNA). Compared with control WT cells (shRNA against luciferase, WTshLuc) and the parental WT cells, the WT cells that stably express the IGF2 shRNA (WT−shIGF2) showed ∼70% down-regulation of IGF2 mRNA expression (Fig. 5A). When WT−shIGF2 cells were coimplanted with A549 cells in SCID mice, significant growth inhibition compared with A549 + WTshLuc tumor was noted (P = 0.001; Fig. 5C). The down-regulation of mouse IGF2 was verified in the xenografts.
(P = 0.004; Fig. 5B). These results are consistent with IGF2 playing an important role in fibroblast-induced enhancement of NSCLC tumor growth.

Discussion

We have demonstrated that integrin α11-expressing stromal fibroblasts have a greater paracrine stimulating effect on the tumor formation of A549 adenocarcinoma cells than fibroblasts deficient in α11 expression, and we have validated the finding in two additional NSCLC cell lines. This finding provides strong evidence to implicate α11 integrin as another stromal factor that may modulate the growth of carcinoma cells during tumor formation. Furthermore, we have shown that this stromal–tumor cell interaction is mediated uniquely by the ability of α11 to regulate the expression of IGF2 in fibroblasts. Considering that >80% of NSCLCs overexpress α11 (19), stromal α11 could play an important role in the malignant progression of NSCLC.

Integrins are one of the most important mediators of cell–extracellular matrix protein interaction. They are localized mainly at focal adhesions and transmit both biochemical and mechanical signals from the matrix proteins to the cytoskeletal machinery of cells. They also activate signal transduction cascades that are important in cellular proliferation, movement, and survival (14, 24). Integrins may also affect these cellular functions through interactions with other transmembrane proteins, including growth factor receptors (25, 26). Studies on the role of integrins in cancer development and biology have mainly focused on their expression of tumor or endothelial cells (15, 24). The patterns of expression changes reported in various tumor types are complex and often involve cell type or disease-specific effects (27). Although most such effects result from direct interaction of integrins on tumor cells and the stromal matrix proteins, our study suggests that altered integrin expression in tumor stromal fibroblasts may also play an important role in the growth of carcinoma cells. Among the collagen receptor integrins, over-expression of α1 and α2 have been reported in squamous cell carcinoma and correlated with increased invasion, but the loss of α2 expression has also been associated with tumor progression in breast and prostate carcinoma cells. In lung cancer, the precise role of these two collagen receptor integrins remains ambiguous and often contradictory (28). With α11 being expressed largely by stromal fibroblasts, our results show that α11 provides an indirect and alternate mechanism for interstitial collagen to influence the growth of tumor cells. We have also demonstrated that one of the mediators of such a mechanism is its ability to tightly regulate the fibroblast expression of IGF2, a potent growth stimulator of epithelial tumor cells.

Goel et al. (29) recently reported that expression of β1C, but not β1A, integrin up-regulates the expression of IGF2 mRNA and protein in β1-null GD25 mouse fibroblasts and in PC3 prostate cancer cells. This finding was associated with the promotion of cell adhesion to laminin-1 and inhibition of proliferation. Here we have shown that similar induction by collagen-binding α-chains is α11-specific because α1 and α2 were expressed equally in WT and KO fibroblasts. Also, contrary to the conclusion of Goel et al. (29, 30), we implicate IGF2 as a potent stimulator of tumor growth for NSCLC cells. The activities of integrin-α1 and β2 subunits are mediated by the heterodimer that constitutes their functional receptor. Although the α1 and β2-chain expressions are regulated independently and β2-chain is usually present in abundance, the α1-chain level can be rate-limiting for the signaling activities and specificities of the heterodimer. Paradoxically, β1C-induced IGF2 expression in GD25 cells was associated with the loss of IGF1 receptor autophosphorylation (34). This result was putatively mediated by β1C-induced activation of the growth factor receptor binding protein 2-associated binding-2, recruitment of SH2-containing protein tyrosine phosphatase-2 (29), and activation of phosphatidylinositol 3-kinase (PI3-kinase) pathway (34). Because β1C is not found in mouse cells (31) and the predominant variant β1A does not affect IGF2 level (29), our data suggest that the regulation of IGF2 expression by α11 in fibroblasts can be mediated by the cytoplasmic domain of α1-chain. The precise mechanism of this regulation requires further studies, but some integrins have been reported to elicit Src-dependent signaling in an α-chain-specific manner (32, 33).

IGF2 is commonly overexpressed in human cancer, including breast (34), colorectal (35), liver (36), esophageal (37), intestinal (38), and prostate cancers (39). The most commonly posited mechanism for its overexpression is through loss of imprinting. However, other regulatory mechanisms of IGF2 expression have been reported, including its induction by prolactin in breast carcinoma (34) and PTEN in hepatoma cells (40). The concordant elevated levels of α11 and IGF2 in CAF suggest that, in primary NSCLC, their expression is closely associated. This result, as well as our finding of α11-regulated IGF2 expression in fibroblasts, suggest that α11 is also an important stromal factor that mediates the tumor growth-enhancing activity of CAF in NSCLC.

The α-subunits of the collagen receptor integrins are distinguished from other family members by the presence of a 200-aa insertion (I/A domain) between blades 2 and 3 of the surface propeller-like structures. Among the four subunits, α2 and α11 show the greatest binding affinity for fibrillar collagen I, whereas α1 and α10 show preferential affinity for network-forming collagens such as collagen IV and collagen VI (41, 42). This difference could be the reason for the observed compensatory elevation of α2 in KO cells. The observed finding also argues against the role of α2 as a stromal factor to promote tumorigenicity. The function of α10 is currently unclear, although its expression is normally restricted to cartilage tissue (43). Its compensatory up-regulation in KO cells suggests that it may have a similar function as α11, although α10 protein expression still requires verification.

In summary, our study clearly indicates that stromal α11 may promote the tumorigenicity of NSCLC cells, and this finding can
influenced by the MEFs, NSCLC cells derived from hIGF2 and IGF1R expression levels were not.

Materials and Methods

Tissue Samples. NSCLC and corresponding nonneoplastic lung parenchymal tissues were harvested and/or banked with informed consent from lung cancer resection specimens. The Research Ethics Board of the University Health Network approved the use of these excess tissues for this project. Banked specimens were snap-frozen and stored in liquid nitrogen until use.

MEF Cell Lines. SV40 immortalized MEF cell lines were derived from WT and α11-deficient (KO) embryonic day 14.5 mouse embryos as previously reported (18) and cultured in Dulbecco’s supplemented with 10% FBS. To reexpress the integrin α11 in KO fibroblasts, a full-length human integrin α11 cDNA (3.5 kb) cloned into the pBJ-1 vector (18) was cotransfected with a puromycin resistance vector. Clone 14 (KI) with confirmed stable expression of integrin α11 was isolated and used in subsequent experiments.

Isolation of CAF. Five independent lung cancer resection specimens were used to establish primary cultures of CAFs and noncancer fibroblasts from the corresponding nonneoplastic lung parenchyma >5 cm distant from the tumor. Tissues were minced into small pieces and digested for 1 h at 37°C in DMEM containing 10% FBS and 1 mg/ml collagenase type IV (Roche Molecular Biochemicals, Burlington, ON, Canada). The suspension was centrifuged at 180 × g for 5 min, and the pellet was resuspended in the serum containing DMEM.

Cell Culture and Xenograft Studies. A549 adenocarcinoma, NCI-H520 squamous cell carcinoma, and NCI-H460 large-cell lung carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA) and routinely cultured in RPMI 1640 supplemented by 10% FBS. All cell lines were cultured at 37°C in a humidified 5% CO₂ atmosphere. The tumorigenicity of cell lines was tested in 6-week-old male SCID mice. Fifty microliters of 10⁶ of A549 cells, WT, KO, or KI cells were injected s.c. into the subcutaneous tissue of the flank at 5 cm distant from the tumor. Tissues were harvested and/or banked with informed consent from lung cancer resection specimens. The Research Ethics Board of the University Health Network approved the use of these excess tissues for this project. Banked specimens were snap-frozen and stored in liquid nitrogen until use.

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RNA Isolation and Quantitative Assay. Tumor cells and their surrounding stroma were isolated from two primary NSCLCs by laser-captured microdissection (46). RNA isolation and cDNA synthesis were as described previously (46). Gene expression level was quantitated by q-RT-PCR and normalized by housekeeping genes (RPS13 for human and GAPDH for mouse and rat). Details are provided in SI Materials and Methods.

Gene Expression Profiling of Tumors. The expression of human and mouse genes in tumors was profiled by using the Affymetrix human U133A and MOE430A chips (Affymetrix, Santa Clara, CA). The cDNA synthesis, hybridization, washing, and scanning were performed by standard protocol provided by Affymetrix. The raw microarray data from both the MOE430A and HGU133A arrays were preprocessed by using the RMA algorithm, and the log-scale expression levels of WT versus KO and As49 WT versus As49 KO were compared.

shRNA Down-Regulation of IGFl in MEF Cells. Mouse IGFl expression in WT fibroblast was silenced by using the shRNA method. Hairpin sense CCGGGACCAGGGTCTTTATTCTTTACAGAGA-CTGAAAGTAGA-AGCCCGGTCAGTTTTT and antisense AATTCAAAGGACGGGCATTCTTCTAGTCTTT-GAACTGAAGTAGAAGCCGCGGTC oligos against IGFl2 (Sigma–Aldrich, St. Louis, MO) were annealed and subcloned into pLKO.1 yellow fluorescent protein vector. This construct, or the control shRNA against luciferase (47) (10 μg), was transfected into 293T cells, together with 5 μg each of the packaging plasmids pMD.G, pMDLg/pRRE, and pRSv-Rev (48) by using the calcium phosphate method. Forty-eight hours later, the viral supernatant was collected and used to infect MEFs. Yellow fluorescent protein-positive cells were sorted by Dako MoFlo flowcytometry (Dako North America, Carpinteria, CA).

We thank Drs. Stewart, Novina, and Weinberg (Whitehead Institute, Cambridge, MA) for their gift of pLKO.Puro; Dr. Naldini for packaging plasmids pMD.G, pMDLg/pRRE, and pRSv-Rev; and Dr. G. Nolan (Stanford University, Stanford, CA) for the Phoenix-Eco cell line. This work was supported by grants from the Canadian Cancer Society (M.S.T. and L.Z.P.), Helse Vest, Research Council of Norway Grant 172330/V00 (to D.G.), and Swedish Research Council Grant VR NTK 2002–4309 (to D.G.). E.R.S.B. is a Fellow of Aventis Canada, S.N.P. is the recipient of a postdoctoral scholarship from the Research Council of Norway, and D.B.-L. is a Fellow of the Canadian Institutes of Health Research.

Supplemental Figure S1. Protein expression of mouse integrin subunits $\beta_1$, $\alpha_1$, $\alpha_2$, and $\alpha_{11}$ in WT, KO and KI MEFs. The absence of expression of $\alpha_{11}$ in KO and the presence of $\alpha_{11}$ protein in WT and KI were confirmed by western blot.
Supplemental Figure S2. Co-implantation studies of H460 and H520 cells with mouse embryonic fibroblasts (MEF). (A) Low mouse integrin alpha-11 (a11) expression in tumors formed by H460 and H520 co-implanted with MEF lacking a11 (KO) compared to those formed with wild type (WT) fibroblasts. (B-E) Histological confirmation of the resultant xenografts showing no apparent differences in relative abundance of carcinoma cells compared to spindle fibrosarcoma cells between H460+WT and H460+KO and between H520+WT and H520+KO tumors.
A. H460+WT

B. H460+KO

C. H520+WT

D. H520+KO