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CAR mediates efficient tumor engraftment of mesenchymal type lung cancer cells

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The coxsackie-adenovirus receptor (CAR) is a developmentally regulated intercellular adhesion molecule that was previously observed to be required for efficient tumor formation. To confirm that observation, we compared the tumorigenicity of clonally derived test and control cell subsets that were genetically modified for CAR. Silencing CAR in lung cancer cells with high constitutive expression reduced engraftment efficiency. Conversely, overexpressing CAR in lung cancer cells with low constitutive expression did not affect tumor formation or growth kinetics. A blocking antibody to the extracellular domain of CAR inhibited tumor engraftment, implicating that domain as being important to this process. However, differences in adhesion properties attributable to this domain (barrier function and aggregation) could not be distinguished in the test groups *in vitro*, and the mechanisms underlying CAR's contribution to tumor engraftment remain elusive. Because high CAR cells displayed a spindle-shaped morphology at baseline, we considered whether this expression was an accompaniment of other mesenchymal features in these lung cancer cells. Molecular correlates of CAR were compared in model epithelial and mesenchymal type lung cancer cells. CAR expression is associated with an absence of E-cadherin, diminished expression of α- and γ-catenin, and increased Zeb1, Snail, and vimentin expression in lung cancer cells. In contrast, epithelial type (NCI-H292, Calu3) lung cancer cells show comparatively low CAR expression. These data suggest that if the mesenchymal cell phenotype is an accurate measure of an undifferentiated and invasive state, then CAR expression may be more closely aligned with this phenotype of lung cancer cells.

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CAR, the coxsackie-adenovirus receptor, is both a regulator of the Notch signaling pathway[1,2] with roles in embryonic development, and an epithelial intercellular adhesion molecule with reported contributions to mucosal integrity and barrier function.[3,4] It is a single-pass transmembrane protein initially described as the high affinity attachment receptor for coxsackie and group C adenoviruses (Ad).[5] CAR is essential and embryonic stem cell-CAR knockouts are nonviable secondary to abnormal myocardial development.[6–8]

The epithelial–mesenchymal transition (EMT) is a phenotypic shift in which epithelial cells break down cell–cell and cell–extracellular matrix connections and then migrate to other locations in the body. These processes are known to be important for embryonic development, and are believed to contribute to repair of tissue injury and to invasive and metastatic tumor growth. EMT is modeled in epithelial cells by exposure to certain cytokines (eg, TGFβ1), or by exogenous expression of various transcription factors (eg, Twist or Snail), which can increase the invasive behavior of epithelial cells.

The dynamics of CAR expression in epithelial transitions is not clear. CAR is believed to recruit the tight junction (TJ) protein ZO-1 to the apical junctional complex.[3] Imaging analyses localize CAR to the TJ in T-84 colon cancer cells,[3] or to the adherens junctions (AJ) in A549 lung cancer cells.[4] Although structurally distinct,[9] functional analogies of CAR to E-cadherin or E-selectin have been suggested.[4,10] CAR overexpression in CHO cells resulted in cellular aggregation;[3] overexpression in MDCK cells evidenced increased transepithelial electric resistance (TEER).[3]

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At intercellular junctions, the extracellular CAR N-terminal domains are believed to homodimerize to mediate homotypic cell adhesion and barrier function. The CAR intracellular C terminus contains PDZ-binding motifs for the initiation of structural and/or signaling scaffolds,[11] but to date, signaling cascades emanating from CAR interactions have not been reported. Screens for CAR molecular interactions have been inconsistent; putative binding partners vary based on the assay system (deletion analyses, yeast two-hybrid screens, affinity chromatography or immunoprecipitation assays) as well as the cell model system that have been used.[1–4,11–15] Proteins identified as putative CAR-binding partners include several PDZ domain-containing proteins (ZO-1, MUPP-1, Ligand-of-Numb protein X, MAGI-1b, and PICK-1), and the functional implications of these interactions are currently under investigation.

CAR's contribution to the malignant phenotype is also under study. CAR is variably expressed in diverse tumors of both epithelial and mesenchymal origins.[16–27] However, CAR intracellular localization, partners in protein interactions, physiological function in epithelia, or aberrant role in epithelial tumors remain largely unexplored. There have been several reports that have sought to link or correlate CAR expression with differentiation, tumorigenicity, and/or tumor progression. For example, based on cell culture models, a group of investigators has postulated that CAR is a global tumor suppressor in prostate and bladder cancer.[16–18] Yet another group reports upregulated CAR expression with both tumor progression[28] and with the inhibition of EMT[29] in model systems. Another group evidenced enhanced CAR expression in the transition from preneoplastic lesions to invasive adenocarcinoma in a breast cancer model.[30] These observations have led some to propose that CAR is a marker of tumor differentiation.[31,32] However, we speculate that collectively, these paradoxical observations are reflective of a complex temporally limited expression of CAR in epithelia and in epithelial transitions.

We previously determined that (1) a soluble CAR ectodomain could be isolated from the tumor milieu (primary malignant pleural effusions),[33] and (2) silencing CAR in human lung cancer cells impaired subcutaneous tumorigenesis in a *scid* mouse model.[34] This impairment in tumor formation was not associated with significant differences in proliferation or induced apoptosis (by serum starvation) between control and test (CAR-silenced) cells. To confirm and expand upon our initial observation, we genetically modified selected lung cancer cells to derive clonal test and control populations that differed in CAR expression. After validation, these groups were tested in tumorigenicity and aggregation assays. Here, we report that silencing CAR in lung cancer cells with high CAR expression (NCI-H1703 and NCI-H157 cells) significantly impairs their ability to form tumors *in vivo*; whereas overexpressing CAR in cells with low constitutive CAR expression (NCI-H2122) does not have a measurable effect on tumorigenicity. Similarly, blocking antibody to the CAR ectodomain inhibits tumor formation by cells with high CAR, but not cells with low CAR. The spindle-shaped morphology of cells that showed high CAR contrasted with epitheliod features of cells that showed low CAR prompted us to consider whether CAR is associated with EMT in lung cancer cells. We compared the NCI-H1703 and NCI-H157 (high CAR) cells with validated lung epitheliod models (NCI-H292 and Calu3) for correlating CAR with the morphological and molecular features of EMT. Our data suggest that high CAR expression is closely associated with mesenchymal markers.

MATERIALS AND METHODS

Cells

NCI-H1703, NCI-H157, NCI-H2122 non-small-cell lung cancer (NSCLC) cells (gifted by Dr Herbert Oie, NCI), and NCI-H292 and Calu3 NSCLC cells (gifted by Dr K Krysan, Dubinett lab, UCLA Lung Cancer Research Program) were maintained in RPMI 1640 (Irvine Scientific, Santa Ana, CA, USA) with 10% fetal bovine serum (FBS; Gemini, Woodland CA, USA) and penicillin (100 U/ml)/streptomycin (100 μg/ml) (complete medium). Morphological and phenotypic features relevant to the NCI-H1703, NCI-H157, and NCI-H2122 cells are described in the 'Results' section. The epitheliod cell models (NCI-H292 and Calu3 NSCLC cells) were chosen because they are reported to form functional junctions, and both lines have been extensively used as models of pulmonary epithelia. H292 cells are polarized in culture,[35] and express E-cadherin with functional AJ.[36–38] Calu3 cells express functional TJs (with circumferential ZO-1 expression, high TEER, and low paracellular permeability characteristics),[39] along with zonulae adherens, and desmosomes.[36,40] Accordingly, Calu3 cells are a model of a tight lung epithelium.

Vectors and Clonal Selection

For *CAR* gene modification, we used an amphotropic retroviral vector encoding the full-length CAR open reading frame in forward and reverse orientations. Briefly, the full-length CAR cDNA insert was digested (*Bam*H1, *Xho*1) out of pcDNA3-CAR (gifted by Joanne Douglas, UAB Gene Therapy Center, Birmingham, AL, USA), and ligated into a linearized pLEGFP-N1 fragment in forward or reverse orientations downstream of the CMV promoter/enhancer sequence (Clontech, Becton Dickenson Biosciences, Palo Alto, CA, USA). Plasmid DNA was isolated from transformed DH5α, and insert orientations were confirmed with *Xba*1 digestion. Vectors were amplified and transfected (Effectene reagent; Qiagen, Valencia, CA, USA) into the PT-67 retroviral packaging cell line (Clontech, Becton Dickenson Biosciences). Producer cell supernatants were used to stably transduce lung cancer cells, and pooled populations were selected following G418 selection (500 μg/ml) over 4 weeks. Clones were derived by limited dilution (1 cell per ml seeded as 200 μl aliquots in 96-well plates). Of these, 15–40 distinct colonies were characterized for CAR by immunoblot and/or

flow cytometry (see below), and selected clonal populations were expanded for these studies.

Characterization of *CAR* Gene-Modified Cells

Transduction was confirmed by the presence or absence of *CAR* gene amplification by DNA–polymerase chain reaction (PCR) primed by sequences flanking the multiple cloning sites in the pLEGFP-N1 vector. *CAR* expression was also functionally validated by Ad transduction in some cells; Ad vectors (Ad5LacZ) were amplified in the UCLA—Jonsson Comprehensive Cancer Center and stored at −20°C in a nonfreezing solution containing 25% glycerol, 0.05% BSA, 4 M CsCl, 50 mM NaCl, 0.5 mM $MgCl_2$, and 5 mM Tris buffer as described.[19] Vectors were gel-filtered (G-50 Sephadex; Boehringer Mannheim, Indianapolis, IN, USA) and eluted into growth medium for transduction of target cells at a multiplicity of infectious units of 100. All gene-modified clones were validated for CAR by PCR, immunoblot of cell lysates, and/or by surface CAR expression by flow cytometry. For RT–PCR, total RNA was extracted from control or CAR-silenced clones using TRIzol reagent (Life Technologies, Grand Island, NY, USA). RT was performed using the Super Script One Step RT–PCR system (Gibco-BRL, Grand Island, NY, USA). Primers for CAR expression were as follows: sense: 5′-ATGCCCACTTCATGGTTAGC-3′; antisense: 5′-TAGGG GCAGCTACCTTAGCA-3′. For GAPDH control, sense: 5′-C CACCCATGGCAAATTCCATGGCA-3′, antisense: 5′-TCTAG ACGGCAGGTCAGGTCCACC-3′ were used. The RT–PCR protocol was as follows: cDNA synthesis (one cycle: 54°C for 30 min) followed by 94°C for 2 min. DNA amplification followed (25 cycles: 94°C for 15 s, anneal 55°C for 30 s, extend 72°C for 1 min) with a final extension at 72°C for 8 min.

For flow cytometry, surface CAR was labeled[34] using RmcB,[41] a murine monoclonal antibody that recognizes the extracellular N-terminal region of CAR. Briefly, 1×10^6 cells were preincubated in 100 μl 0.1% BSA (in PBS for 20 min at room temperature) before primary antibody (RmcB; mouse IgG1, 1:100 dilution in PBS/0.1% BSA for 90 min at room temperature) was admixed on an orbital shaker. Cells were then sedimented and washed three times with PBS/0.1% BSA, incubated with secondary (PE-conjugated sheep anti-mouse $(Fab')_2$; Sigma, St Louis, MI, USA, 1:200 dilution in PBS/0.1% BSA for 30 min at room temperature in the dark), washed three times and resuspended in 500 μl PBS for flow cytometry by FACScan using CellQuest software (Becton Dickinson, Mountain View, CA, USA). For all data acquisition, gates were based on the forward and side scatter profiles of unstained cells, and surface expression of target proteins was normalized to cells that had been incubated with secondary antibody alone.

Antibody-Mediated CAR Neutralization and Tumorigenesis Assays

For comparative tumorigenesis, 6-week-old *nu/nu* or *scid/scid* mice were injected with 5×10^6 to 8×10^6 cells per mouse subcutaneously, in the flank region, in groups of 4–6, under an institutionally approved protocol. The predefined primary outcome measurement was tumor volumes at the time point when the first mouse in either the control or test groups met institutional criteria for killing due to tumor burden. Tumor dimensions were estimated based on bisecting diameters measured with a caliper, and the tumor volume was approximated using the formula 0.4 (ab^2) where a is the long measured axis of the tumor and b the short measured axis. For CAR-blocking antibody (RmcB) blockade experiments, 5×10^6 NSCLC cells were co-injected into the right flank subcutaneous region of female *nu/nu* mice (four or five mice per group) with 2 μg of RmcB or control murine IgG1. Tumor volumes were assessed every 24–48 h until mice in either test or control cohort met criteria for killing, or the experiment was terminated after a maximum of 90 days.

Cell Aggregation Assays

Cell aggregation assays were performed with minor modifications of methods described earlier.[42,43] Briefly, cells grown to 85% confluence in complete medium were washed with 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 5 mM $CaCl_2$, and 1 mM $MgCl_2$ (aggregation buffer), and then detached with 0.01% trypsin (Invitrogen, Carlsbad, CA, USA) in the same buffer on a gyratory shaker at 75 r.p.m. At the end of the incubation period, trypsin was quenched with 20% FBS/RPMI, and cells were sedimented (200g for 5 min). The cell pellet was resuspended in the aggregation buffer containing 1% BSA; cell suspensions (density of 5×10^4 cells per 0.5 ml) were added to individual wells of a 24-well plate and incubated at 37°C for 1 h on a gyratory shaker at 75 r.p.m. Cells were observed for aggregation in a Leica DMIL microscope equipped with Hamamatsu ORCA-ER digital camera. Photomicrographs were captured at ×40 magnification using MetaMorph software. E-cadherin and CAR neutralization was performed based on published methods.[19,33,34,41,44] Briefly, cells were incubated with blocking antibodies (10 μg/ml of anti E-cadherin; Zymed Laboratories or 5 μg/ml RmcB) for 30 min before starting the aggregation. Cell aggregation was quantified by the measured decrease in the free or individual particle number (individual particles defined as aggregates ≤ 3 cells) in high-powered microscopic fields. For these analyses, 10 μl of a 5×10^4 cells per 0.5 ml cell suspension (therefore, containing 1000 cells) was resuspended in 50 μl volume of aggregation buffer, and the total number of free/individual particles was counted. The percent aggregation was calculated as 1−((number of free/individual particles)/(initial particle number or 1000)) × 100).

Molecular Characterization of EMT Markers Using Immunoblots

Coordinated cell cultures were lysed following the end of log phase growth (post-confluent state, 24 h after culture confluence) using Mammalian Cell-PE LB medium

(Genotechnology Inc., St Louis, MO, USA) at 4°C containing Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA). Insoluble debris was pelleted by centrifugation and total protein in the supernatant was quantified using the Bio-Rad Assay Kit (Bio-Rad, Hercules, CA, USA). Protein (40 μg) was suspended in 1 volume of Laemmli sample buffer (Bio-Rad), and the volume of the samples was brought to 50 μl with Mammalian Cell-PE LB medium for loading. The samples were heated at 95°C for 5 min, resolved by 10% SDS–PAGE, and transferred onto polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA, USA) on ice. After blocking the membrane with NAP-Sure Blocker (Genotechnology Inc.), the membrane was washed three times in PBS and 0.1% Tween 20 (PBST) over 30 min, and probed using the following primary antibodies (diluted in PBST as recommended by the manufacturer, overnight incubations at 4°C): polyclonal anti-CAR H300, anti-Zeb H-102, anti-Occludin H-279, anti-β-tubulin H202, and anti-vimentin V7 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-E-cadherin (BD Biosciences PharMingen/Transduction Laboratories, San Jose, CA, USA), anti-β-catenin and anti-snail (Cell Signaling, Danvers, MA, USA), anti-α-catenin (Chemicon International, Temecula, CA, USA), anti-γ-catenin and anti-ZO-1 (Zymed/Invitrogen Laboratories, Carlsbad, CA, USA). Following three washes in PBST, the primary antibody was labeled with the appropriate horseradish-peroxidase-conjugated secondary antibody (1:5000 dilution, 1 h at room temperature; Amersham Biosciences, Piscataway, NJ, USA). After repeated washes in PBST, specific bands were detected by chemiluminescence (Amersham Biosciences).

Statistical Methods

The mean and standard deviation of categorical values (percent transduction, tumor volumes at time of first killing) were compared for differences using the unpaired *t*-test or one-way ANOVA, using GraphPad InStat software. A statistically significant difference was defined as $P<0.05$ between the groups compared.

RESULTS

Silencing CAR in Lung Cancer Cells with High Expression Impairs their Ability to Form Tumors

NCI-H1703 and NCI-H157 lung cancer cells (selected as models because they highly express CAR constitutively) were genetically silenced for CAR, and clones were isolated (1 cell per ml seeded as 200 μl aliquots in 96-well plates). Colonies were expanded and clonally derived cell populations were validated for altered CAR expression. For the NCI-H1703 subset, we used Ad transduction as a functional screen for CAR expression, because CAR is the cellular attachment receptor for Ad,[5] and because CAR expression is a primary determinant of Ad transduction efficiency of lung cancer cells *in vitro* and *in vivo*.[20,45] CAR-silenced clones exhibited significant reductions in Ad transduction efficiency (Figure 1a) when compared with parental or vector controls. The decrease in Ad transduction efficiency was corroborated by marked reductions in CAR expression as directly measured by CAR-mRNA transcript by semiquantitative RT-PCR (Figure 1b), and by validation of CAR expression by flow cytometry (Figure 1c). Surface expression of CAR in the parental and vector control groups was confirmed to be significantly higher than the CAR-silenced cells, and significantly lower than the CAR-overexpressing cells.

Clonally derived populations of both CAR-silenced lung cancer cells (NCI-H1703, NCI-H157) were significantly inhibited in their ability to form subcutaneous xenografts, as compared to control populations (Figures 1d and e). For example, in the NCI-H1703 cohort, CAR silencing was associated with a reduction in the efficiency of tumor formation with two distinct clones (1/4 with H1703-AS5 and 1/5 tumors with H1703 AS6 cells), compared to parental and vector control (4/4 and 5/5, respectively). In addition, the aggregate tumor volume (Figure 1d) in the CAR-silenced groups was significantly reduced (at 40 days, when the control subset first met criteria for killing because of tumor burden). Silencing CAR in NCI-H157 cells also decreased engraftment efficiency (1/5 with H157 CAR-silenced clone, *vs* 4/4 with H157 vector control) and the aggregate tumor volume ($P=0.008$ by the unpaired *t*-test), compared to vector control (Figure 1e). These results indicate that silencing CAR in lung cancer cells that display high constitutive expression impairs the engraftment efficiency of those cells. Tumors that arose from NCI-H157 overexpressing CAR (CAR+) followed a kinetic trend similar to that of the vector control cells (Figure 1e).

Interestingly, we observed the slow emergence of two tumors in mice that had been injected with the CAR-silenced (AS5 and AS6) cohorts, and the tumor cells from these late tumors showed high CAR expression (Figure 1f). Thus, when the delayed-onset tumors were extirpated (at day 101 or 118 after implantation), the surface CAR expression in the rescued cancer cells was similar to that of wild-type control, rather than that of the cells that were originally implanted (Figure 1f). These results seem to suggest that although CAR-silenced cells were initially inhibited in their ability to form tumors *in vivo*, the delayed tumors arose from phenotypic revertants that had possibly inactivated the CAR-silencing vector, and thus displayed surface CAR expression again.

CAR Overexpression in NCI-H2122 Lung Cancer Cells does not Effect Subcutaneous Tumor Formation

Similarly, *CAR* gene-modified clones were generated from lung cancer cells with very low constitutive CAR expression (H2122 cells);[20,45] representative flow scattergrams of the grouped clonal cell populations are presented (Figure 2a). Silencing CAR in H2122 cells resulted in a cell population

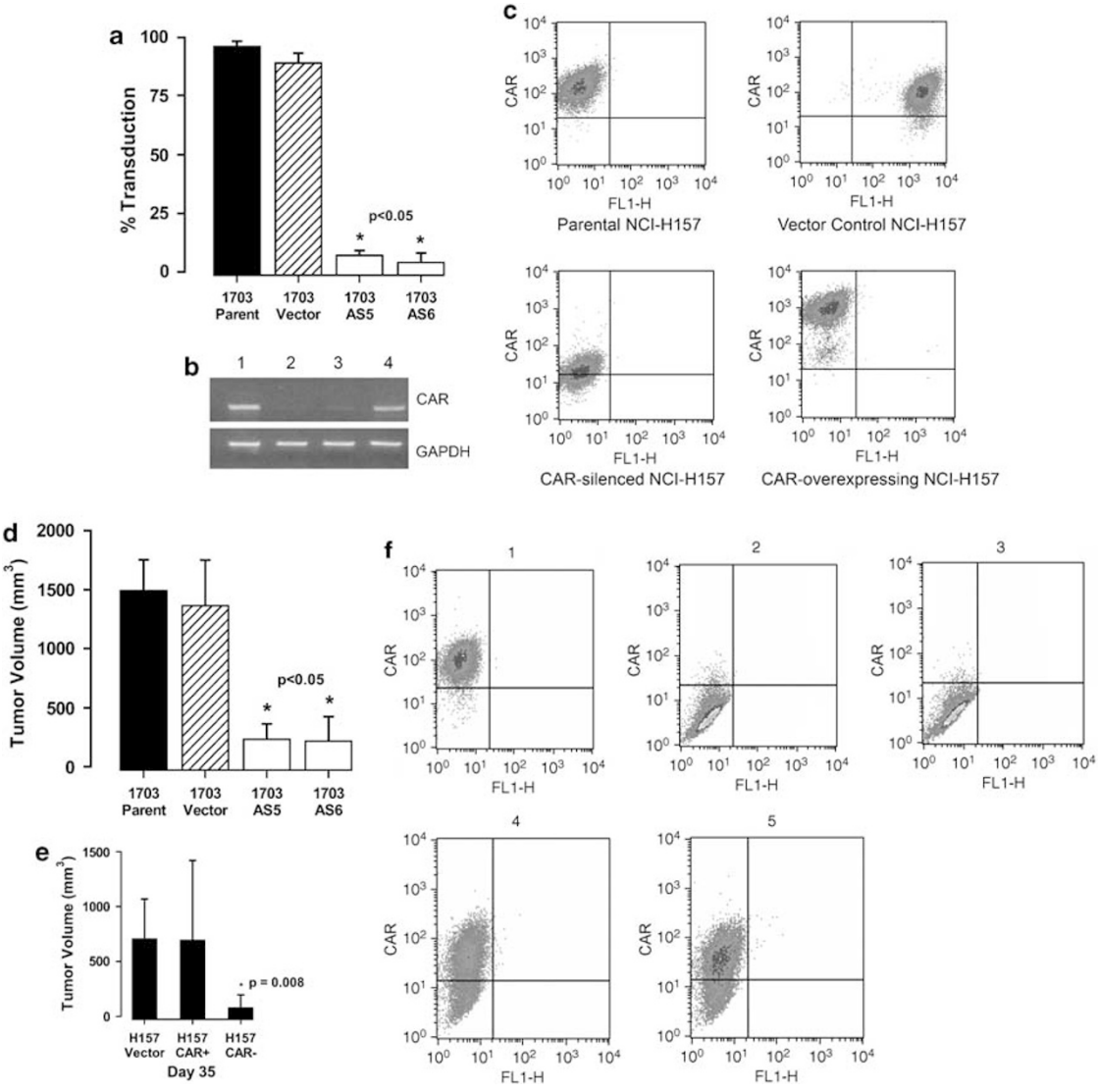

Figure 1 Silencing CAR in NCI-H1703 and NCI-H157 cells impairs their ability to form subcutaneous tumors: clonal test (CAR-silenced, CAR-overexpressing) or control (NCI-H1703, NCI-H157, or vector control) populations are validated for CAR expression by Ad transduction, RT–PCR, and/or flow cytometry. Following, the ability of these cell cohorts to generate subcutaneous xenografts in *scid* mice is evaluated. (**a**) Compared to parental and vector controls, CAR-silenced clones (1703 AS5 and 1703 AS6) are significantly more refractory to Ad transduction. (**b**) Compared to parental (lane 1) and vector (lane 4) controls, CAR-silenced clones display reduced levels of the CAR transcript. (**c**) For flow cytometry, 1×10^6 cells are labeled with primary antibody (RmcB), sedimented and washed, and detected with PE-conjugated sheep anti-mouse $(Fab')_2$. Surface expression of CAR was normalized (at $>$99th percentile) to cells that had been incubated with secondary antibody alone. Depicted is comparative CAR expression in the NCI-H157 (parental, vector control, CAR-silenced, and CAR-overexpressing) clones. CAR expression is on the ordinate scale; the green channel fluorescence (abscissa) in the vector control cells is attributable to the expression of the enhanced green fluorescent protein (EGFP) in control construct. (**d**) CAR-silenced NCI-H1703 clones (AS5 and AS6) are inhibited in their ability to engraft in *scid* mice. Depicted are the tumor volumes (mm^3) $\pm$ standard deviation at day 40 after implantation. $^*P<0.05$ for a significant difference between tumor formation by the vector control *vs* CAR-silenced cells. (**e**) CAR-silenced NCI-H157 clone (CAR$-$) are inhibited in their ability to engraft in *scid* mice. There is no significant difference in tumor growth between the control and CAR-overexpressing (CAR$+$) clonal cohort. $^*P=0.008$ for a significant difference between the vector control *vs* CAR-silenced cells at day 35 after implantation. (**f**) Delayed-onset tumors mice that were engrafted with CAR-silenced cells have cells that display high CAR. (f1), Wild-type (H1703 cells) CAR expression (mean channel fluorescence intensity or MCF $=$ 108) in; (f2), CAR-silenced H1703 AS5 clone (MCF $=$ 5) at time of implantation; (f3), CAR-silenced H1703 AS6 clone (MCF $=$ 5) at time of implantation; (f4), CAR in tumor cells extirpated from H1703 AS5 xenografts (integrated MCF $=$ 45); and (f5), CAR in tumor cells extirpated from H1703 AS6 xenografts (integrated MCF $=$ 41). These results suggest that reemergence of CAR expression may be associated with delayed tumor formation in these mice.

that could not be differentiated from the parental or vector control groups on the basis of the surface phenotype. Comparatively, there was significantly higher surface expression of CAR (MCF $=$ 404) in the H2122 CAR-overexpressing population. However, overexpressing CAR in these cells did not impact either engraftment efficiency (100% in every

group) or tumor volumes (Figure 2b) in relation to controls, and the tumor kinetic curves were virtually superimposable across the groups (data not shown). Thus, genetically silencing or overexpressing CAR in cells with low constitutive expression has no measurable impact on engraftment efficiency or tumor growth.

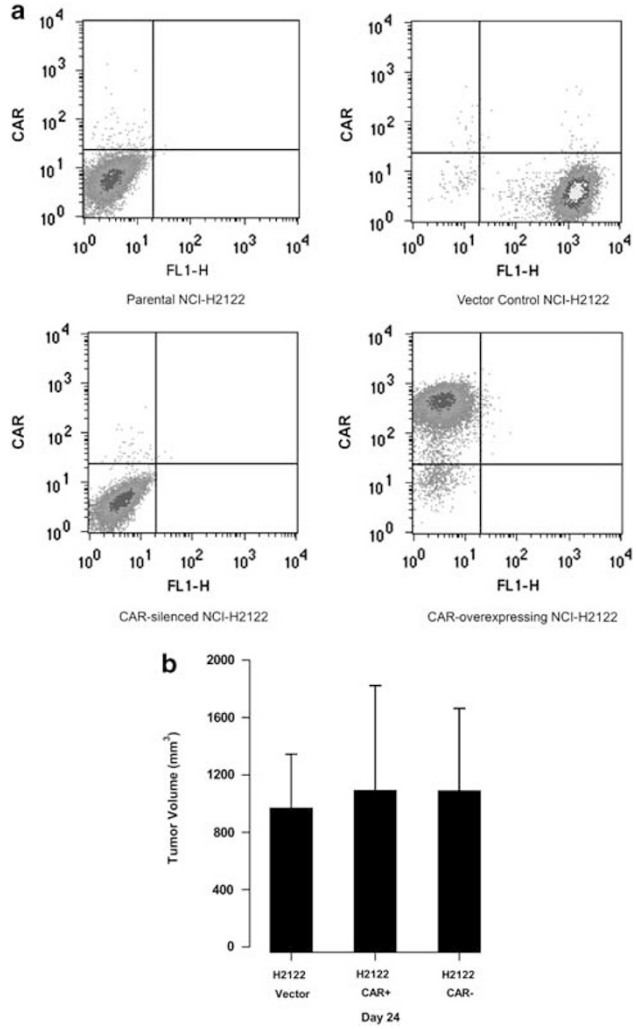

Figure 2 CAR overexpression in NCI-H2122 lung cancer cells does not effect subcutaneous tumor formation. (**a**) Clone of *CAR* gene-modified NCI-H2122 cells was propagated and validated for surface CAR expression. Depicted are representative flow scattergrams of NCI-H2122 (parental, vector control, and CAR-silenced and CAR-overexpressing) clonally derived cell populations, with CAR expression represented in the ordinate scale. Parental group (MCF = 7), vector control (MCF = 4), CAR-silenced (MCF = 4), and CAR-overexpressing (integrated MCF = 404) cells. (**b**) Overexpressing or silencing CAR in lung cancer cells that have low CAR expression (NCI-H2122 cells) does not change their ability to form tumor xenografts. Vector control, CAR-overexpressing (H2122CAR+), and CAR-silenced (H2122CAR−) NCI-H2122 cells were injected into the right flank region of *scid/scid* mice. Tumor volumes were monitored every other day until the time when the first mouse in either the control or test groups met criteria for killing. Depicted are the mean tumor volume ± standard deviation within each group of animals at day 24 after implantation.

Blocking Antibody to CAR Extracellular Domain Impairs Tumor Formation in NCI-H1703 Cells, but not in NCI-H2122 Cells

The distal extracellular domain of CAR on adjacent cells is envisioned to homodimerize and function in homotypic epithelial cell adhesion.[46] This domain also mediates binding to coxsackievirus and subgroup C adenovirus with high avidity (Kd ~ 1 nM),[5,41,47] a process which is blocked using the murine monoclonal antibody RmcB.[5,41] Thus, we used RmcB to block cell–cell interaction mediated by the distal extracellular domain of CAR. Compared to control murine IgG1, co-injection of RmcB with lung cancer cells unfailingly impaired tumorigenesis when those cells highly expressed CAR (Figures 3a,b). By contrast, RmcB neutralization of CAR–CAR interactions did not significantly alter the tumor kinetics of NCI-H2122 cells, which have low CAR expression (Figure 3c). The difference in the tumorigenicity was not attributable to differences in complement-mediated lysis of antibody-coated CAR+ cells (H1703 and H157) *vs* CAR-deficient H2122 cells. In this regard, there was no difference in rabbit HLA-ABC complement-mediated lysis with RmcB *vs* mIgG1 *in vitro* (data not shown). Notably, injecting RmcB into established tumors did not induce tumor regression, and growth of established tumors proceeded as with control mIgG1 (data not shown). Together, these results suggest that blocking of CAR extracellular domain function (previously reported by others as homotypic adhesion) may serve to block the initial engraftment of CAR-expressing tumors *in vivo*, but blocking CAR interactions when tumors have developed does not lead to tumor regression.

CAR Adhesion Function cannot be Evidenced by *In Vitro* Measures of Barrier Function or Cell Aggregation in Lung Cancer Cells

CAR overexpression in model epithelia has been shown to increase TEER,[3] and heterologous expression in CHO cells is reported to bring about cell aggregation through homotypic cell adhesion.[3,46] Because blocking the activity of the CAR extracellular domain reduced engraftment efficiency *in vivo*, we investigated whether we could develop a functional correlate of this activity *in vitro*, using lung cancer cell models. To control for the activity of E-cadherin in these functional analyses, we included the immunoblockade of E-cadherin into the assay design. CAR expression was not associated with the establishment of a transepithelial electrical resistance (TEER) in the lung cancer cell models (whereas coordinated cultures of MDCK cells generated peak resistances of 1361 Ω cm^2 and plateau of 300–600 Ω cm^2 for up to 2 weeks after the gradient had been established). In addition, we did not find CAR expression to be associated with increased cell aggregation in the lung cancer cells tested. Thus, the H1703 cells aggregated to a similar degree as the CAR-silenced cohorts derived from those cells (Figures 4a and b), and there was no difference in cell aggregation between cohorts of NCI-H2122 cells and clones that were genetically modified to

overexpress CAR (NCI-H2122S13) (Figures 4c and d). The suggestion that CAR was not significantly contributing to the *in vitro* aggregation function was also buttressed by blocking experiments in the NCI-H2122 cell cohort. NCI-H2122 cells express E-cadherin;[48] thus, we tested the extent to which the observed aggregation was attributable to E-cadherin *vs* CAR in H2122 cells overexpressing CAR (NCI-H2122S13) cells. Whereas the incubation of neutralizing antibody to E-cadherin largely blocked NCI-H2122S13 cell aggregation, blocking CAR did not have a significant effect on this function (Figures 4e and f). Despite differences in CAR expression, NCI-H1703 cells and their CAR-silenced derivatives aggregated (as defined by cell clusters ≥ 4 cells) approximately 20% of the time, whereas both the NCI-2122 cells and their CAR-overexpressing derivatives aggregated 80% of the time. These results indicate that changes in CAR expression cannot be measured by changes in paracellular ion flux (barrier electrical function) or *in vitro* cell aggregation in lung cancer cells. However, these data certainly do not exclude the possibility that CAR adhesion functions are important to tumor engraftment *in vivo*.

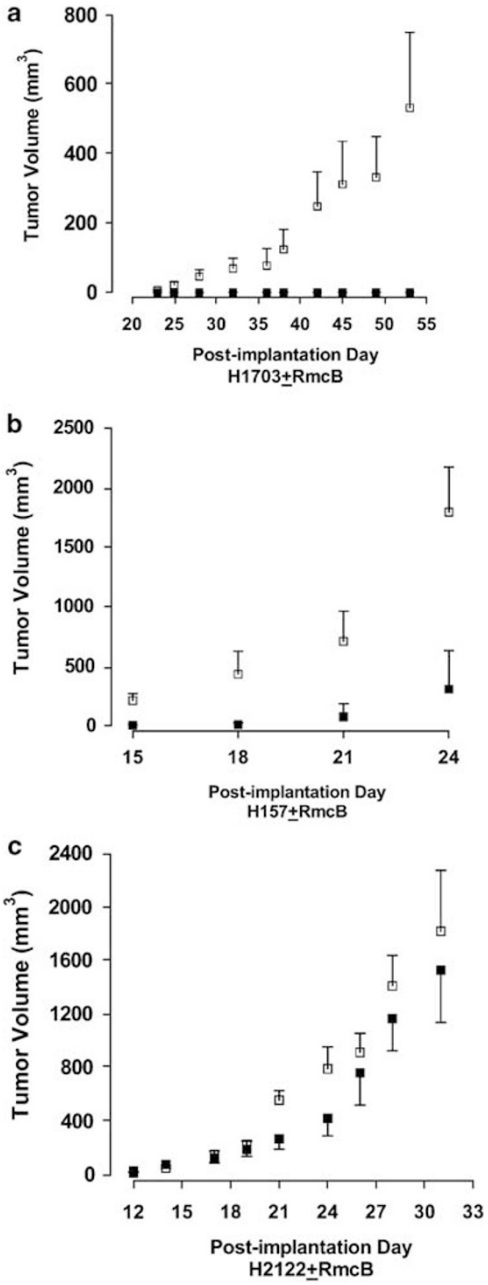

Figure 3 Blocking antibody to CAR extracellular domain impairs tumor formation by NSCLC cells with high constitutive CAR expression (NCI-H1703, NCI-H157), but not of cells with low CAR (NCI-H2122): RmcB, a murine monoclonal antibody that recognizes the CAR extracellular domain, was used to block CAR–CAR homotypic binding at the time of tumor implantation. A total of 5×10^6 NSCLC cells + 2 μg per 10^6 cells of RmcB or control murine IgG1 were implanted into the right flanks of female *nu/nu* mice. Implanted lung cancer cells (open squares) or cells + RmcB (solid squares) were monitored for tumor formation over time. Depicted are the tumor-growth curves (mean + standard deviation tumor volume) to a time point of first killing. (**a**) NCI-H1703 cell cohort (**b**) NCI-H157 cell cohort, (**c**) NCI-H2122 cell cohort.

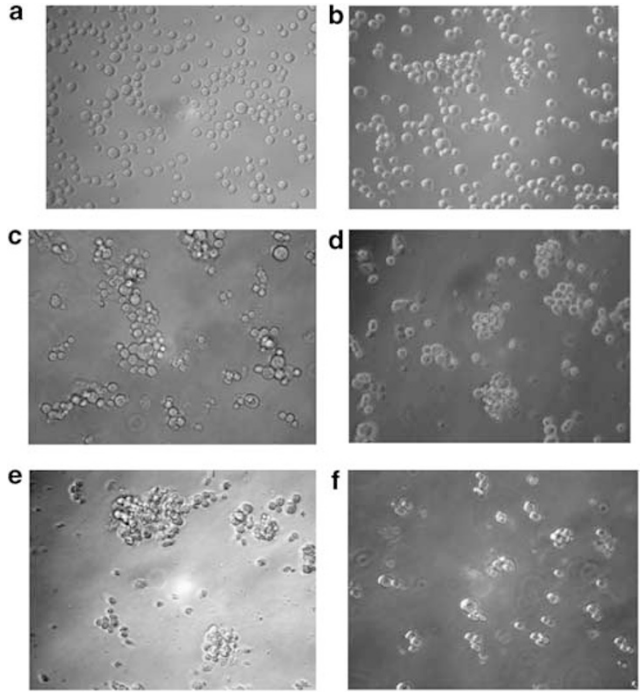

Figure 4 CAR expression is not associated with cell aggregation in lung cancer cells. Paired clones of *CAR* gene-modified lung cancer cells (NCI-H1703 and NCI-H1703 AS6; NCI-H2122 and NCI-H2122S13) were compared for cell aggregation in gyrating suspensions. The relative contribution of CAR or E-cadherin to cell aggregation in the NCI-H2122S13 cells (which express both of these molecules) was assessed with blocking antibodies. Cell aggregation was quantified by the measured decrease in the free particle number in high-powered microscopic fields as described in the Materials and methods section. Depicted are photomicrographs following 1 h in a gyrating suspension. (**a**) NCI-H1703 cells, (**b**) NCI-H1703AS6 (CAR-silenced) cells, (**c**) NCI-H2122 cells, (**d**) NCI-H2122S13 (CAR-overexpressing) cells, (**e**) NCI-H2122S13 cells + RmcB (anti-CAR), (**f**) NCI-H2122S13 cells + anti-E-cadherin. CAR expression does not contribute to NSCLC cell aggregation in this *in vitro* assay.

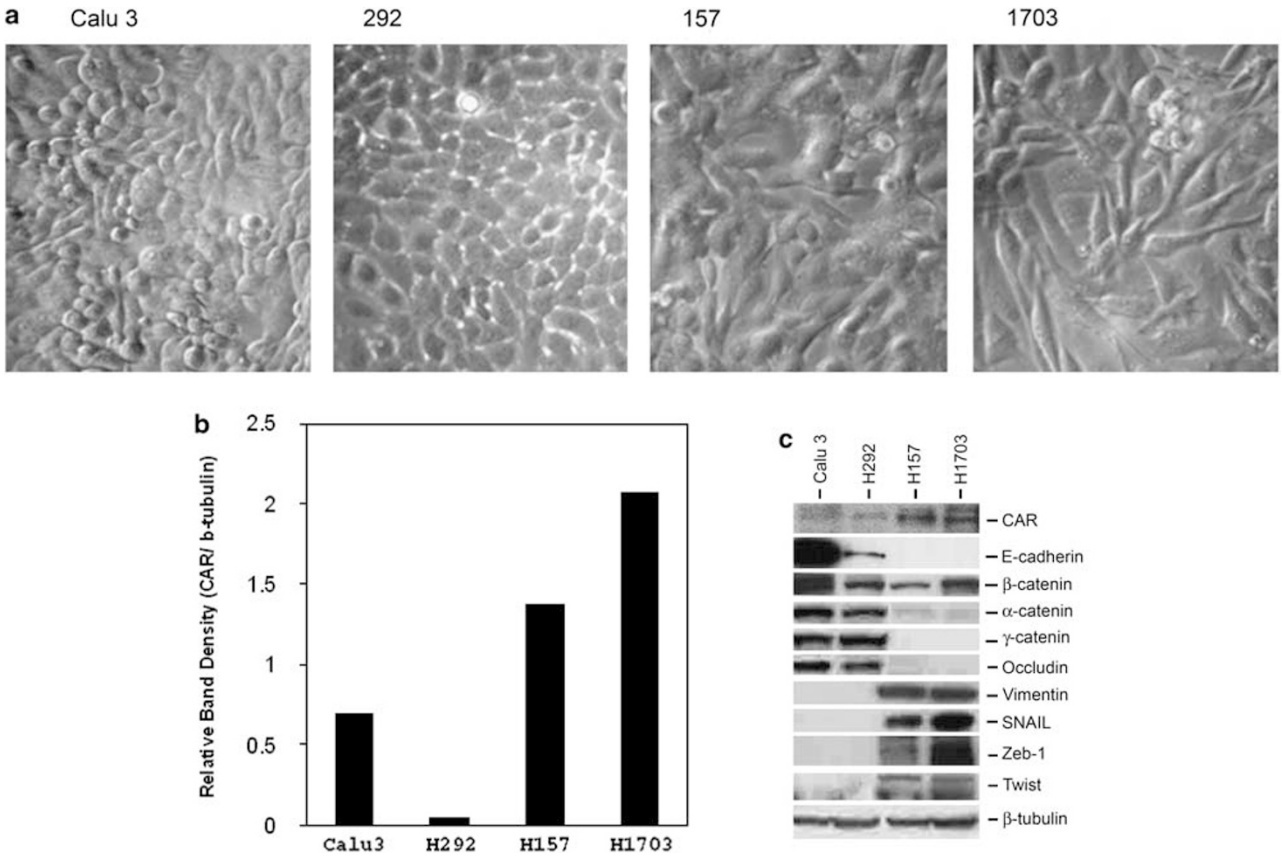

Figure 5 CAR expression is more closely associated with markers of the mesenchymal rather than the epitheliod phenotype of lung cancer cells. Lung cancer cells were grown to a post-confluence state, at which time cell lysates were extracted and quantified for total protein. Protein (40 μg) was separated on a 10% SDS–PAGE gel and probed for markers associated with mesenchymal or epitheliod phenotypes. (**a**) Photomicrographs of confluent cultures of NCI-H292 and Calu3 (epitheliod morphology) *vs* NCI-H1703 and NCI-H157 (mesenchymal morphology) lung cancer cells. (**b**) Graphical depiction of differences in CAR expression, as indexed to control β-tubulin, between the lung cancer cell models. (**c**) Comparative immunoblots of epitheliod *vs* mesenchymal markers in the lung cancer cell models.

CAR Expression Correlates with a Molecular Signature Suggesting a Mesenchymal Phenotype

The function of CAR in conferring the malignant phenotype is unclear. Whereas increased CAR expression has been associated with the malignant conversion of breast epithelium,[28,30] the prevailing view is that CAR is a marker of tumor differentiation.[22,31,32] In lung cancer cells that display high constitutive expression, we have associated CAR with successful engraftment *in vivo*[34] (Figures 1 and 3). Moreover, we were struck by the observation that lung cancer cells that exhibit high CAR are morphologically more akin to mesenchymal rather than epitheliod tumor cells, and the NCI-H2122 cells, which express low CAR, are more representative of epitheliod cells.[49] This observation prompted us to consider the extent to which CAR is evident in lung cancer cells that have been functionally characterized as epithelial. Both NCI-H292 and Calu3 lung cancer cells have been extensively used as experimental models of pulmonary epithelia; these cells polarize in culture, and express functional tight and/or adherens junctions.[35–40] All protein lysates in the lung cancer models were extracted when cultures had reached a post-confluence state (Figure 5a), to minimize the impact of density-dependent differences in marker expression. CAR expression was highest in mesenchymal type (fibroblastoid) cells (NCI-H1703 and NCI-H157 cells; Figure 5a,b), and was directly related to mesenchymal markers (vimentin and transcriptional repressors of E-cadherin) (Figure 5c). Conversely, the confluent epitheliod cell lines poorly expressed CAR by comparison. As a result, CAR expression inversely correlated with junctional (E-cadherin, occludin, and α- and γ-catenin) (Figure 5a and b) protein expression. Total β-catenin expression was not significantly different between the epitheliod or fibroblastoid cells. These data suggest that the intercellular adhesion molecule CAR is more highly expressed in the lung cancer subset that shows mesenchymal characteristics.

DISCUSSION

Malignant transformation and tumor progression is associated with acquired alterations in cell–cell interactions, which impact the organization and functionality of epithelium. Tumors differ from tissues of their epithelial origin by

using proteins that function out of context with tissue development, that have perturbations in the expression and/or cellular localization, or that show abnormalities in physiological (protein–protein) interactions. These processes underlie tumor growth as well as progression to invasion and metastases.

CAR is an immunoglobulin-like single-spanning transmembrane intercellular adhesion molecule that has an essential function in development. CAR's role has primarily been investigated in the context of Ad gene delivery; however, its physiological and pathological functions (eg, in malignancy) remain unclear. Recently, several studies that examine the expression of CAR in human epithelial tumors have been published. For example, a group of investigators has compared CAR immunohistochemistry in prostate epithelium and prostate cancer in a grade-specific and metastases-associated manner. They evidence high CAR expression in prostate epithelium, diminishing CAR with increasing grade of primary prostate cancer, and the re-emergence of high CAR expression in metastases.[22] Their interpretation of these results is that CAR expression may be analogous to E-cadherin with respect to epithelial function and cellular differentiation.[22] Our data and speculative hypotheses are not consistent with this vision of CAR biology (see below). In another study, archived breast cancer tissues showed higher amounts of CAR transcripts when compared to background, and CAR expression trended with an increase tumor grade and metastases.[50] Paradoxically, however, the report also suggested that levels of CAR positively correlated with survival.[50] Similarly, in lung cancer, Wang *et al*[51] suggest that CAR expression may be subtype associated, a finding that is consistent with an observation in cell culture models reported over a decade ago.[19] Thus, whereas squamous-cell and small-cell lung cancers highly expressed CAR; only a minority of adenocarcinomas showed detectable expression. Intensity of CAR labeling did not appear to correlate with tumor grade, and importantly, CAR expression in inflammatory or hemorrhagic lung injury was not observed to be increased.[51] However, in adenocarcinomas of a different tissue origin, a majority of endometrial tumors displayed CAR expression, with both a membranous and a cytoplasmic pattern of distribution.[52] Accordingly, a consensus agreement regarding CAR's role in epithelial function and/or pathological contributions is difficult to glean from these diverse observations and interpretations.

Personal discussions with an investigator (Dr David Beer, University of Michigan) who has molecularly profiled resected lung cancer specimens, combined with our own unpublished preliminary data seem to suggest that CAR is variably expressed in lung cancer cells. Across a large panel of resected lung cancers (thus, representing a sampling bias toward well-differentiated and circumscribed lung cancers), CAR expression is highly variable, not unlike that of E-cadherin. In fact, CAR expression seems to be directly correlated with E-cadherin expression in resected adenocarcinomas, but not lung squamous-cell carcinomas. We speculate that differences in CAR expression are reflective of varying degrees of intratumoral heterogeneity among various tumors. If that speculative hypothesis is correct, then perhaps the most relevant question is not 'what subtype of tumors highly express CAR?', but rather 'what are the distinguishing features of CAR-expressing cells in an individual tumor?' We speculate that the CAR-expressing cell subset may have unique properties that impact clinical behavior (eg, tumor progression and/or metastases). Given our long-standing focus on lung cancer, in which intratumoral heterogeneity is the rule rather than the exception, we seek to examine the functional impact of differences in CAR expression in future studies.

Likewise, the function that CAR has in mediating *in vivo* gene delivery by Ad vectors is being recognized to be increasingly complex. Our earlier work, using model cell lines, indicated that CAR expression is an important variable for efficient gene delivery by Ad vectors.[20] However, we and others have also previously reported that Ad interactions with high-abundance plasma proteins (albumin and globulins) or stromal components possibly impact the bioavailability of the Ad vector *in situ*.[33] Adding to this complexity is a recent report that suggests that CAR expression on erythrocytes possibly serves as a sink to diminish systemic delivery of the vector to target sites (eg, specific organ or tumor).[53] Finally, even if bioavailability challenges can be overcome by altering the viral capsid or the host environment, in light of recognized tumor heterogeneity in lung cancer, we speculate that only a subset of cells within any tumor are likely expressing sufficient CAR and other co-receptors to mediate efficient Ad entry. Thus, even under optimal circumstances, we speculate that only a fraction of tumor cells will be targeted by any Ad or retargeted Ad approaches; however, it remains to be determined whether the fraction of cells that are transduced in this manner will yield a therapeutic benefit.

In this report, we functionally validate an earlier observation[34] that blocking CAR expression in lung cancer cells with high expression of this molecule inhibits their ability to form tumor xenografts in mice (Figures 1, 2, and 3). Thus, our data from lung cancer cells suggest a role for CAR in tumor engraftment, though the mechanism underlying this role remains elusive. This is in part due to the experimental realization that assays that are typically used for ascertaining biological functions (eg, aggregation or barrier function) yield inconclusive results when lung cancer cells are used (Figure 4), and frustrate the effort to definitively ascertain CAR biology in these cells. However, if the mesenchymal cell phenotype is an accurate measure of an undifferentiated and invasive state, then CAR expression seems to be more closely aligned with this phenotype of lung cancer cells (Figure 5). Thus, if these results are an accurate reflection of CAR's biological role, then they implicate a putative role for CAR during EMT, and lend credibility to dynamically testing CAR expression in transitioning models

of epithelia as a better indicator of CAR biology in tumor tissues.

That an cell adhesion molecule like CAR would participate in tumor progression is not surprising, and there is ample precedence for the aberrant expression or activity of adhesion molecules associated with tumor progression.[54] For example, occludin and other TJ components are displaced in *ras*-transformed MDCK cells,[55] and claudin 1 proteins are frequently dysregulated and overexpressed with tumor progression.[56–60] Among the single-pass epithelial transmembrane TJ proteins, JAM, which recruits occludin, claudin, ZO1, and interacts with the PAR complex for junctional assembly and epithelial polarity, is the closest structural analogue of CAR.[61–63] Like CAR, JAM is also suggested to be involved in the EMT during embryonic development.[64] In adult tissues, JAM participates in epithelial–endothelial and epithelial–stromal cell interactions,[65] which can lend themselves to participate in pathological processes, including tumor progression. Thus, JAM-C expression may mediate metastatic spread,[66] and antibody neutralization of JAM-C reduces tumor growth, decreases macrophages infiltration, and inhibits angiogenesis.[67] Interestingly, recent studies suggest that JAM isoforms also interact with CAR in a heterophilic manner to mediate heterotypic cell interactions.[10,68]

Thus, accruing data seem to suggest a more promiscuous function for CAR in mediating interactions between distinct cellular species and tissue origins. Based on these data, we suggest that the characterization of CAR as a homotypic adhesion molecule needs to be broadened.[3,4] How this role is subverted during malignant progression is unknown, but to associate CAR with the 'differentiated state' (because it is an epithelial adhesion molecule in polarized epithelia) is likely incomplete, or incorrect. To suggest that CAR functions solely as a mediator of barrier function in polarized epithelia also does not appear to be consistent with its emerging portrait as a developmentally expressed adhesion molecule in the mammalian brain,[69–71] heart,[8,72,73] and skeletal muscle,[74] nor with its characterization as a mediator of heterotypic cell interactions.[10,68] The fact that CAR is ubiquitously expressed in both epithelium- and mesenchyme-derived tissues (apparently including hematopoietic precursors[53]) during development suggests a more generalized role for CAR. In the adult organism, perhaps, the reexpression of this molecule in epithelial tissues is indicative of a recapitulation of ontogeny following epithelial injury, and/or tumor progression.

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