Published in final edited form as:

Mol Imaging Biol. 2012 August ; 14(4): 480–488. doi:10.1007/s11307-011-0518-y.

Orthotopic expression of Noggin protein in cancer cells inhibits human lung carcinoma growth *in vivo*

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Abstract

Purpose—We explored the effect of Noggin protein expression on tumor growth *in vivo* by using fluorescence imaging.

Procedures—Human lung carcinoma MV522 cells were transduced by using bicistronic (*EGFP/Nog*) or a control (*EGFP*) lentivirus at >95% efficacy. The transduced cells were implanted in athymic mice either individually, or after mixing with DsRed2-expressing MV522 cells.

Results—The expression of Noggin protein was demonstrated in EGFP+/Nog+ but not in EGFP+ cell lysates and conditioned media. Noggin did not inhibit tumor cell proliferation *in vitro*. Implantation of EGFP+ resulted in rapid tumor growth, whereas mice implanted with EGFP+/Nog+ either failed to develop tumors, or developed smaller slowly proliferating ones. In the case of tumors grown from mixtures with DsRed2+ cells only Noggin-expressing cells resulted in decreased tumor volumes with low vascular density and poorly developed stroma.

Conclusion—The effect of Noggin protein expression is a consequence of inhibition of stromal and/or endothelial proliferation *in vivo*.

INTRODUCTION

The majority of current antiangiogenic strategies in the treatment of cancer utilize inhibitors of VEGF-mediated signaling (reviewed in [1, 2]). However, there are alternative pathways that support the formation and expansion of vascular networks. For example, it has been shown that some members of the bone morphogenetic protein (BMP) family enhance the formation and outgrowth of an immature vascular system [3]. It has also been established that BMP-2 and -7 have direct pro-angiogenic roles in prostate cancer [4]. It has been recently reported that BMP-2 in gastric cancer promotes motility and invasion of cancer cells by activating PI3 kinase/Akt concomitant with the changes in epithelial-mesenchymal transition (EMT) markers expression [5]. It has been shown that by treating these cells with Noggin protein the changes in EMT markers in cancer cells were restored [5]. In addition,

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BMPs modulate the expression of stromal cell-derived factor-1 (SDF-1 or CXCL12) by altering transcription levels in cancer-associated fibroblasts [4]. In turn, SDF-1 promotes vascular tube formation by activating microvascular endothelial cells, i.e. SDF-1 provides stimulatory support for very early events directly related to angiogenesis and vasculogenesis [6, 7]. It is known that inhibitors of CXCL12/CXCR4 inhibit both tumor cell migration and vasculogenesis *in vitro* and *in vivo* [8, 9]. Therefore, we hypothesized that due to a powerful inhibition of BMP-mediated signaling the expression of BMP antagonists in tumors can potentially result in the inhibition of tumor growth.

We have recently explored the effects of one of the BMP antagonists, i.e. Noggin protein, using *in vivo* imaging [10]. Noggin is a glycosylated cysteine-knot protein which functions as an extracellular negative regulator of transforming growth factor (TGF- β) superfamily members that include BMPs [11]. Noggin inhibits vasculogenesis even in the presence of pro-vasculogenic VEGF and FGF-2. We demonstrated that orthotopic expression of Noggin in lentivirally transduced HUVECs results in the inhibition of cell migration and prevents the formation of endothelial cords *in vitro* [10]. Culture medium, conditioned by Nog+ HUVECs and containing secreted Noggin protein, disrupted the formation of cords by non-transduced wild type HUVECs. Moreover, using magnetic resonance imaging in live animals, we demonstrated that the formation of functional blood vessels *in vivo* was blocked in adoptively xenotransplanted human Noggin-expressing endothelial cells but not in control endothelial cells [10].

The goal of the current study was to determine the effects of Noggin expression on the proliferation of highly vascularized tumors resulting from transduced non-small cell carcinoma (NSCLC, MV522 line) [12]. Specifically we (a) identified *in vitro* and *in vivo* the effects of stable orthotopic Noggin expression and secretion; (b) imaged changes in tumor growth *in vivo* associated with Noggin expression in MV522 cells; and (c) imaged the effects of neighboring Noggin expressing cells on tumors to assess the potential “bystander effect” of Noggin protein. The latter was achieved by implanting mixed cell populations, i.e. Nog+MV522 and MV522 cells tagged with DsRed2 marker protein. This was accomplished by transfecting MV522 cells with bicistronic lentiviral vectors encoding Noggin and EGFP marker protein, then using single and dual channel fluorescence imaging to track tumor growth *in vivo*.

MATERIALS AND METHODS

Transduction of MV522 cells with *EGFP* and bicistronic *EGFP/Nog* lentiviral vectors

Drug resistant NSCLC MV522 cells [12] were cultured in RPMI containing 10% fetal bovine serum. *EGFP* (enhanced green fluorescent protein encoded in transfer vector CSCGW2) [13] and bicistronic *EGFP/Nog* (CSCW2-hNoggin-IG) lentiviral vectors were kindly provided by Dr. Miguel Sena-Estevés (Department of Neurology, UMASS Medical School). The cells were plated in T25-flasks and transduction was performed when cells reached 50% confluence. *EGFP* lentiviral vector or *EGFP/Nog* bicistronic lentiviral vectors were added to the flask at 100 MOI in the presence of polybrene (final concentration - 8 μ g/ml) and incubated overnight followed by growth medium change. The cells were detached with 0.5 $\times$ trypsin/EDTA and sorted via FACS based on EGFP expression as

markers. The EGFP positive cells were cultured in RPMI containing 10% fetal bovine serum and used for further experiments. Further experiments were carried out with four types of MV522 cells; untreated MV522 (i.e., WT), DsRed2+, EGFP+, and EGFP/Noggin-expressing (EGFP+/Nog+) MV522. The DsRed2-expressing MV522 cell line was previously described [14].

Detection of Noggin expression

Noggin expression in EGFP+/Nog+ MV522 cells was confirmed by FACS using rabbit polyclonal anti-Noggin antibodies (Santa Cruz Biotech, CA). MV522 cells were detached by incubating with enzyme-free cell dissociation buffer (Invitrogen Co.) at 37°C. Before immunolabeling, the cells were permeabilized with 0.5% saponin, 1% horse serum in DPBS (4°C, 30 min) followed by fixation in 4% buffered formaldehyde/0.1% glutaraldehyde. Monofunctional N-hydroxysuccinimide esters of cyanine dyes were obtained from GE-Life Sciences. The following fluorescent dye labeled antibodies were used: 1) for flow cytometry Cy5.5-linked mouse anti-rabbit IgG1 was used as secondary antibody; 2) for fluorescent microscopy of fixed and permeabilized cells anti-rabbit PE conjugate (Pierce) was used; 3) for immunofluorescent detection of Noggin on frozen tissue sections, primary anti-Noggin antibodies were covalently labeled using digoxigenin (DIG) hydroxysuccinimide ester (Roche, Indianapolis IN) and anti-DIG F(ab')₂ (Roche) labeled with Cy3 were used as secondary antibodies.

Western blotting

Cell lysates were prepared by using 0.1% Igepal, 0.01% SDS in 0.1 M Tris, pH 8 in the presence of protease inhibitors mixture (Sigma-Aldrich, St. Louis, MO). Conditioned media were obtained by using 80% confluent cells. After 48–72 h, the media were aspirated and fractionated on centrifugal filters (Amicon® Ultra-4), the fractions were passed through a 50,000 MWCO membrane and were concentrated on 10,000 MWCO, which yielded proteins with molecular weights in the range of 10,000–50,000. Proteins in cell lysates and conditioned media were treated with 5 mM dithiothreitol and resolved in gradient SDS-PAGE plates (4–15%). Western blotting of cell lysates and conditioned media was performed by probing the PVDF membranes with rabbit anti-Noggin antibody (Santa Cruz Biotech, CA) followed by horseradish peroxidase-labeled goat anti-rabbit antibody (Pierce).

In vitro cell proliferation assay

The different types of MV522 ($3 \cdot 10^4$ cells/well), or mixtures of EGFP+MV522 and DsRed+MV522, as well as EGFP+/Nog+MV522 and DsRed+MV522 (equal numbers of cells at $1.5 \cdot 10^4$ cells/well) were plated in 24 well-plates. Cells were trypsinized and counted every 12 hr (n=4, at 6 time points).

Tumor xenograft model

The protocol below was approved by UMASS Medical School Institutional Animal Care and Use Committee in accordance with Federal Regulations for Animal Research. Five suspensions of MV522 cells including EGFP+ (G), EGFP+/Nog+ (GN), DsRed2+ (R), a mixture of EGFP+ with DsRed2+ (GR, 1:1 by number, $5 \cdot 10^5$ cells of each type) and EGFP

+Nog+ with DsRed2+ (GNR, 1:1 by number, $5 \cdot 10^5$ cells of each type) were prepared. Wild-type MV522 (WT) were used as a control. A total of $1 \cdot 10^6$ MV522 cells in serum-free RPMI were subcutaneously injected into the right posterior flanks of female nu/nu mice (NCI) (weight of 19~22 g, n=5) using tuberculin syringes.

Monitoring of tumor growth

Monitoring of tumor growth was initiated 7 days after the implantation of MV522 using two methods: 1) caliper measurements of tumor sizes and 2) measurements of fluorescence of EGFP and DsRed marker proteins by using a Xenogen IVIS100 (XFO-12 fluorescence imaging option, Caliper Life Sciences, Hopkinton MA) and appropriate background-corrected excitation filters. During optical imaging, the mice were anesthetized with 1.5% isoflurane in oxygen. Fluorescence images were displayed in two channels (EGFP and DsRed2) using Live Image (Xenogen) software-generated radiance (p/s/cm²/sr) maps. After in vivo imaging the animals were euthanized and tumors were excised, washed in DPBS and imaged using IVIS. To reduce variability due to field illumination ex-vivo imaged tumors were compared using fluorescent efficiency images. Tumor volumes were calculated with an ellipsoid volume approximation $wl^2/2$ (w ; shorter diameter, l ; longer diameter).

Histology

Dissected tumors were imaged to determine the distribution of EGFP and DsRed fluorescent signals in tumor sections. Microvascular densities in MV522 frozen sections were measured as previously described [14] by using DIG-labeled rat anti-mouse CD31 monoclonal antibody (BD Pharmingen). Histology images were recorded using a color CCD camera-equipped microscope. The images were subjected to segmentation analysis by using IP Lab Spectrum software (Scanalytics Inc., Rockville MD) and the number and the area of segments corresponding to endothelial cell content per tumor tissue section were determined using three non-consecutive sections obtained from two tumors (total n=6/group). The immunodetection of smooth muscle actin (SMA α) and SDF-1 expression was performed by using rabbit polyclonal antibodies (Abcam, Cambridge MA) followed by anti-rabbit-alkaline phosphatase conjugate (Sigma, St. Louis MO). For counterstaining we used Nuclear Fast Red (Vector Labs).

RESULTS

Cell transduction and Noggin expression

We initially transduced MV522 cells with high-titer lentiviruses ($6\text{--}8 \cdot 10^7$ TU/ml). The virus was produced in 293T cells using a CSCGW2 transfer vector [13] encoding either a CMV minimal promoter-driven expression cassette containing human *Noggin* and *EGFP* sequences separated by the internal ribosome entry site (IRES), or a cassette containing *EGFP* sequence only (control lentivirus).

The expression of EGFP (cell transduction efficacy) was initially verified by flow cytometry. The optimized transduction of MV522 cells resulted in 90% cell transduction efficacy for both lentiviruses (Fig. 1A,B). These cells were then subjected to preparative sorting and expansion in culture to further eliminate low-fluorescent sub-fraction.

Immunofluorescent microscopy of saponin-permeabilized EGFP+/Nog+ MV522 cells showed the presence of strong cytoplasmic red fluorescence of anti-Noggin/anti-rabbit-PE conjugate indicating the presence of proteins reactive with polyclonal anti-Noggin antibody (Fig. 1C). The fluorescence of anti-Noggin/anti-rabbit-PE conjugate in the cytoplasm was absent in wild-type (plain MV522 cells), control EGFP+ or DsRed2+ MV522 cells (not shown).

Co-expression of Noggin and EGFP in transduced MV522 cells was analyzed by using flow cytometry of permeabilized cells incubated with anti-Noggin antibody followed by Cy5.5-conjugated secondary antibody. The permeabilized wild-type and EGFP+MV522 cells did not bind anti-Noggin antibodies (Fig. 1D, F). The cells transduced with bicistronic Noggin/EGFP lentivirus showed high levels of Noggin expression (ca. 80% of cells were Cy5.5 positive, Fig. 1E), of these cells about a half was EGFP-positive (Fig. 1E). A comparison of the cytometry results shown in Fig. 1B and 1E suggested a potential loss of EGFP from permeabilized and fixed MV522 cells. Fluorescent microscopy of fixed EGFP+/Nog+ MV522 cells confirmed a partial loss of EGFP marker from these cells since the fraction of these cells was clearly devoid of green fluorescence after the permeabilization with saponin (Fig. 1C). Flow cytometry showed variable and abundant levels of DsRed2+ expression in 97% of stable transfected DsRed2+ cells (Fig 1G).

Noggin protein expression (27 kD band, glycosylated form) was identified by immunoblotting of EGFP+/Nog+ MV522 cell lysate (Fig. 2). Since polyclonal anti-Noggin antibodies reacted also with an additional 42 kD protein band in all MV522 lysates, we used a lysate of EGFP+/Nog+ transduced endothelial cells [10] as a positive control. The protein band in this positive control lysate showed migration similar to that of EGFP+/Nog+ MV522 cells. The non-specific antibody binding was observed only in SDS-denatured lysates treated with DTT. We also observed the presence of Noggin in concentrated and fractionated cell culture medium conditioned by EGFP+/Nog+ MV522 cells but not in EGFP+ MV522 cell-conditioned medium (Fig. 2).

***In vitro* cell proliferation**

We studied the potential inhibitive effect of Noggin expression on MV522 cell proliferation by determining cell numbers in control and Noggin- overexpressing cells in culture. No statistically significant differences in cell numbers measured over 12 h intervals were detected in either Noggin-expressing or control MV522, as well as in the mixed cultures containing equal numbers of Noggin+ and Noggin-negative cells. The doubling time of all cell lines was approximately 44 h (43.3–45.5 h, Fig. 3A).

***In vivo* effects of Noggin-expressing MV522**

To study the potential *in vivo* tumor inhibiting activity of Noggin protein, we prepared five suspensions of MV522 cell variants including wild-type MV522 (WT), EGFP+ (G), EGFP+/Nog+ (GN), DsRed2+ (R), and mixtures of EGFP+ with DsRed2+ (GR) and EGFP+/Nog+ with DsRed2+ (GNR). The abbreviations shown in parentheses above were used in labeling the corresponding groups of mice. The mixtures of cells (GR and GNR) contained equal numbers of each cell type. The mixed cell tumors were grown for assessing the

potential “bystander effect” of Noggin expression. One week after the implantation, the presence of tumor growth was detected in groups WT, G, R and GR by measuring *in vivo* fluorescence using an IVIS camera. Small areas of fluorescence were detected in the lower flanks of all mice of these groups. In addition, fluorescence in these groups of mice was detected in the anterior abdominal region. Caliper measurements were unreliable at the early phase of tumor growth. Tumors in three groups of animals progressed very rapidly and reached volumes of approximately 1.5 cm³ in 14 days (Fig. 3B). The comparison of caliper-measured tumor volumes in live animals (G: 1045±195 mm³, R: 1410±60 mm³, GR: 1215±190 mm³) showed that the difference in tumor volumes between these three groups of tumor-bearing animals was not statistically significant ($P>0.5$).

In contrast, in groups GN and GNR, i.e. in animals injected either with pure or mixed Noggin-expressing MV522 cells, tumors grew at a slow rate, and in some animals, tumor volume was not measureable until 4 weeks post implantation (Fig. 3B). Nonetheless, tumors containing EGFP+/Nog+ cells could be detected by fluorescence imaging. Histology analysis showed that EGFP+/Nog+ MV522 tumors were positive for both cytoplasmic EGFP and Noggin, which was detected in vesicles of the majority of EGFP-positive cells in the field of view (Fig. 3D). The control EGFP+MV522 tumor did not show detectable Noggin expression (Fig. 3C).

The GNR tumors could be identified during the early phase of growth primarily by the presence of weak DsRed2 fluorescence. The areas of tumor fluorescence observed *in vivo* (Fig. 4A) were remarkably smaller than those of tumors in groups G, R and GR. The average caliper-measured volumes of GN (Noggin+) tumors were 30 times lower than G (Noggin-) tumors. The ratio of EGFP fluorescence efficiencies measured in live mice was approximately 450 (G:GN). The “bystander effect” of EGFP+/Nog+ MV522 cells resulted in a 10-times lower efficiency of DsRed2 fluorescence in the GNR group as compared to the GR tumor group. In GNR group of mice the ratio of areas measured in the red channel was 10:1 if compared to GR. Approximately the same ratio of fluorescent tumor areas was measured in green fluorescence channel in G and GN groups (9.4:1). Fluorescence efficiency measurements showed that the ratio of EGFP to DsRed2 fluorescence efficiency in mice implanted with EGFP+ and DsRed2+ cells was 1:1.

After animals were euthanized, we performed *ex vivo* imaging of the dissected tumors (Fig. 4B), which was mostly confirmatory of our *in vivo* observations. The differences between fluorescence efficiencies measured in Noggin+ and control tumors were on the average, lower than those measured *in vivo* due to the presence of additional light-absorbing and scattering layers of tissue in live animals (Table 1). Figure 4B shows fluorescence images of MV522 tumors obtained from two animals in each group in two channels: green (EGFP) and red (dsRed2). Our results suggest that Noggin+ cells exhibited a strong anti-proliferative bystander effect that resulted in smaller areas occupied by red fluorescent cells and smaller tumors in animals from GNR tumor group, an indicative of a decreased number of DsRed2 expressing cells both detectable using fluorescence imaging *in vivo* and *ex vivo*.

To compare microvascular densities and morphology of blood vessels in Noggin- and Noggin+ MV522 tumors, we performed the analysis of tumor frozen sections after staining

with anti-mouse CD31 monoclonal antibody (Fig. 5B,D). The tumors grown by implanting EGFP+ and DsRed2+ cells that did not express Noggin had a high density of blood vessels and overall larger vascular diameters (Fig. 5A,B). In tumors harvested from GN and GNR mice, overall vascular density appeared lower than in G and R mice and contained narrowed blood vessels with considerably fewer endothelial cells spreading away from the vascular lumina. These observations were supported by quantitation of two parameters reflecting vascular density (MVD) and endothelial migration in EGFP+/Nog+ MV522 tumors and comparing them to the wild-type as well as EGFP+ control MV522 tumors (Fig. 5E). We performed segmentation of histology images by using CD31- positive staining as an image segmentation marker in order to quantify the number and area occupied by blood vessels. We determined that on the average MVD in EGFP+/Nog+ tumors was 4-times lower and the tumor section area occupied by vessel-associated CD31+ endothelial cells was 10-times lower than in control tumors (Fig. 5E).

We performed staining of tumor sections for SMA α and SDF-1 antigens in attempt to correlate effects of Noggin expression in tumor cells with morphology of tumor stroma. Tumor xenografts grown by implanting EGFP+MV522 cells contained large areas of stroma populated with tumor-activated myofibroblasts as evident from strong immunoreactivity with the antibody to smooth muscle actin alpha (SMA α) (Fig. 5F). We also observed a strong staining with the antibody against SDF1 α in multiple areas adjacent to stroma in these tumors (Fig. 5H). In MV522 tumors expressing Noggin protein we also detected SMA α positive cells. However, these cells expressed less SMA α , were not organized in stroma and were scattered around the periphery of blood vessels (Fig. 5G). The expression of SDF1 α in Noggin-expressing MV522 tumors was also much lower than in control ones (compare Fig. 5H and 5I).

DISCUSSION

During vertebrate development the expression of Noggin protein plays a critical role in regulating signal transduction mediated by bone morphogenetic proteins (BMPs), members of the transforming growth factor-beta superfamily [15]. There is ample evidence that Noggin is involved in regulation of vasculogenesis and angiogenesis [16, 17]. For example, ectopic implantation of Noggin-secreting cells was shown to inhibit local vascular development [18]. Moreover, under conditions of oscillatory shear stress, the co-expression of Noggin and BMP-4 appears to be involved in controlling pro-inflammatory signaling in endothelial cells [19].

We previously investigated the ability of human endothelial cells transduced with *Noggin*-encoding lentivirus, to form cords on extracellular matrix and thus support the formation of human blood vessels after implanting these cells in athymic animals [10]. In the present study, we set forth to investigate whether orthotopic expression of Noggin in NSCLC cells influences the ability of these tumor cells to form tumor xenografts *in vivo*. We hypothesized that since Noggin secretion appears to interrupt the development of functional vessels in a variety of tissues, a similar lack of functional blood vessel formation around NSCLC cells would result in a delay of xenograft tumor growth *in vivo*.

As in the case of primary human venous endothelial cells that we reported previously [10], it was apparent that the expression of Noggin in MV522 carcinoma cells was neither toxic to transduced cancer cells, nor did the secretion of Noggin cause changes in the rate of proliferation of non-transduced cells *in vitro* (Fig. 3A). Since Noggin is a powerful BMP antagonist [20], our finding suggests that BMP inhibition was not toxic to tumor cells. However, our *in vitro* observations were markedly different from the effects observed after implanting Noggin+ or control cells into mice, in that Noggin showed inhibitory effects on tumor xenograft growth *in vivo* (Fig. 3B). To study the effects of Noggin expression in tumor cells *in vivo* in more detail we further employed fluorescence imaging to follow the progression of EGFP+ or dual fluorescent (EGFP+ and DsRed2+) mixed tumors in live animals.

Despite certain limitations of detecting fluorescence in the visible range in animals due to limited incident and emitted light, the chosen approach was sensitive enough for detecting fluorescent proteins in subcutaneous tumors. The main advantage of fluorescence imaging *in vivo* was in enabling dual channel detection of fluorescent proteins. The feasibility of simultaneous imaging of red and green fluorescent proteins has been previously investigated for planar imaging of gene expression [21] as well as for fluorescent tomography [22]. In our study imaging of EGFP and DsRed2 was necessary for assessing both the direct effect of Noggin expression on overall tumor growth and the effects of Noggin on the growth of tumors from implanted MV522 cells tagged with DsRed2 fluorescent protein (i.e., the “bystander effect”). The potential bystander effect of Noggin+ tumor cells on tumor growth was anticipated since we identified secreted Noggin protein in cell culture medium of EGFP+Nog+ MV522 cells (Fig. 2) and detected Noggin-positive vesicles in the cytoplasm of cells in EGFP+Nog+MV522 tumor sections suggesting that Noggin is secreted by MV522 cells *in vivo* as well (Fig. 3). Since EGFP+Nog+ MV522 did not affect the proliferation rates of DsRed2+MV522 cells in mixed cultures *in vitro*, and the expression of EGFP alone in control EGFP+MV522 did not affect tumor growth, the inhibition of tumor growth as a result of Noggin expression *in vivo* clearly resulted from an indirect “bystander effect”. Given the fact that both microvascular density and the area occupied by endothelial cells in Noggin+ tumors was lower than control tumors (Fig. 5), we concluded that the effect of Noggin expression is potentially a consequence of the inhibition of endothelial migratory activity *in vivo* [3, 10, 18] which was confirmed by semi-quantitative immunohistochemistry (Fig. 5E). In addition, the inhibition of tumor growth by Noggin *in vivo* could have been caused by direct Noggin-mediated interruption of the downstream BMP-mediated signaling in cancer-associated fibroblasts [4]. BMP signaling is important in tumor fibroblasts that secrete SDF-1, a chemokine that supports cancer cell taxis and adhesion [23]. Our immunohistochemistry results suggest that carcinoma-activated tumor fibroblasts (myofibroblasts) were present in both control (EGFP+) and EGFP+/Nog+ tumors. However, there was a striking difference in stroma organization and the number of stroma-associated myofibroblasts (Fig. 5) suggesting that Noggin expression in MV522 tumors interferes with carcinoma-mediated fibroblast activation. Therefore, our *in vivo* results confirm that Noggin expression in carcinomas has an inhibitory role on tumor progression via the disruption of stroma development [16, 24].

Therefore, our results and the results of others [11] indicate that the mechanism of Noggin-mediated BMP antagonism is complex and that the role of BMP antagonists in tumorigenesis should be viewed strictly in the context of tissue type and microenvironment. For example, it has been recently shown that transgenic overexpression of Noggin directed to epidermis in mice results in spontaneous skin tumors as a result of interruption of Wnt and Shh signaling pathways in keratinocytes [25].

In conclusion, we demonstrated that by using *in vivo* fluorescence imaging we could assess the effects of Noggin expression in xenografted lung carcinoma cells. The use of dual fluorescence protein markers enabled the assessment of both direct and bystander effects of Noggin protein expression. Taken together, the results of our study clearly demonstrate that orthotopic expression of *Noggin* potentially inhibits invasive growth of MV522 xenografts *in vivo* due to Noggin-mediated interference with the development of vascular supply and supporting stroma in these tumors.

Acknowledgments

This work was supported in part by RO1 EB000858 grant (A.B.). The authors are grateful to Dr. Miguel Esteves (Department of Neurology, UMASS Medical School) for providing high-titer lentiviral vectors for this study, and to Dr. Mary Mazzanti for editing the text.

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Figure 1. Detection of Noggin and EGFP marker protein in virally transduced MV522 cells
A, B- flow cytometry histograms showing transduction efficacy of *EGFP* (A) and *EGFP/Nog* (B) lentivirus; **(C)** immunofluorescence microscopy of fixed and saponin-permeabilized EGFP+/Nog+ MV522 cells stained using rabbit polyclonal anti-Noggin antibody (Santa Cruz Biotechnology, Santa Cruz CA, USA) followed by anti-rabbit PE conjugate, green – EGFP, blue- DAPI, bar = 10 μ m; **(D–G)** flow cytometry of MV522 (“wild type”, D); EGFP+/Nog+ (E); EGFP+ (F), and DsRed2+ (G) cells. In flow cytometry experiments Noggin expression was detected by using rabbit polyclonal anti-Noggin antibody followed by anti-rabbit-Cy5.5 antibody conjugate.

Figure 2. Immunoblotting of MV522 cell lysates and of conditioned medium

The proteins were detected using a polyclonal anti-Noggin antibody followed by anti-rabbit-peroxidase conjugate (DAB staining). EGFP+/Nog+ lentivirus-transduced human umbilical vein endothelial cells (HUVEC) were used as a positive control. Molecular weight markers are shown at the left (MW). The position of Noggin protein migration is shown with the arrowhead. Arrow - nonspecific binding of polyclonal antibody (NS) which was detected in MV522 cell lysates.

Figure 3. Cell proliferation *in vitro*, tumor growth and Noggin expression *in vivo*
 (A) MV522 cells and their variants (see figure legend) were plated at the total of 3×10^4 cells/well in 24-well plates. The mixtures of EGFP+MV522 and DsRed+MV522, as well as EGFP+/Nog+MV522 and DsRed+MV522 (equal numbers of cells) were plated in 24-well plates. Cells were trypsinized and counted every 12 hr ($n=4$, at 6 time points)-monoexponential fitting, $R^2 > 0.95$. (B) Caliper measurements reflecting tumor growth in athymic mice ($n=4-6$). Tumor volumes were calculated as described in Materials and Methods. All curves were fitted using biexponential growth function ($R^2 > 0.90$) with the

exception of GN tumors (open diamonds) and GNR tumors (DsRed2+ cells mixed with EGFP+/Nog+ cells, closed circles). (**C, D**) fluorescence microscopy of G (**C**) and GN (**D**) tumor frozen sections stained using DIG-labeled anti-Noggin antibodies. Anti-DIG F(ab')₂ labeled with Cy3 was used as secondary antibody (red). Green - EGFP expression; blue – nuclear staining with DAPI. Bars = 100 μ m.

Figure 4. Fluorescence imaging of MV522 tumors *in vivo* and *ex vivo*

(A) Representative fluorescence (live) images of athymic mice bearing MV522 variant tumors (two mice/group, group names are shown above mouse images) obtained by using Xenogen IVIS100. Fluorescence channels are indicated on the left. Images were taken in green (above) and red (below) fluorescence channels; (B) Representative fluorescence images of dissected MV522 tumors (in fluorescence radiance units, p/s/cm²/sr). Images were taken in green (above) and red (below) fluorescence channels, as indicated. G – EGFP+; GN

– EGFP⁺/Nog⁺; R – DsRed2⁺; GR – a mixture of EGFP⁺ and DsRed2⁺ cells; GNR – a mixture of EGFP⁺/Nog⁺ and DsRed2⁺ cells.

Figure 5. Immunohistochemistry of MV522 tumors

Frozen sections of : (A) G; (B) GN; (C) R and (D) GNR tumors. The detection of blood vessels on frozen sections was performed by using DIG-labeled anti-mouse CD31 monoclonal antibody followed by anti-DIG F(ab')₂-alkaline phosphatase conjugates; (E) a comparison of microvascular densities (MVD, left Y axis) and the area occupied by CD31-positive cells (right Y axis) on frozen sections between two controls (MV522 wild type tumors, G tumors) and the GN experimental tumors. The differences in means between two controls were insignificant. The differences in both mean values (MVDs and CD31+ areas) between any of the control groups and the experimental group were significant ($P < 0.05$). Image analysis of 6 sections per group (3 different tumors/ group) is presented; (F, G) immunohistochemical detection of SMA α - positive cells in: (F) G and (G) GN tumors. Frozen sections were stained by using anti-mouse smooth muscle actin (SMA α) antibody. H, I – staining for SDF-1 expression in: (H) control G and (I) experimental GN tumors. Anti-SDF-1 was detected by using a rabbit polyclonal antibody followed by anti-rabbit-antibody-alkaline phosphatase conjugate. Bars = 100 μ m. MV522 tumor samples: G – EGFP +; GN – EGFP+/Nog+; GNR – a mixture of EGFP+/Nog+ and DsRed2+ cells.

Table 1

The results of *ex vivo* tumor fluorescence and size measurements in athymic mice xenografted with single-type (G, GN, R) and mixed type (GR, GNR) MV522 cell suspensions.

Group	Fluorescent efficiency (EGFP) 10 ⁻⁴ *	Fluorescent efficiency (DsRed2) 10 ⁻⁴ *	Area (cm ²)	Wet tissue weight, mg	Number of animals/group
G	9.01±0.07	-	0.85±0.02	1045±194	5
GN	0.02±0.01	-	0.09±0.01	34±12	6
R	-	8.41±0.10	2.03±0.47	1410±60	4
GR	4.77±0.67	4.23±0.85	2.17±0.12	1215±191	5
GNR	0.11±0.02	3.90±1.57	0.09±0.01 **0.25±0.02	263±159	5

* Data presented as mean±SD. Data was obtained using fluorescent efficiency images with the use of background correction filters. Fluorescence efficiency is the fractional ratio of fluorescent emitted photons per incident excitation photon.

** Green fluorescent area represented a minor fraction of total tumor area.