Supplemental Material and Methods

Transplantation of SCLC cell lines. For s.c. transplantation experiments, cells were injected in both flank of mouse and cell lines were injected as single-cell suspensions either as pure populations (NE 5×10^5 cells, NonNE 1×10^5 cells) or mixed at a ratio 4:1 (NE 4×10^5 + NonNE 1×10^5 cells). Balb/c nude mice were imaged on the IVIS200 (CCD camera; Biocompare) once a week. Tumor growth was allowed until a maximum volume of 1500 mm3, and mice were euthanized and tumors and tissues (lung and liver) processed for further analysis. For i.v. transplantation experiment, 5 × 105 cells of NE and/or NonNE cells were injected as pure and mixed populations. After 3 weeks, mice were imaged on the IVIS200, and mice were euthanized and tumors and tissues were isolated for further analysis.

Histology and Immunohistochemistry. Tissues were isolated and fixed in formalin. For histological analysis, fixed tissues were subsequently dehydrated, embedded in paraffin and sections prepared and stained by hematoxylin and eosin (H/E). For immunohistochemistry, tissues were rehydrated, blocked in BSA containing PBS, and incubated with specific primary antibodies, and then stained with biotin-conjugated secondary antibodies (DAKO). Streptavidin-peroxidase (DAKO) or Powervision poly-HRP (Leica Microsystems), were used for visualization. The following antibodies were used: anti-Ncam1 (rabbit polyclonal, Millipore), anti-synaptophysin (rabbit polyclonal, Abcam), anti-vimentin (rabbit polyclonal, Cell signaling). Substrate was developed with DAB (DAKO).

Mouse and Human SCLC cell lines. Clonal cell lines derived from *Trp53*$^{F/F}$;*Rb1*$^{F/F}$ SCLC tumors has been previously described (Calbo et al. 2011). Cells are maintained in modified HITES medium DMEM/F12 (1:1) (GIBCO) supplemented with 4µg/ml Hydrocortisone (Sigma), 5 ng/ml murine EGF (Invitrogen), Insulin-Transferrin-Selenium mix/solution (GIBCO) and 10% Fetal Bovine Serum (GIBCO). Human NIH-H446 cells are maintained in DMEM/F12 (1:1) (GIBCO) and 10% Fetal Bovine Serum (GIBCO). Cells are cultured at 37ºC in a humidified atmosphere of 5% CO_2.

Harvest conditioned medium. For the generation of conditioned medium, NonNE cells, NE cells and Mlg cells were grown in complete modified HITES medium DMEM/F12 (1:1) to 70% confluence. Then the medium was changed and left on the cells for an additional 48 hrs. The NonNE-conditioned medium was harvested, centrifuged to remove any cell debris and filtered through 0.22µm filters (Millipore).

In vitro invasion assay. The invasive ability of NE cells was assessed using BD Biocoat Matrigel Invasion Chambers (24-well, 8 µm pore size; BD Biosciences Discovery Labware) according to the manufacturer's instructions. The lower chamber of the transwell plates were filled with 750 µl of either 10% FBS/DMEM/F12 medium or conditioned medium from NonNE cells diluted with 10% FBS/DMEM/F12 medium or different amount of mouse Fgf2 (R&D Systems) containing 10% FBS/DMEM/F12 medium, and the plate was incubated at 37ºC with 5% CO_2 for 48hrs. NE cells invading the lower surface of the membranes were fixed, stained with crystal violet, and counted. The cell number was non-invading cells were removed from the upper surface of the membrane with cotton swabs. At least four separate microscopic fields were counted per membrane, each performed in triplicate and statistical significance was determined using the student t-test.

Gene expression analysis. RNA extraction and all subsequent procedures were performed at the Central Microarray Facility of the Netherlands Cancer Institute following validated and standardized protocols that are available online (http://microarrays.nki.nl). 32K mouse oligoarray (Operon Biotechnologies Inc.) were used for the relative quantification of mRNA expression profile, taking the Universal Mouse reference RNA (Stratagene) as reference.

Overexpression or Knockdown experiments. For stable overexpression of the Pea3 gene, the fragment encoding the full-length sequence-verified cDNA of Pea3 was obtained from Open Biosystems was cloned into the pMSCV-puro retroviral vector (Invitrogen). Knockdown of target gene was achieved through the use of lentiviral vector mediated shRNA interference using MISSION RNAi system clones (shPea3-1, TRCN0000295522; shPea3-2, TRCN0000295465; shPea3-3, TRCN0000306806; shFgf2-1, TRCN0000067283; shFgf2-2, TRCN0000067287; shControl, TRCN306806; non-target shRNA control, SHC002) from Sigma-Aldrich. Plasmid DNAs were transfected either PlatE 293T retrovirus packaging cell line or 293FT lentivirus packaging cell line (Invitrogen) along with lentiviral packaging plasmids. Positive NE cells

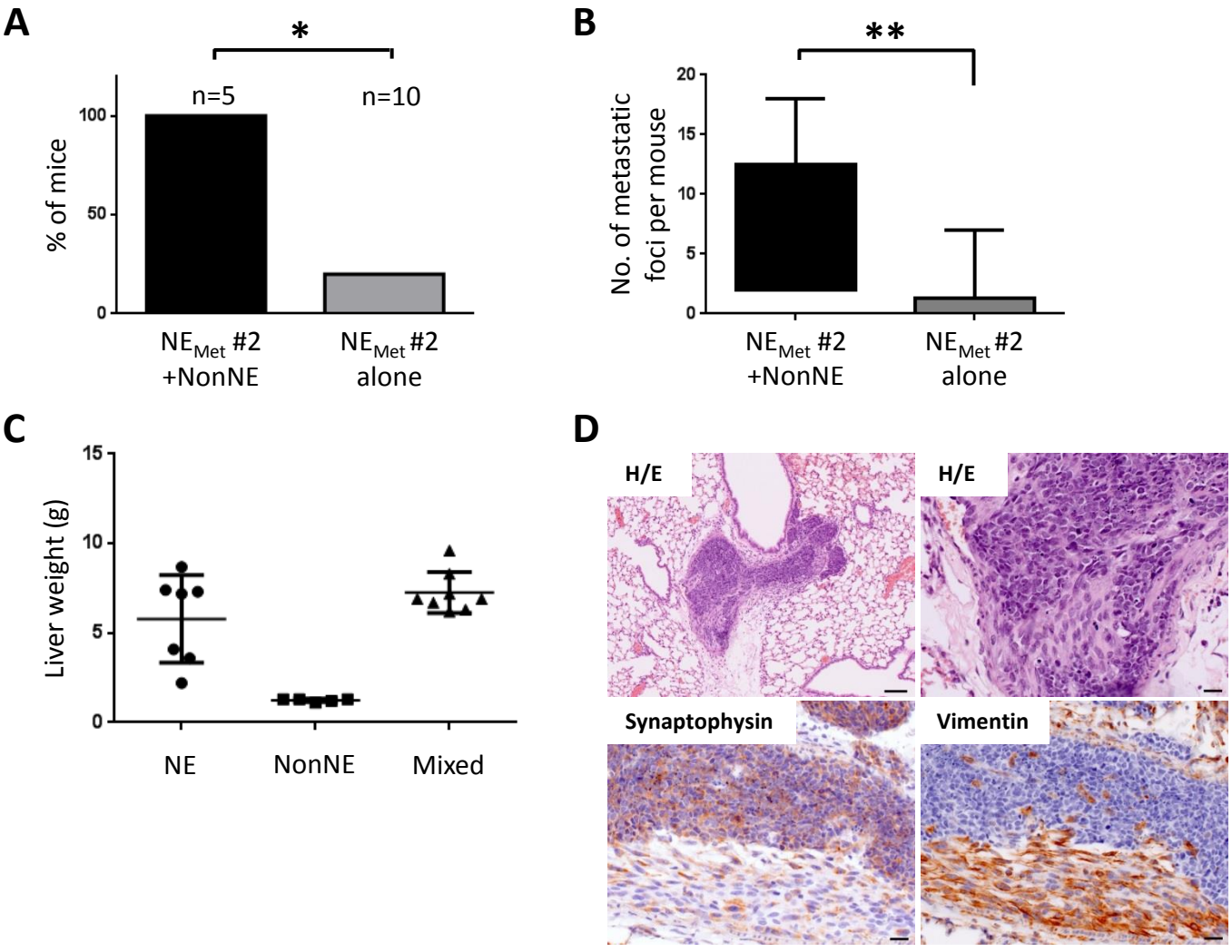
expressing Pea3 cDNA or Pea3 shRNA cassette were selected by 1.8 μg/ml puromycin (P8833, Sigma-Aldrich) after infection of either retrovirus or lentivirus.

WB analysis. For western blot analysis, whole cell lysates were mixed with IP buffer [50 mM HEPES/NaOH (pH 7.5), 3 mM EDTA, 3 mM $CaCl_2$, 80 mM NaCl, 1% Triton X-100, 5 mM DTT] and separated by 4-12% NuPAGE Bis-Tris Gel (Invitrogen). Proteins were transferred to a nitrocellulose membrane and visualized by Supersignal West Pico Chemiluminescent substrate (Thermo Scientific) using following primary antibodies against: Pea3 (rabbit polyclonal; Abcam); HA hemagglutinin tag (mouse monoclonal; Covance Research Products Inc.); β-tubulin (mouse monoclonal; Klinipath). Erk (rabbit polyclonal; Cell signaling) and Phospho-Erk (mouse monoclonal; Cell signaling) were visualized and quantified using the Odyssey Infrared Imaging System (LI-COR, Inc.). For Odyssey, membranes were incubated with fluorescent-labeled secondary antibodies (IRDye 680 Goat anti-Rabbit IgG and IRDye 800CW Goat anti-Mouse IgG; Westburg).

qPCR analysis. Total RNA from cells was extracted using an RNeasy Mini Kit (Qiagen) and cDNA was prepared using the Superscript II reverse transcriptase (Invitrogen) according to manufacturer's instructions. For real-time PCR, cDNA was subjected to 40 cycles of amplification either using Applied Biosystems TaqMan gene expression assays (mouse Pea3, Mm00476696_m1; human Pea3, Hs00383361_g1; Actin, Mm00607939_s1) or the Applied Biosystems SYBR Green expression assay (Pea3, 5'- agccccctatcaacaccaac-3' and 5'-agccataacccatcactcca-3'; Actin, 5'- aaatcgtgcgtgacatcaaa-3' and 5'-aaggaaggctggaaaagagg-3'; Fgf2, 5'- ccaagcagaagagagaggagtt-3' and 5'- tgtccaggtcccgttttg-3'; Fgf7, 5'- tgcttccacctcgtctgtct-3' and 5'- cctttcactttgcctcgttt -3'; Fgf10, 5'- tgttgctgcttcttgttgct-3' and 5'- ctgaccttgccgttcttctc -3'), as per the manufacturer's instructions. Data on the quantity of target RNA was normalized using the data for Actin in each sample.

ELISA experiment. NonNE cells were cultured for 48 hrs in serum-free DMEM/F12 medium and the conditioned medium samples (cell free supernatant) were analyzed for concentrations of mouse Fgf2 using mouse Fgf2 ELISA kit (Abcam). ELISA was performed as per the manufacturer's instructions.

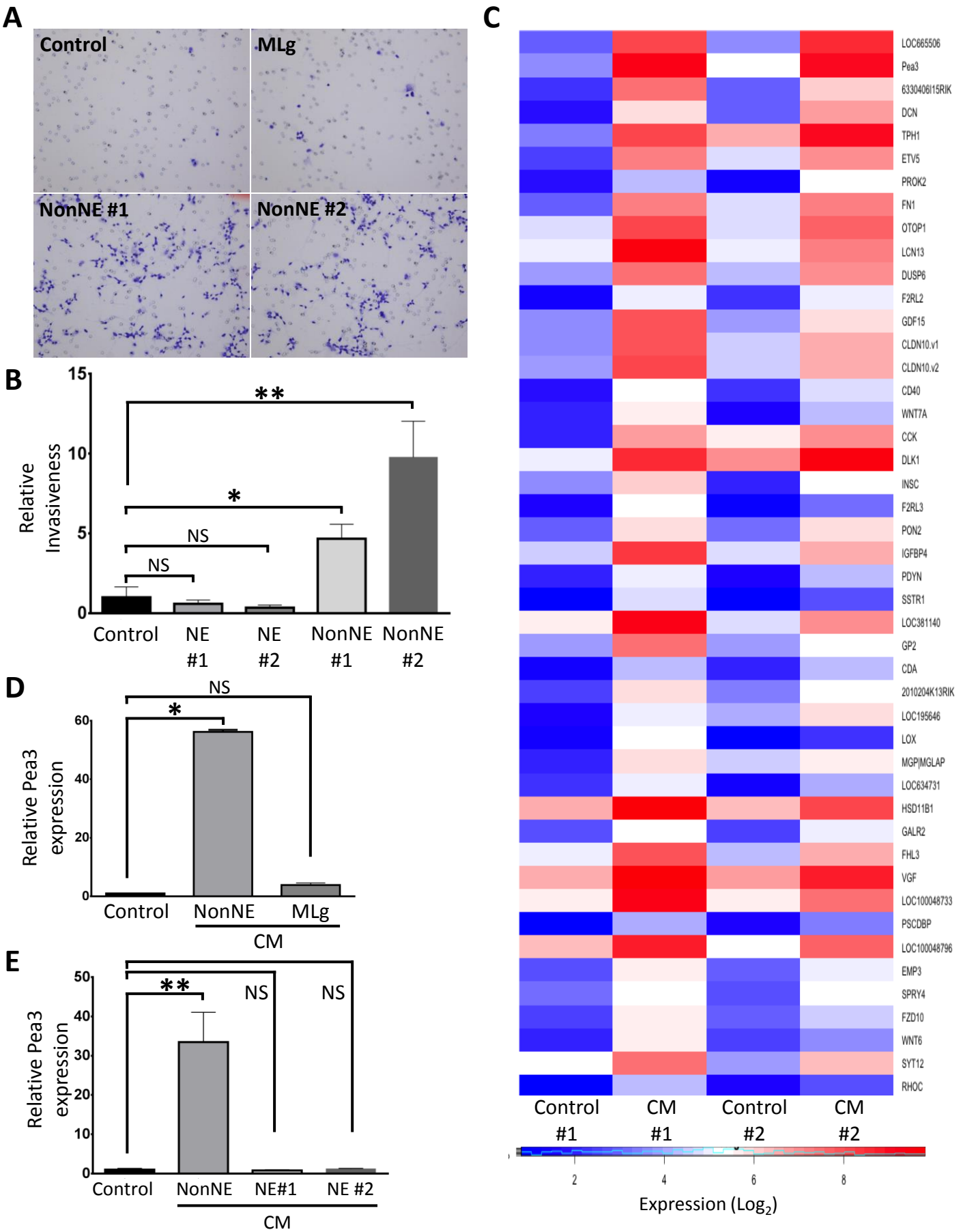
Kwon et al. Supplemental Fig. 1



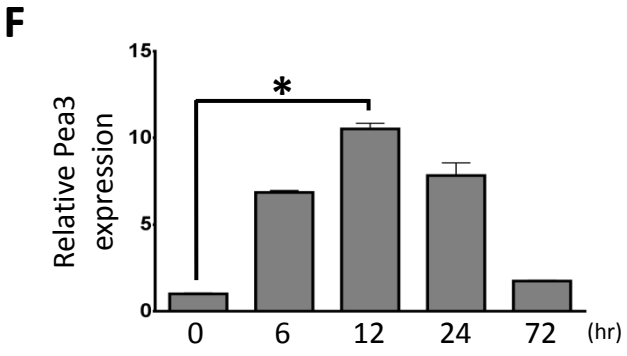
Supplemental Figure 1.

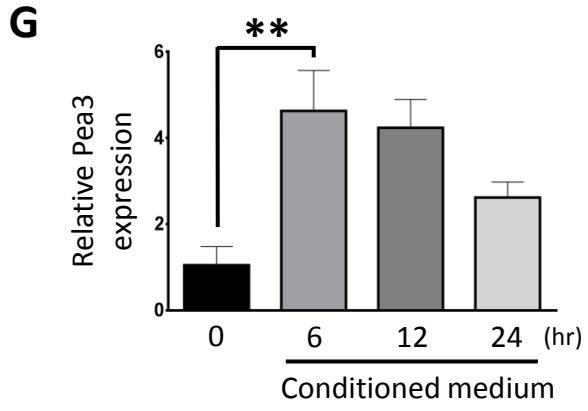
(A, B) Different NE cells from liver metastasis were tested for their metastatic potential. Either NE_{MET} cells alone or NE_{MET} cells mixed with NonNE cells were subcutaneously injected and mice with liver metastasis were counted (A). Number of metastatic foci per mouse was calculated (B). Error bars indicate SD. *$p < 0.005$, **$p < 0.05$. (C) Weight of liver from each mouse was measured and large tumor burdens were seen in both the NE cells only and mixed cells injected intravenously. Error bars indicate SD. (D) Rare example of a lung metastasis with outgrowth of both NE and NonNE cells after IV injection of the mixture (upper images) and existence of NE cells (Synaptophysin) and NonNE cells (Vimetin) was confirmed by the immunohistochemistry with the specific antibodies. Scale bars are 100 µm, upper left and 20 µm, upper right, lower left and right.

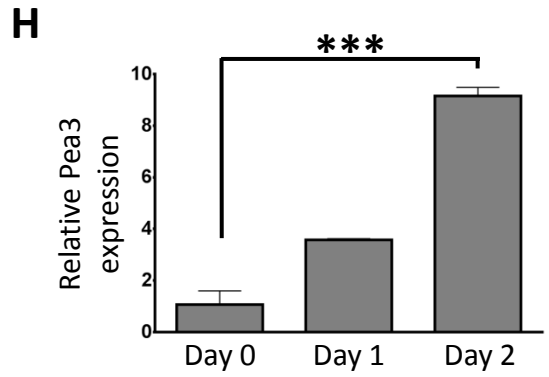
Kwon et al. Supplemental Fig. 2

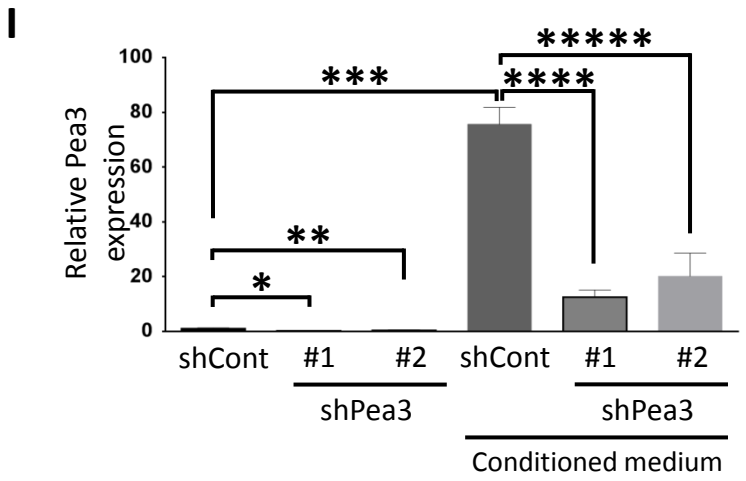


Kwon et al. Supplemental Fig. 2 (continue)





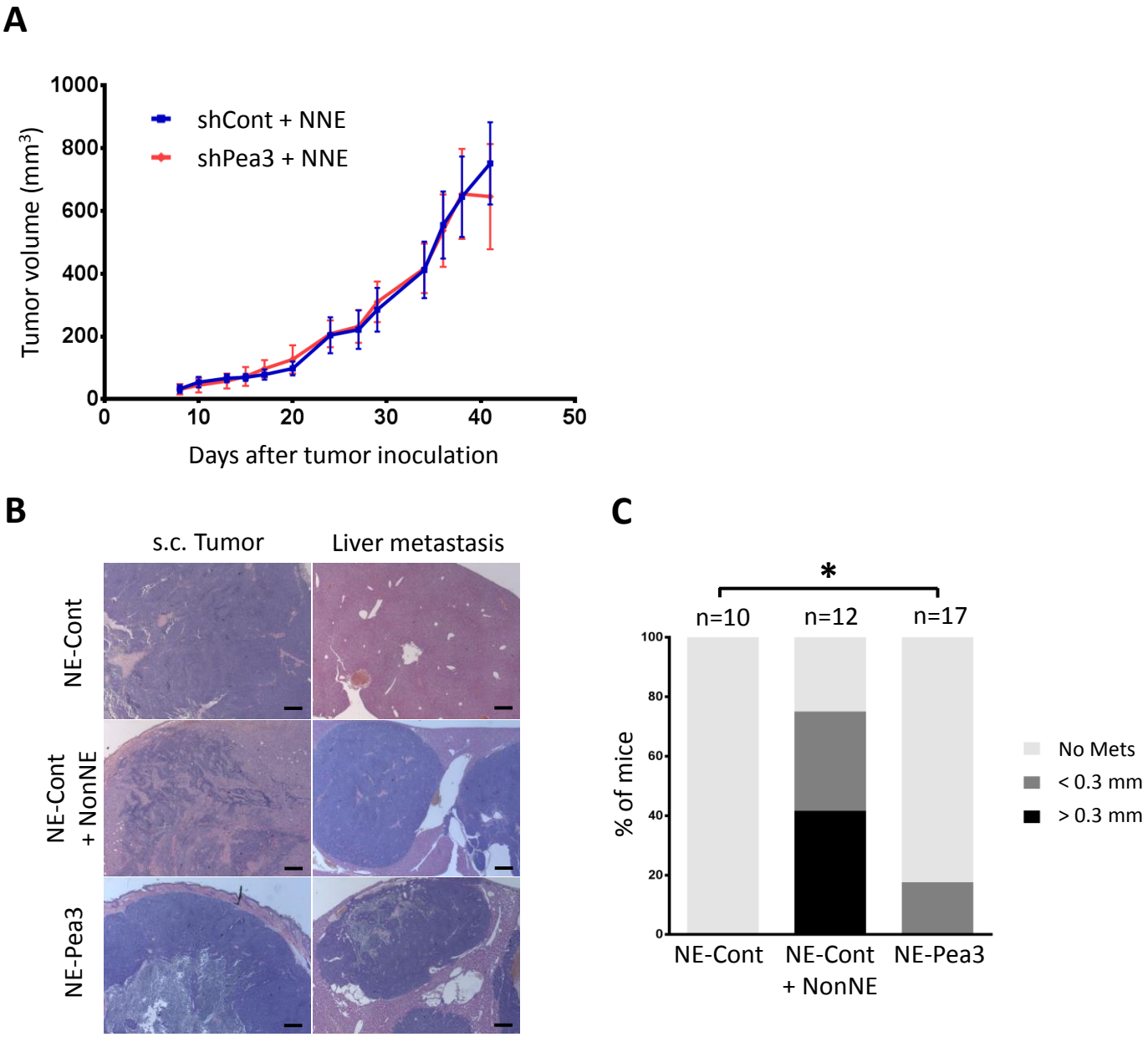




Supplemental Figure 2.

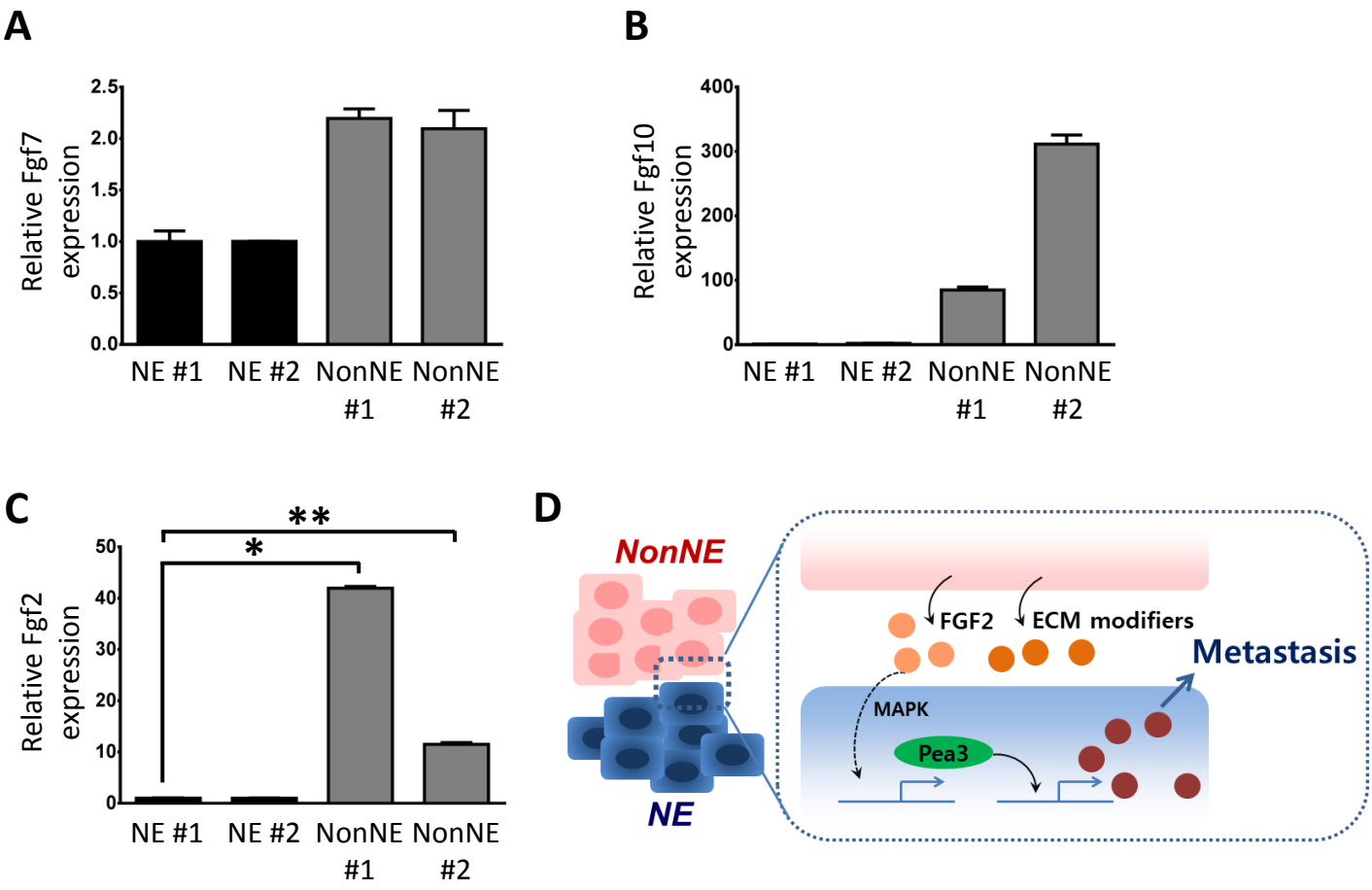
(A) Typical field of a Matrigel invasion insert showing the increased number of invading NE cells upon co-culture of NonNE cell clones or mouse lung fibroblasts (MLg) in the lower compartments of matrigel coated modified Boyden chambers. Note the lack of invasion activity of NE cells by conditioned medium from MLg mouse lung fibroblast cells (upper right panel) (A). (B) Conditioned medium from NE cells did not promote the invasion of NE cells, while conditioned medium from NonNE cells significantly increased the invasion activity of NE cells. NS, not significant. Data are representative of two independent experiments. *$p < 0.001$, **$p < 0.0005$. (C) The expression profile from two NE cell clones upon treatment with conditioned medium from NonNE cells. Heat map representing the genes induced at least 5 average fold in comparison with complete medium treated control NE cell clones. Note that Pea3 transcription factor shows the second highest fold induction. (D, E) NE cells were cultured with conditioned medium (CM) from indicated cell lines, harvested and Pea3 mRNA expression was measured. Relative expression levels were calculated with complete medium treated NE cells as reference (Cont). Note that conditioned medium from MLg mouse lung fibroblast cells (MLg) and NE cells did not induce Pea3 expression. Data are representative of two independent experiments. NS, not significant. *$p < 0.0001$, ** $p < 0.0001$. (F) Dynamic induction of Pea3 expression by CM from NonNE cells. Data are representative of two independent experiments. (G) Previously established CD44- NE cells and CD44+ NonNE cells from human NIH-H446 SCLC cell line (Calbo et al. 2011) was used to examine the Pea3 expression upon treatment of conditioned medium of human NonNE cells. Note that human NE cells also showed the increased expression of Pea3 in conditioned medium from human NonNE cells. Data are representative of two independent experiments. (H) Mixed NE + NonNE (4:1) cell cultures were sorted by removing NonNE cells using binding to magnetic beads coated with anti-CD44 antibodies and Pea3 expression of isolated NE cells was measured after indicated time of co-culture. Data are representative of two independent experiments. *$p < 0.001$, ** $p < 0.005$, *** $p < 0.001$. (I) Pea3 knockdowned NE cells were treated with conditioned medium and expression of Pea3 was measured. Data are representative of two independent experiments. *$p < 0.05$, ** $p < 0.05$, *** $p < 0.0001$, **** $p < 0.0001$, ***** $p < 0.001$.

Kwon et al. Supplemental Fig. 3



Supplemental Figure 3.
(A) Measurement of tumor volume after subcutaneous injection of shRNA control and shPea3 expressing NE cells with NonNE cells. Data are presented as mean ± SD. (B) Representative H&E staining images of s.c. tumors and liver metastasis sections after inoculation of NE cell alone (upper), mixed NE-Cont and NonNE cells (middle), and HA-Pea3 overexpressing NE cells (lower). Scale bars are 200 μm. (C) Same experiment as in Fig 5D, but with different NE cell clone. *p < 0.3.

Kwon et al. Supplemental Fig. 4



Supplemental Figure 4.
(A and B) Expression of Fgf7 (A) and Fgf10 (B) was measured by qRT-PCR from various NE and NonNE cells. (C) NonNE cell clones express higher levels of Fgf2 compared with NE cell clones as measured by qRT-PCR analysis. Data represent mean ± SEM. Data are representative of three independent experiments. *$p < 0.0005$, **$p < 0.0005$. (D) Scheme depicting the Fgf2/MAPK/Pea3 signaling pathway between NE and NonNE cells for the metastasis of NE cells. Fgf2, one of the secreted factors from NonNE cells, and activation of MAPK signaling pathway induces the expression of Pea3 and enhances the invasiveness of NE cells. However, only half of the mice subcutaneously grafted with Pea3 overexpressing NE cells showed liver metastasis indicating that Pea3 is required but not sufficient to fully substitute for the factors contributed by NonNE cells. Interestingly, LC-MS/MS secretome analysis revealed that NonNE cells secret many extracellular matrix (ECM) remodeling factors such as Protein-lysine 6-oxidase (LOX) (Barker et al. 2012), Cathepsin (Tan et al. 2013), and Plasminogen activator inhibitor 1 (PAI-1) (Croucher et al. 2008) that could modify the tumor microenvironment and facilitate tumor cell invasion and metastasis (MC, Kwon et al., unpublished data). Lack of such ECM modifiers in a clonal population of Pea3 overexpressing NE cells might therefore dampen their metastatic spread from subcutaneous primary tumors.

Supplemental References

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