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Horizontal transfer of tumor DNA to endothelial cells *in vivo*

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Tumor endothelial cells have long been regarded as genomically stable and therefore less likely to develop resistance to antiangiogenic therapies. However, recent findings have challenged this notion. We have shown that DNA can be transferred between cells through phagocytosis of apoptotic bodies by adjacent viable cells. Propagation of the ingested DNA is prevented by the activation of the p53–p21 pathway. In this study, we examined whether concomitant transfer of tumor DNA with genes that inactivate the p53 pathway could overcome the barrier to tumor DNA propagation. Our results demonstrate that fibroblasts and endothelial cells are capable of acquiring and replicating tumor DNA when the apoptotic tumor cells contain the SV40 large T antigen. Analysis of the tumor stroma of xenotransplanted tumors in severe combined immunodeficient mice revealed that a sub-population of the endothelial cells contained tumor DNA. These cells maintained the ability to form functional vessels in an *in vivo* assay and concurrently express tumor-encoded and endothelial-specific genes.
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Malignant and non-malignant cells are dependent on a functional blood circulation for the supply of oxygen and nutrients.[1] Evidence from transgenic mouse models demonstrate that tumors initially lack the ability to induce blood vessel formation but subsequently acquire the capacity to recruit vessels from the adjoining stroma.[2] This 'angiogenic switch' coincides with increase in tumor mass and invasiveness. Tumor cells secrete a plethora of angiogenic factors that induce sprouting angiogenesis (i.e. the formation of new capillaries from pre-existing capillaries) and the differentiation of hematopoietic stem cells into endothelial cells, which then contribute to tumor vessel formation.[3]

A potential advantage in targeting the endothelial cells of the tumor vasculature is that these cells are regarded as diploid and genetically stable. However, recent data have challenged this notion. Uveal melanomas have been reported to form their own circulatory network by the formation of vascular channels lined by tumor cells.[4] These vasculogenic channels apparently link directly to normal vessels without evidence of angiogenesis. Streubel *et al.*[5] analyzed the cellular origin of the microvascular endothelial cells of B-cell lymphomas in patients. Chromosomal translocations specific for the tumor cells were detected in the tumor endothelium, suggesting a close relationship between the two cell types. Furthermore, analyses of human tumors transplanted in severe combined immunodeficient (SCID) mice demonstrate that the endothelial cells of solid human tumors grown in mice are genetically unstable and exhibit aneuploidy with multiple

chromosomes and centrosomes.[6] Cumulatively, these studies potentially imply that tumor endothelial cells exhibit genetic instability albeit the mechanisms by which genetic alterations are induced are presently unclear.

Our previous studies and those of others have demonstrated that viral and chromosomal DNA can be efficiently transferred between somatic cells through the uptake of apoptotic bodies.[7–10] However, replication of the DNA is effectively blocked by the activation of the p53–p21 pathway.[10–12] We have also reported that mouse embryonic fibroblasts (MEFs) deficient in the *p53* gene or its transcriptional target *p21* differ from normal cells as they are able to replicate DNA taken up from apoptotic bodies.[10–12] Recent preclinical and clinical findings indicate that the tumor stroma carry p53 mutations or alterations.[13–19] This observation further supports the likelihood that gene transfer occurs between tumor cells and stroma.

In this study, we explore whether tumor DNA can be transferred to endothelial cells within the tumor microenvironment. We demonstrate that DNA from apoptotic cells from rat fibrosarcoma cells expressing H-ras^{v12}, human c-myc and SV40 large T (SV40LT) is transferred to and transforms primary fibroblasts and endothelial cells *in vitro*. Analysis of tumor stroma from fibrosarcoma tumors grown in mice revealed that mouse stromal cells contained tumor DNA. Purified murine tumor endothelial cells expressed rat fibrosarcoma-derived SV40LT and were immortalized when grown in culture. Injection of these tumor-derived endothelial cells together with Matrigel revealed that they could generate a

functional vascular network. These data indicate that functional gene transfer between tumor cells and endothelium may be a novel mechanism by which tumors promote angiogenesis.

Results

DNA can be horizontally transferred *in vitro* from apoptotic tumor cells to fibroblasts and endothelial cells. In a previous report, we have demonstrated that DNA from apoptotic cells may be salvaged and reutilized by phagocytosing cells. The horizontally transferred DNA persists in normal diploid cells for up to 2 months but it is not replicated.[7] Apoptosis induction in the donor cells emerges to be a critical event preceding gene transfer as no horizontally transferred DNA was detected when living or senescent cells were used.[7–9] An essential role of the p53 pathway is revealed by the findings that DNA from apoptotic

tumor cells is transferred and replicated in p53- or p21-deficient recipient cells.[10,11] We examined whether transfer of tumor oncogenes that dominantly inactivate p53 would allow replication of transferred genes in normal cells. For this purpose, rat fibrosarcoma tumor cells expressing H-ras[v12] and c-myc were stably co-transfected with the SV40LT oncogene (which inactivates the Rb and p53 tumor suppressor proteins) together with the gene encoding puromycin resistance (REFs transfected with ras, myc and SV40LT and the gene encoding puromycin resistance (REFrmLTp[r])).[20] As controls, the same rat fibrosarcoma tumor cells were stably transfected with or without the gene encoding puromycin resistance (named REFs transfected with ras, myc and the gene encoding puromycin resistance (REFrmp[r]) and REFs expressing the H-ras[v12] and human c-myc genes (REFrm), respectively). Protein expression and integration of SV40LT in REFrmLTp[r] were verified by immunofluorescence and fluorescence *in situ* hybridization (FISH) analyses (Figure 1a and b). Apoptosis was induced
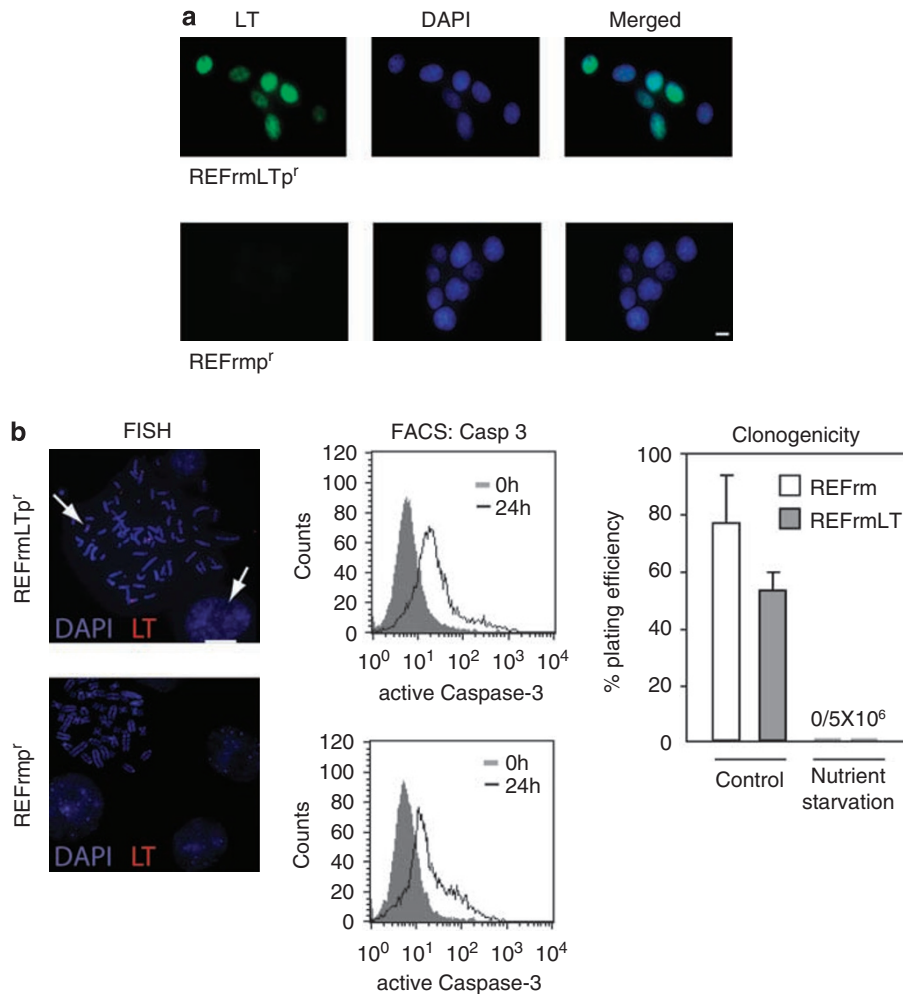

Figure 1 REFrmLTp[r] cells exhibit SV40LT integrated into their genomes, express SV40 large T and undergo apoptosis similarly to REFrmp[r] cells upon nutrient depletion. Rat embryonic fibroblasts stably expressing H-ras[v12], c-myc, SV40LT and puromycin resistance (i.e. REFrmLTp[r] cells) express the large T (LT) antigen (a). Fluorescence *in situ* hybridization (FISH) analyses with an SV40LT-specific probe demonstrate integration of SV40 LT into the genome of REFrmLTp[r] cells, indicated by arrows (b). REFrmLTp[r] cells undergo apoptosis in a similar manner as REF stably transfected only with puromycin resistance (i.e. REFrmp[r] cells) upon nutrient depletion, as illustrated as shown by caspase 3 activation and annexin V positivity (b; Supplementary information) and cell survival by clonogenicity (representative picture from three independent experiments) (b). Size bar = 10 μm

by nutrient depletion, leading to the activation of caspase 9 and positivity for annexin V (Figure 1b; Supplementary Figure 1). No difference in the levels of apoptosis between the SV40LT-expressing REFrmLTp[r] cells or the SV40LT-deficient REFrmp[r] cells could be detected (Figure 1b). Overnight nutrient depletion led to 100% cell death as analyzed in a clonogenic assay (Figure 1b).

To evaluate transfer of tumor DNA to MEFs or bovine aortic endothelial cells (BAEs), we induced apoptosis in our transfected rat fibrosarcoma cells by nutrient depletion and added the resulting apoptotic bodies to the recipient cells. Functionality of transferred genes was assessed either by selection with puromycin or by the loss of contact inhibition and consequent formation of foci. The number of puromycin-resistant colonies was counted after 3 weeks of selection. The REFrmLTp[r] cell line gave rise to a mean of 156 ± 27 puromycin-resistant colonies per 10-cm Petri dish when MEF cells were used as recipient cells and 1494 ± 235 per 10-cm Petri dish when BAE cells were used as recipient cells (Figure 2a). No colonies were formed when apoptotic REFrm cells were used as donors. Immunofluorescent staining confirmed that the colonies expressed SV40LT (Figure 2b). The cellular origins of the puromycin-resistant colonies after co-cultivation of REFrmLTp[r] and MEF were determined using a mouse-specific genome probe (Figure 2c). To control specificity of FISH, we analyzed these mouse/rat genomic DNA mixtures on the slides, which were made from a mixture of MEF and REF cells. Positive and negative controls were used in all experiments and no cross-hybridization was detected in the approximately 10 000 cells analyzed per slide (Figure 2d).

We also investigated whether transfer of DNA from REFrmLTp[r] apoptotic cells could induce loss of contact inhibition. Consistent with our previous findings,[10] the REFrmp[r] cells failed to affect focus formation in MEF cells (Figure 2e). In contrast, addition of REFrmLTp[r] apoptotic bodies resulted in a mean of 12 ± 6 foci per 10-cm Petri dish. Analysis of metaphase spreads of cells derived from these foci using rat- and mouse-specific probes revealed uptake of entire rat chromosomes, chromosomal fragments reminiscent of double minutes, as well as rat–mouse chromosomal fusions (Figure 2f).

Tumor DNA can be horizontally transferred to stromal cells *in vivo*. We subsequently analyzed whether DNA could be spontaneously transferred in a similar mode between tumor and stromal cells *in vivo*. With this intent, REFrmLTp[r] or REFrmp[r] cell lines were injected subcutaneously into SCID mice. Tumor engraftment occurred in all of the injected animals that were killed when the tumor diameter reached 15 mm. Tumor cryosections were analyzed with a mouse genome-specific probe in combination with an SV40LT DNA probe to delineate whether the murine cells contained tumor-derived SV40LT. Approximately 4–5% of cells were noted that hybridized with the mouse genomic probe and were positive for SV40LT (Figure 3a). To eliminate artifacts ensuing from DNA smearing during cryosectioning, we analyzed tumor and stromal cells isolated by collagenase digestion. FISH analysis established that 5.6% of the cells that hybridized

the murine-specific probe were also positive for SV40 LT (Figure 3b and d). These data reflect gene transfer that has occurred *in vivo* as we could not detect any horizontal gene transfer under similar conditions *in vitro* where BrdU-labeled tumor cells were incubated with primary mouse endothelial cells (Supplementary Figure 2). To estimate the amount of tumor DNA in the murine host cells, we used a combination of mouse and rat genome-specific probes. The results demonstrated that rat–mouse double-positive nuclei contained whole chromosomes of both species. Interestingly, similar to our *in vitro* findings in Figure 2d, in metaphase chromosomal spreads we could detect fusions of mouse and rat chromosomes (Figure 3c).

Isolation and propagation of endothelial cells containing tumor DNA. We further investigated whether DNA was transferred to tumor endothelial cells and whether they could maintain an endothelial phenotype *in vitro*. For this purpose, we immunomagnetically enriched endothelial cells from collagenase-treated tumor cell suspensions using a mouse-specific CD31 rat monoclonal antibody. The isolated cells were cultured in endothelial cell medium supplemented with vascular endothelial growth factor (VEGF) as described in Materials and Methods. The resulting colonies were harvested and examined by flow cytometry to establish their cellular origin. The cells showed positivity for the endothelial markers PECAM1 (CD31), sialomucin (CD34), vascular endothelium (VE)-cadherin (CD144) and VEGF-R2. No positive staining could be detected with the stem cell marker CD133, thus suggesting that the cells were mature endothelial cells and not endothelial progenitor cells (Figure 4a). We further analyzed whether expression of the tumor-derived SV40LT was detectable in the tumor-associated endothelial cell lines (T-EC) by dual staining with antibodies against SV40LT and CD31. The cloned cell lines exhibited dual positivity for SV40LT and PECAM1, as determined by flow cytometry and immunofluorescent staining (Figure 4b and c). The REFrmp[r] cells and immortalized mouse endothelial cells (PmT-EC) served as positive and negative controls, respectively. FISH analysis performed on the isolated clones with probes specific for the mouse and rat genomes demonstrated a blend of DNA from both the species with both intact chromosomes and fusions between mouse and rat chromosomes (Figure 4d). Although these cells contained tumor-derived DNA, they were not tumorigenic when inoculated in SCID mice (data not shown).

SV40LT endothelial cells form functional vascular networks *in vivo*. An important issue pertains to the functional ability of the endothelial cells containing tumor DNA to actively participate in the formation of a microcirculatory network. Tube-forming capacity of T-EC *in vivo* was examined using an assay in which Matrigel plugs are implanted subcutaneously into SCID mice.[21] T-ECs were added to Matrigel as a single-cell suspension. In one group, fibroblast growth factor-2 (FGF-2) was also included in the matrix to promote the recruitment of host vessels. The Matrigel plugs were retrieved 7 days after injection, photographed and the histology was analyzed by light microscopy. T-EC injected with Matrigel formed small
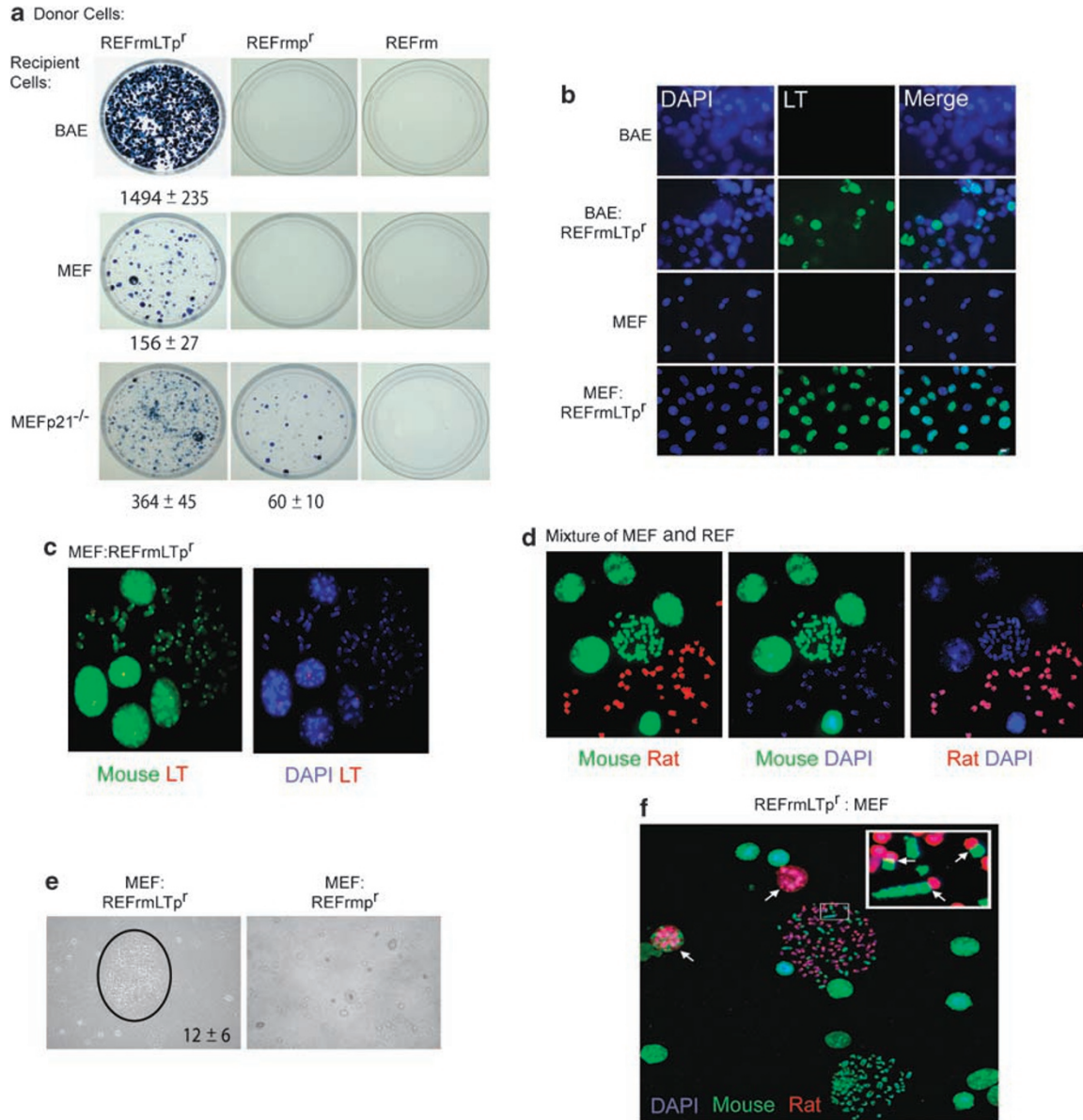
Figure 2 Stable transfer of puromycin resistance into primary MEF and BAE cells through apoptotic cells. Mouse embryonic fibroblast (MEF) or bovine aortic endothelial (BAE) cells were co-cultured with apoptotic REFrmLTp^r or REFrmp^r cells. After selection with puromycin for 3 weeks, clones were stained with Coomassie blue. Puromycin-resistant colonies were formed when donor cells harbored both the SV40LT gene and the puromycin resistance gene (**a**) but not if the donor cell line lacked the SV40LT gene or the puromycin resistance gene. MEF p21$^{-/-}$ were used as a positive control. Number of colonies is the average from three independent experiments, $\pm$ indicates S.D. Clones from the co-cultivation of MEF and apoptotic REFrmLTp^r or BAE and apoptotic REFrmLTp^r were picked under a microscope and expanded. All clones isolated expressed SV40LT (**b**). The cellular origins of the puromycin-resistant colonies after co-cultivation of REFrmLTp^r and MEF were determined using a mouse-specific genome probe (**c**). In parallel, mouse/rat chromosomal spreads were analyzed for cross-hybridization of the genome-specific probes. No cross reactivity could be detected in 10–20 000 cells analyzed (**d**). Co-cultivation between MEF and apoptotic REFrmLTp^r cells without puromycin selection resulted in foci formation (**e**) and FISH analysis of these revealed with the presence of whole chromosomes as well as fusion chromosomes between mouse and rat, indicated by arrows (**f**). Number of foci is the average from three independent experiments, $\pm$ indicates S.D. Size bar = 10 μm

blood-containing cysts (Figure 5a). The inclusion of FGF-2 resulted in massive vascularization with large blood-filled vessels visible to the naked eye. Addition of primary mouse lung endothelial cells (L-ECs) did not promote angiogenesis compared with control plugs.

The Matrigel plugs were sectioned, stained with hematoxylin and eosin and examined by light microscopy. Plugs containing T-EC showed cell-lined vessels filled with erythro-cytes, signifying that the vessels had anastamosed with the host circulatory system (Figure 5b). Evidence that the structures seen in the Matrigel plugs were derived from T-EC rather than recruited cells was obtained by labeling the membranes of the T-EC with a green fluorescent dye before incorporation into Matrigel and injection into mice. The presence of the fluorescent label on the cells lining the blood-filled spaces established that they were derived from
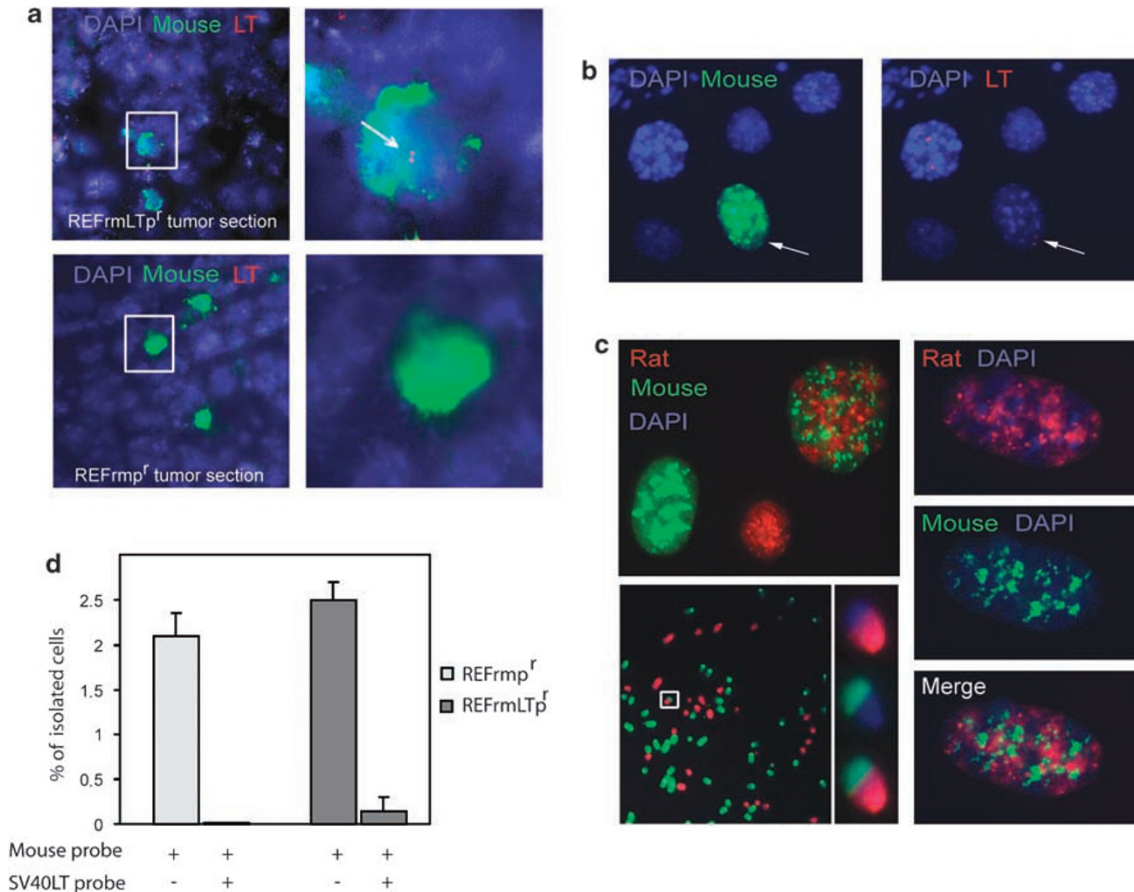
Figure 3 FISH analysis of REFrmLTp[r] tumors reveals transfer of SV40LT into infiltrating mouse cells. Rat embryonic fibroblast cell lines REFrmLTp[r] and REFrmp[r] were injected into SCID mice and the resulting tumors were sectioned and analyzed by fluorescence *in situ* hybridization (FISH). Mouse cells are stained green with a mouse-specific FISH probe and SV40LT is stained red with an SV40LT-specific FISH probe. Double-positive cells were seen in cryosections from REFrmLTp[r] tumors but not in tumors from REF cells lacking SV40LT (i.e. REFrmp[r] cells), illustrating that tumor specific genetic material was transferred to mouse cells (**a**). Similarly, double-positive cells were observed in single cell spreads of REFrmLTp[r] tumors but not of REFrmp[r] tumors, indicated by arrows (**b**). When rat and mouse probes were used simultaneously, a mixture of rat and mouse DNA could be detected in interphase nuclei as well as whole chromosomes, and fusions between rat and mouse chromosomes were detected (**c**). Quantification of number of cells positive for the mouse genomic probe and SV40LT. Over 3000 nuclei were scored per section from three independent experiments (**d**). Error bars indicate standard deviation

the injected T-EC (Figure 5c). These data indicate that functional ability of the endothelial cells to form new blood vessels was maintained following horizontal DNA transfer.

Discussion

The results of the study provide direct evidence that tumor DNA may be transferred to vascular endothelial cells *in vitro* and *in vivo*. Transfer of tumor DNA resulted in immortalized endothelial cells, which maintained the ability to form functional vessels *in vivo*. The findings represent the first account of a novel mechanism by which tumors may promote growth of their own vascular network.

In previous studies, we have provided extensive evidence that DNA may be transferred to phagocytes through the uptake of apoptotic cells.[7,8,10–12] Although normal cells may take up DNA by horizontal gene transfer, it is not propagated due to the activation of the p53–p21 pathway. In this study, we demonstrate that the transfer of SV40LT may overcome this protection, presumably due to the inactivation of p53 that

allows the transfer and replication of transferred DNA in normal host cells. It was observed that SV40LT could be transferred *in vitro* within the chromosome of the apoptotic donor cell or cross over to the chromosomes of the new host. This phenomenon facilitated the investigation whether mutations could be transferred in mice from tumor cells to the stroma and propagated. In tumors from rat fibroblasts transformed with H-ras[v12] and c-myc carrying SV40LT, a sub-population (6%) of the stromal cells were noted to contain both mouse and rat DNA. The purification and subsequent FISH analysis of tumor endothelial cells illustrated that these cells harbored both mouse and rat chromosomes. Parallel to our previous observations with cells propagating transferred DNA, many endothelial cells in this study contained rat–mouse fusion chromosomes reminiscent of translocations in human tumors. The present findings are similar to those reported by others including Harris *et al.*[22] who as early as 1971 described fusion hybrids of tumor with non-malignant cells *in vivo*.[23] In this study, FISH analysis on more than 20 distinct endothelial cell lines isolated from SV40LT tumors
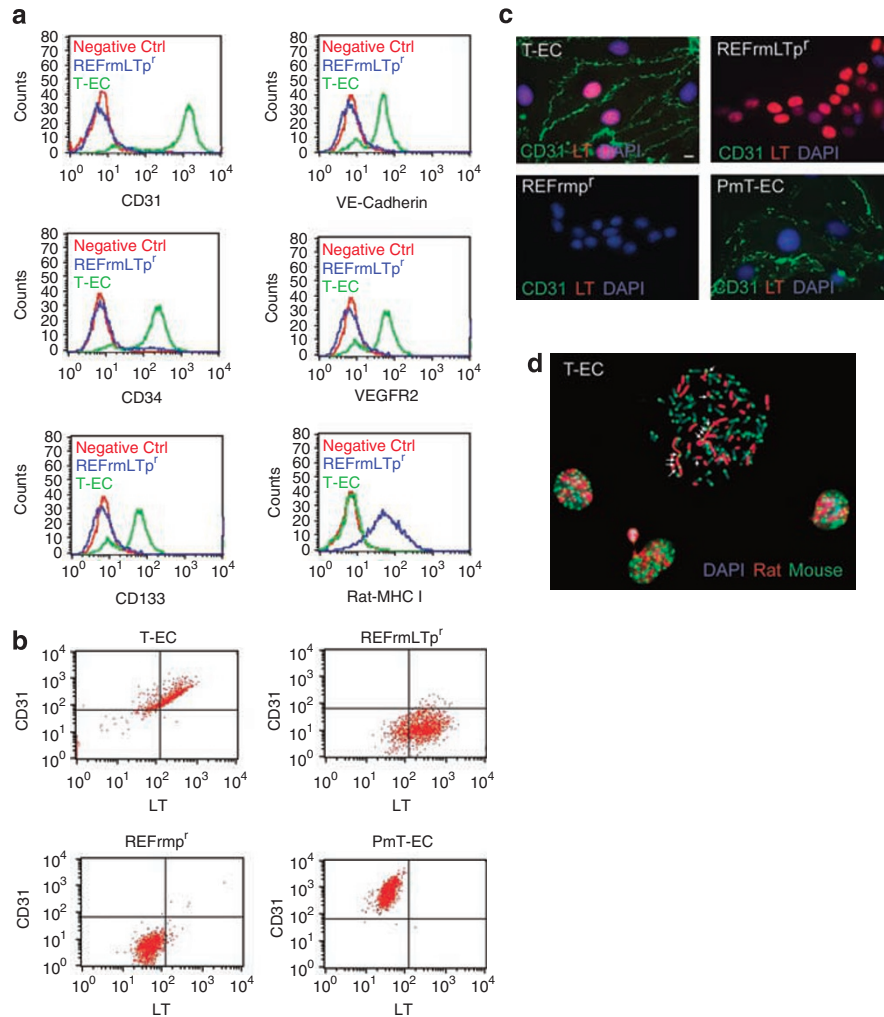
Figure 4 Tumor endothelium from REFrmLTp[r] tumors are double-positive for CD31/PECAM1 and LT. Magnetic beads coated with CD31/PECAM1 were used to isolate tumor endothelial cells (T-EC) from tumors established from rat embryonic fibroblasts stably expressing H-ras[v12], c-myc, puromycin resistance and SV40 large T (i.e. REFrmLTp[r] tumors). Twenty isolated clones were positive for the endothelial surface markers CD31/PECAM1, CD34, VE-cadherin and VEGFR2, but negative for the immature hematopoietic stem cell marker CD133 and rat-MHC I ((**a**) shows data from one representative clone). Negative control was unstained T-EC. Double staining with CD31/PECAM1 and SV40 large T (LT) revealed that the isolated clones were double positive (**b**). The fluorescent-activated cell sorting (FACS) analysis results were verified by immunofluorescence staining (**c**). Rat and mouse genomic probes were used simultaneously; chromosomal level fusions between rat and mouse chromosomes are indicated with arrows (**d**). Size bar = 10 μm

revealed that the rat DNA (and SV40LT) persisted in the endothelial cells even after 30 passages *in vitro*. These cells maintained their ability to form functional vessels within the Matrigel matrix, *in vivo* and the neovascularization activity was enhanced further by the addition of FGF-2.

Streubel *et al.*[5] discovered that the endothelial cells of human lymphomas bear genetic alterations that are shared by neighboring tumor cells. This led to several possibilities regarding the relationship between the tumor and its endothelium. One possibility is that tumors trans-differentiate into endothelial cells, which is unequivocally excluded in our model system as the tumor endothelial cells contained DNA derived from the tumor. In addition, we did not detect any cells of rat origin that expressed endothelial markers. The concept of trans-differentiation is further challenged by reports that transplanted bone marrow cells form hybrids by cell–cell

fusion rather than developing into specialized cells through trans-differentiating.[24–26] The data presented here are consistent with these findings.

Cell fusion is a fundamental process in morphogenesis and tissue regeneration of normal somatic cells and is restricted to only a few cell types such as cytotrophoblasts and myocytes during development (reviewed in Ogle *et al.*[27]). We argue that in tumors, cell fusion is not distinguishable from horizontal gene transfer. Data presented here and in our previous reports prove that uptake of dying cells results in transfer of whole chromosomes or fragments, leading to the formation of cells similar to those observed *in vivo*.

If *p53* is mutated or deleted from a tumor cell that has taken up DNA from an apoptotic cell, the genetic information can be thereafter transferred vertically, rather than horizontally, and
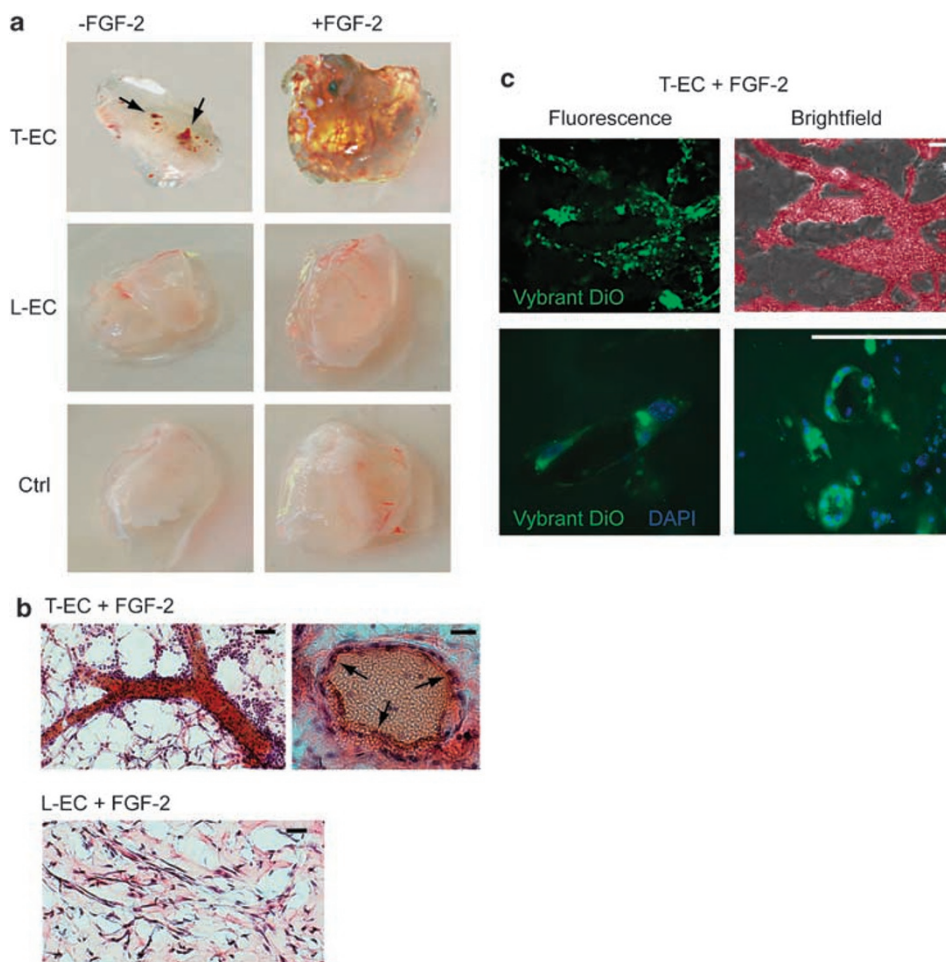
Figure 5 Tumor-associated endothelial cells isolated from REFrmLTp[r] tumors are functional. To assess whether the isolated tumor-associated endothelial cells (T-ECs) were functional, the endothelial cells were mixed with Matrigel and injected into severe combined immunodeficient (SCID) mice. The resulting Matrigel plug showed blood-filled spaces and large blood vessels, indicated by arrows (**a**). In contrast, addition of primary mouse lung endothelial cells (L-ECs) did not promote angiogenesis when compared with control plugs. Sections of the plugs stained with hematoxylin and eosin showed that the blood-filled vessels and spaces were lined with endothelial cells, indicated by arrows (**b**). To verify whether the blood vessel networks formed were made up of recruited endothelial cells or the T-EC, the T-ECs were fluorescently marked with a green fluorescent dye (Vybrant DiO) and injected together with Matrigel into SCID mice (**c**). Erythrocytes were artificially colored red for clarity and all pictures are representative of at least three independent experiments. Size bar = 50 μm

propagated to subsequent generations of tumor cells. This mechanism may cause development of aneuploidy in cancer.[28,29] However, in cells containing wild-type p53, DNA taken up from an apoptotic body will activate p53 once cell cycle ensues. The outcome is cell cycle arrest or apoptosis, which effectively impedes the vertical transmission of this new genetic information. Our results establish that the SV40LT is sufficient to overcome this 'genetic surveillance' both *in vitro* and *in vivo*. These observations are particularly significant in view of several studies providing evidence that the tumor stroma carry genetic mutations and altered DNA copy numbers. Indeed, p53 mutations or alterations have been found in adjoining tissue in diverse malignancies.[13,14–19] Hill *et al*.[30] showed that tumor development in a transgenic prostate cancer model involved the selective loss of p53 in the stromal fibroblasts. Cumulatively, these findings underscore that loss of p53 function in tumor stroma is an essential step in tumor development.

Materials and Methods

Cell lines. REFrm (described in Bergsmedh *et al*.[10]) was transfected with pSVEpR$_4$ plasmid containing the early SV40LT antigen together with a puromycin resistance plasmid or with the puromycin resistance plasmid alone. Transfected cells were selected for 2 weeks in 5 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA). REFrm transfectants expressing SV40LT and the puromycin resistance gene were designated REFrmLTp[r]. Other cell lines utilized in this study were MEFs, derived from p21$^{-/-}$ mice and BAEs. All cell lines were grown in Dulbecco's modified Eagle's medium (Invitrogen Corporation, Carlsbad, CA, USA) with 10% fetal bovine serum, glutamine and penicillin/streptomycin. Experiments were performed in accordance with protocols approved by the Animal Ethics Review Committee of Karolinska Institutet in conformity with national guidelines and regulations.

Fluorescent-activated cell sorting. Apoptosis was induced in REFrmp[r] and REFrmLTp[r] by nutrient depletion, as described previously.[10] Externalization of plasma membrane phosphatidylserine was assessed by the measurement of annexin V fluorescence (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. One million cells were trypsinized (Sigma-Aldrich), washed in PBS and resuspended in incubation buffer (i.e. 10 mM HEPES/NaOH, pH 7.4,

140 mM NaCl and 5 mM CaCl$_2$) containing 1% annexin V. The samples were incubated for 15 min prior to the addition of a further 400 μl of incubation buffer and analysis by flow cytometry (Becton Dickinson (BD), San Diego, CA, USA). Flow cytometry was used to characterize cells isolated immunomagnetically (Dynal M-450) from REFrmLTp^r tumors. Cells were stained for surface antigens without fixation or permeabilization. The antibodies used were: CD31/PECAM1, CD34, CD133, VE-cadherin, VE growth factor receptor 2 (all from BD) and rat MHC I (AbD Serotec, Kidlington, UK). For dual staining, cells were fixed in 0.25% freshly prepared paraformaldehyde (Sigma-Aldrich) and permeabilized with digitonin added to the primary antibody. Antibodies used for dual staining were: PECAM1-APC (Biosite Incorporated, San Diego, CA, USA), active caspase 3 FITC-conjugated and LT (Calbiochem, Darmstadt, Germany).

Co-cultivation experiments. Apoptosis was induced in REFrm, REFrmp^r or REFrmLTp^r cells by nutrient depletion as described previously.[10] Five million apoptotic bodies were added to 1×10^6 MEF, MEFp21$^{-/-}$ or BAE cells in 10-cm Petri dishes. After 48 h, the cells were selected in media containing puromycin (5 μg/ml; Sigma-Aldrich). Nutrient-depleted cells were cultured in parallel and analyzed by flow cytometry. Surviving cells were grown in clonogenic assays. All experiments were performed in triplicate and surviving colonies were either stained with Coomassie blue dye or utilized for FISH analysis.

Tumor sections and isolation of endothelial cells. Approximately 50 000 REFrmp^r or REFrmLTp^r cells were injected into the dorsal subcutaneous space of 6- to 8-week-old SCID mice. Tumor development was monitored by palpation and mice were killed when tumor sizes reached 15 mm in diameter. Sections (5 μm thick) were cut from formalin-fixed, paraffin-embedded tumors and used for immunohistochemistry. Single-cell suspensions of endothelial cells were obtained from collagenase-digested tumor or lung tissue. Endothelial cells were purified immunomagnetically (Dynabeads, Dynal M-450) according to the manufacturer's instructions using anti-CD31/PECAM1 antibody (BDD). CD31/PECAM1-positive cells were grown in Endothelial Cell Basal Medium MV with supplemental pack MV (PromoCell, Heidelberg, Germany).

Immunohistochemistry. Puromycin-resistant clones were harvested, expanded and plated on chamber plates (BD). Immunohistochemistry was performed on cells fixed with methanol/acetone (1 : 1 for 10 min at -20°C) using an anti-SV40LT antibody (Calbiochem). Slides were mounted with Vectashield containing DAPI (Vector Laboratories Inc., Burlingame, CA, USA).

FISH. DNA from MEF and REF cells was isolated using Qiagen columns (Qiagen, Valencia, CA, USA). The DNA isolates and pSVEpR4 plasmid DNA were labeled either with biotin-dUTP or with digoxigenin-dUTP (BIO-Nick Translation Mix or Digoxigenin-Nick Translation Mix; Roche Diagnostics). The differentially labeled DNAs were mixed in equal proportion and sonicated salmon sperm was added in at least two-fold excess to reduce unspecific hybridization. The DNAs were precipitated, dissolved in a solution containing $2 \times$ SSC, 50% formamide, 10% dextran sulfate and 0.1% Tween 20, pH 7.0 and denatured at 85°C for 7 min.

The slides for FISH were prepared according to the standard procedure, from cell suspensions treated with hypotonic solution (0.75 M KCl) and fixed with 1 : 3 acetic acid/methanol. These slides were denatured in $2 \times$ SSC, 70% formamide, pH 7.0 at 73°C, 3 min prior to probing. Probes were hybridized for 1–3 days at 37°C under cover slips in humid chambers. The slides were then washed at 46°C at first with 50% formamide in $2 \times$ SSC and then three times in $2 \times$ SSC alone. The biotin-labeled probe hybridization was detected with Cy3-conjugated streptavidin (Amersham Biosciences, GE Healthcare, Piscataway, NJ, USA) and the digoxigenin-labeled probe hybridization was detected using FITC-conjugated anti-digoxigenin antibodies (Roche Diagnostics). Each slide was then embedded into mounting solution containing DAPI for DNA counterstaining (Vectashield Laboratories Inc.) and analyzed using a fluorescence microscope (Leitz-DMRB; Leica, Wetzlar, Germany) equipped with a Hamamatsu C 4800 cooled CCD camera (Hamamatsu Photonics Norden AB, Solna, Sweden). A minimum of 50 metaphases and 200 nuclei in each sample were examined. The specificity of mouse and rat DNA hybridization for the identification of mouse and rat genomic material was tested on mouse–rat fibroblast cell mix. A slide prepared with such a mix was included with each FISH experiment as a specificity control. A FISH assay was considered technically satisfactory only when all mouse and rat nuclei could be easily distinguished by their respective color probes (see Figure 2d). Images were

captured using the CCD camera, then processed and analyzed using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

Matrigel plug assay *in vivo*. Matrigel is a solubilized basement membrane preparation extracted from Engelbreth–Holm–Swarm murine sarcoma, which is liquid at 4°C but solidifies at 37°C. The Matrigel plug assay was performed as described previously with some modifications.[21] Liquid Matrigel (9.7 mg/ml; Becton Dickinson Discovery Labware), at 4°C, was mixed with 500 ng/ml of FGF-2 (Peprotech) and tumor or lung endothelial cells (3×10^5 cells per 1 ml of Matrigel). The cell suspensions were injected (0.5 ml) into the abdominal subcutaneous tissue of mice along the peritoneal midline. Matrigel plugs were removed on 7th day after implantation, photographed and prepared for histology. In some experiments, cells were tagged using the Vybrant® DIO cell labeling kit (Invitrogen Corporation) prior to injection.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)