nature cell biology

Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells

Khalid Al-Nedawi[1], Brian Meehan[1], Johann Micallef[3], Vladimir Lhotak[2], Linda May[2], Abhijit Guha[3] and Janusz Rak[1,4]

Aggressive human brain tumours (gliomas) often express a truncated and oncogenic form of the epidermal growth factor receptor, known as EGFRvIII. Within each tumour only a small percentage of glioma cells may actually express EGFRvIII; however, most of the cells exhibit a transformed phenotype[1]. Here we show that EGFRvIII can be 'shared' between glioma cells by intercellular transfer of membrane-derived microvesicles ('oncosomes'). EGFRvIII expression in indolent glioma cells stimulates formation of lipid-raft related microvesicles containing EGFRvIII. Microvesicles containing this receptor are then released to cellular surroundings and blood of tumour-bearing mice, and can merge with the plasma membranes of cancer cells lacking EGFRvIII. This event leads to the transfer of oncogenic activity, including activation of transforming signalling pathways (MAPK and Akt), changes in expression of EGFRvIII-regulated genes (*VEGF*, *Bcl-x*$_L$, *p27*), morphological transformation and increase in anchorage-independent growth capacity. Thus, membrane microvesicles of cancer cells can contribute to a horizontal propagation of oncogenes and their associated transforming phenotype among subsets of cancer cells.

Activated cells of various types are known to produce and shed membrane microvesicles into their surroundings. Some of these are also known as microparticles or ectosomes, whereas others are referred to as exosomes, depending on the properties and specific mechanisms of their generation[2]. The biological role of these structures is poorly understood, but may include secretory processes, immunomodulation, coagulation and intercellular communication[3]. Microvesicles may vary in their abundance, size and composition, but they often contain material associated with membrane lipid rafts, including functional transmembrane proteins[4]. For example, procoagulant receptor tissue factor (TF) can be released in this fashion from inflammatory cells and these microvesicles are subsequently incorporated into membranes of platelets, endothelium and other cells where TF exerts its biological effects[4]. Similarly, vesicular transfer of the CCR5 receptor contributes to cellular susceptibility to HIV transmission[5]. The mechanism triggering microvesicle generation by cancer cells is unknown, but loss of the tumour suppressor gene *p53* may, in some instances, influence the release of increased amounts of TF-containing[6] or secretory[7] microvesicles to the blood of tumour-bearing mice, or to the pericellular milieu.

We reasoned that oncogenic receptors often reside within regions of the plasma membrane, from which microvesicles originate in cancer cells (for example, lipid rafts) and therefore they could themselves become included in the microvesicle cargo. This would be of particular interest in malignant brain tumours, where the activation of membrane-associated EGFR (**A000823**) represents a major transforming event, with nearly 50% of cases of glioblastoma multiforme (GBM) expressing amplified EGFR, whereas a large proportion is positive for a distinct mutant known as EGFRvIII[8].

Interestingly, we observed that production of microvesicles by cultured U373 glioma cells lacking the activated EGFR increases markedly with enforced expression of the EGFRvIII mutant (U373vIII cells). Thus, formation of vesicular membrane protrusions was readily detected by scanning electron microscopy of U373vIII cells and the degree of this vesiculation was markedly greater than that of U373 cells. This effect was accompanied by a corresponding increase in the recovery of protein from the microvesicular fraction of the culture medium of the respective cell lines[6,9] (Fig. 1a, b; Supplementary Information, Fig. S1). This material contained flotillin-1, a protein associated with membrane lipid rafts that is often found in raft-related microvesicles[4]. These observations suggest that the expression of EGFRvIII triggers production of microvesicles derived from membrane lipid rafts.

Oncogenic receptor tyrosine kinases, including EGFRvIII, are known to accumulate in membrane lipid rafts of cancer cells. We reasoned that this may cause release of EGFRvIII as cargo of raft-related microvesicles. Indeed, we noticed that EGFRvIII protein was not only readily detectable in lysates of U373vIII cells, but was also present in their derived flotillin-1-containing microvesicles. As expected, U373 cells released microvesicles containing only trace amounts of wild-type EGFR (wtEGFR) and no

[1]Montreal Children's Hospital Research Institute, McGill University, 4060 Ste Catherine West, Montreal, Quebec, Canada H3Z 2Z3. [2]Henderson Research Centre, McMaster University, 711 Concession Street, Hamilton, Ontario, Canada, L8V 1C3. [3]Arthur and Sonia Labatts Brain Tumor Centre, The Hospital for Sick Children, University of Toronto, TMDT-101 College Street, Toronto, Ontario, Canada M5G 1L7.
[4]Correspondence should be addressed to J.R. (e-mail: janusz.rak@mcgill.ca)

Received 11 December 2007; accepted 10 March 2008; published online 20 April 2008; DOI: 10.1038/ncb1725

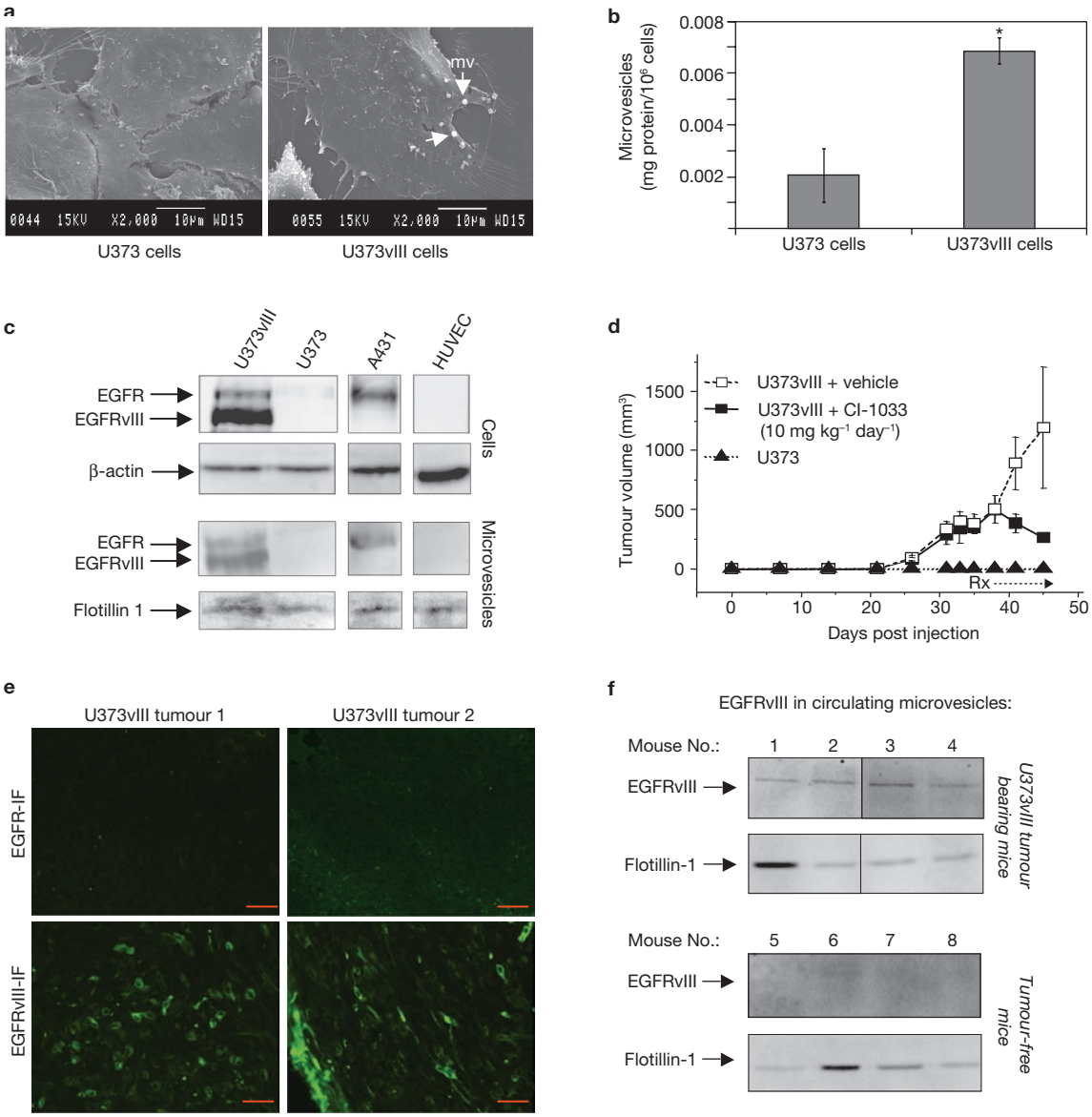


Figure 1 Production of *EGFRvIII* (oncogene)-containing microvesicles by human glioma cells. (**a**) Generation of multiple microvesicular (mv) structures on the surfaces of U373vIII glioma cells with the *EGFRvIII* oncogene (white arrowheads; SEM image) is less pronounced in the case of their indolent parental U373 cells. (**b**) Increase in abundance of the microvesicular fraction of the conditioned medium, as a function of EGFRvIII expression in U373 glioma (measured by total protein content; mean ± s. d.; $*P < 0.001$, *t*-test, $n = 3$). (**c**) Inclusion of oncogenic forms of EGFR in lipid-raft-derived microvesicles released by EGFR-expressing cancer cells. Mutant/activated EGFRvIII is expressed by U373vIII glioma, whereas the A431 squamous cell carcinoma cells harbour endogenously activated wild-type *EGFR* oncogene. Both EGFRvIII and EGFR were detected in the corresponding microvesicle preparations, which were also positive for the lipid-raft marker flotillin 1 (bottom panel). Parental U373 glioma cells or non-transformed HUVECs (top panel) did not express EGFR in the plasma membrane and produced small amounts of EGFR-free microvesicles. (**d**) Dependence of tumorigenic properties of U373vIII cells on the functional EGFRvIII. Tumours did not form in the absence of EGFRvIII (U373), or when this receptor was inhibited by administration of the pan–ErbB inhibitor (CI-1033); data are mean ± s. d. (see Supplementary Information, Methods). (**e**) Immunostaining with a monoclonal anti-EGFR antibody that does not recognize truncated EGFRvIII (top panel) or a specific anti-EGFRvIII antibody (bottom panel) shows predominant expression of EGFRvIII, but not EGFR in U373vIII tumours. (**f**) Release of EGFRvIII-containing, flotillin-1-positive microvesicles to the circulating blood of SCID mice harbouring U373vIII tumours (top panels). Scale bars are 10 μm (**a**) and 50 μm (**e**). Full scans of all gels are shown in Supplementary Information, Fig. S5.

EGFRvIII. These results were validated against EGFR-negative endothelial cells (HUVECs) and A431 cells expressing only wtEGFR, as well as their respective microvesicle preparations (Fig. 1c).

In contrast to parental U373 cells, U373vIII readily form subcutaneous tumours in immunodeficient (SCID) mice, in a manner susceptible to inhibition by daily administration of an irreversible, small molecule pan–Erb inhibitor CI-1033 (ref. 6; Fig. 1d). These U373vIII tumours stain strongly for EGFRvIII, but not for wtEGFR and emit EGFRvIII-containing microvesicles into the systemic circulation (Fig. 1e, f). Thus, expression of the mutant EGFRvIII gene causes increased aggressiveness of glioma cells, coupled with extracellular and systemic release of microvesicles containing an intact EGFRvIII oncoprotein.

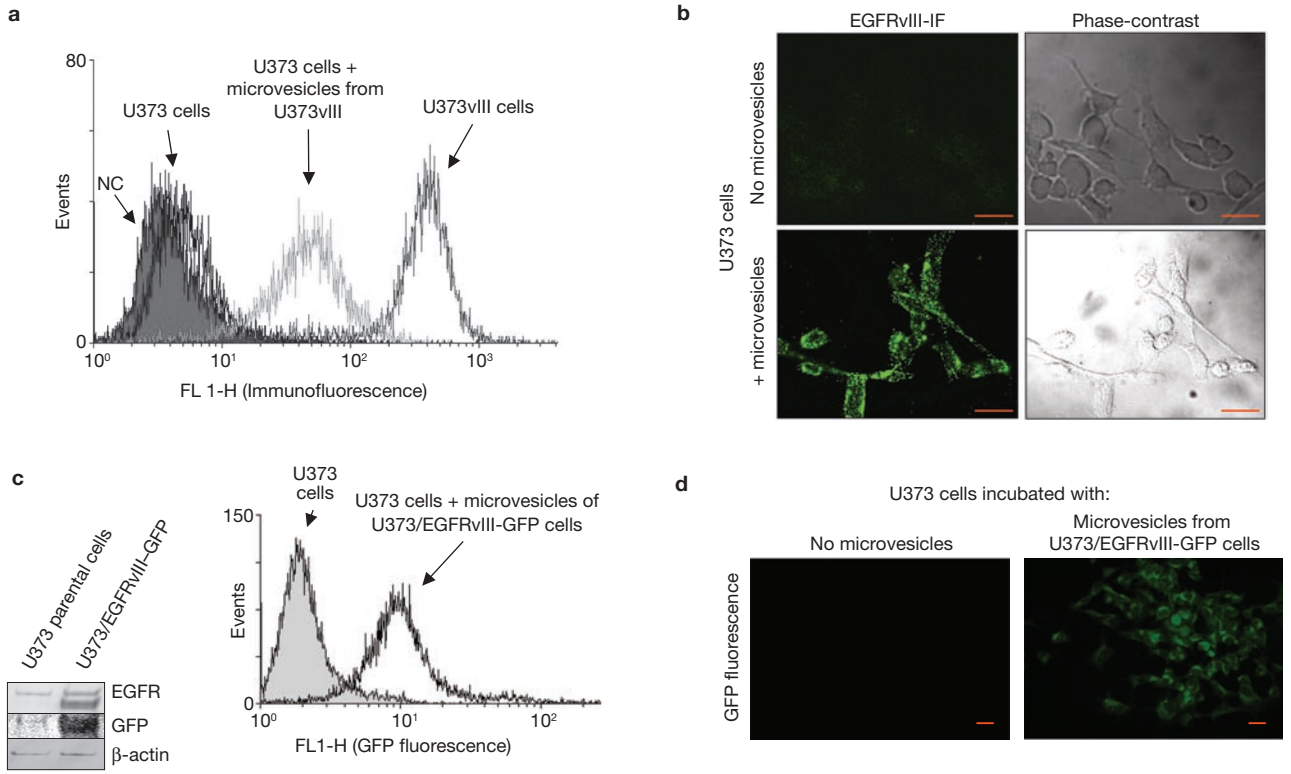


Figure 2 Microvesicular transfer of the oncogenic EGFRvIII between glioma cells. (**a**) Fluorescence-activated cell sorting (FACS) analysis showing that U373 cells incubated with microvesicles released by their EGFRvIII-transformed counterparts (U373vIII) acquired the expression of the EGFRvIII antigen on their surfaces. (**b**) Direct detection of EGFRvIII on the surface of U373 cells incubated with U373vIII-derived microvesicles. Changes in cell shape (transformation) were seen with microvesicle/EGFRvIII uptake. (**c**) Generation of the U373/EGFRvIII–GFP cell line by expression of the GFP-tagged EGFRvIII (EGFRvIII–GFP) in U373 cells (left panel). Detection of GFP fluorescence (FACS) on the surface of viable U373 cells preincubated with microvesicles derived from U373/EGFRvIII–GFP cell line — evidence for intercellular transfer of EGFRvIII–GFP. (**d**) Direct GFP-fluorescence of U373 cells incubated with EGFRvIII–GFP containing microvesicles. All procedures involved several washes, during which non-incorporated microvesicles have been completely removed. All scale bars are 20 μm.

Heterogenous expression of EGFRvIII in human glioma[1] suggests that different tumour-cell subsets could shed EGFRvIII-containing microvesicles into the common intercellular space. As microvesicles can readily fuse with cellular membranes through a phosphatidylserine-dependent mechanism[4], we asked whether oncogenic EGFRvIII could be transferred in this manner from more aggressive to indolent glioma cells. EGFRvIII-negative U373 cells were incubated with preparations of microvesicles obtained from either their U373vIII counterparts (harbouring EGFRvIII), or from U373vIII–GFP cells, engineered to express a green fluorescent protein (GFP)-tagged EGFRvIII variant (EGFRvIII–GFP). This caused an extensive uptake of the microvesicular content by U373 cells, and their *de novo* surface expression of the EGFRvIII antigen, or GFP fluorescence, respectively (Fig. 2a–d).

The apparent intercellular microvesicle-mediated transfer of the ostensibly intact EGFRvIII receptor raises the question as to the signalling consequences (if any) of this event for the 'recipient/acceptor' (U373) cells. To address this question, we examined U373 cells 24 h after their exposure to EGFRvIII-containing microvesicles, for activation of the EGFRvIII downstream signalling pathways, such as MAPK and Akt cascades[8,10]. Indeed, incorporation of EGFRvIII into the U373 plasma membrane resulted in a consistent increase in Erk1/2 phosphorylation and this event was dependent on the transfer of active (phosphorylated) EGFRvIII, as U373-derived microvesicles (containing no EGFRvIII) were ineffective. Moreover, the irreversible blockade of the microvesicle-associated EGFRvIII by preincubation of this (cell-free) material with pan–ErbB inhibitor (CI-1033) markedly reduced its ability to trigger Erk1/2 phosphorylation (Fig. 3a, b). Phosphorylation of Erk1/2 was also abrogated by preincubation of EGFRvIII-containing microvesicles with annexin V (**A000281**), which blocks their exposed phosphatidylserine residues and thereby their uptake by U373 cells[4]. These results suggest the actual (phosphatidylserine-dependent) microvesicle integration and EGFRvIII transfer, and not merely contact between the EGFRvIII-containing microvesicles and the surface of U373 cells, are required for triggering the activation of MAPK pathway in the 'recipient' cell (Fig. 3c). Incorporation of EGFRvIII-containing microvesicles into U373 cells also induced phosphorylation of Akt, in a manner that could be inhibited by annexin V pretreatment (Fig. 3d), and triggered several other events, such as phosphorylation of PDK1 and Raf (data not shown). These events were also related to the transfer of EGFRvIII and not its effectors, such as Erk1/2 and Akt, as the latter were largely undetectable in the microvesicle lysates (Supplementary Information, Fig. S5).

The transforming effect of EGFRvIII-dependent pathways is ultimately mediated by deregulation of several genes responsible for tumour growth, survival and angiogenesis[8,10]. With regard to the latter we noted that U373

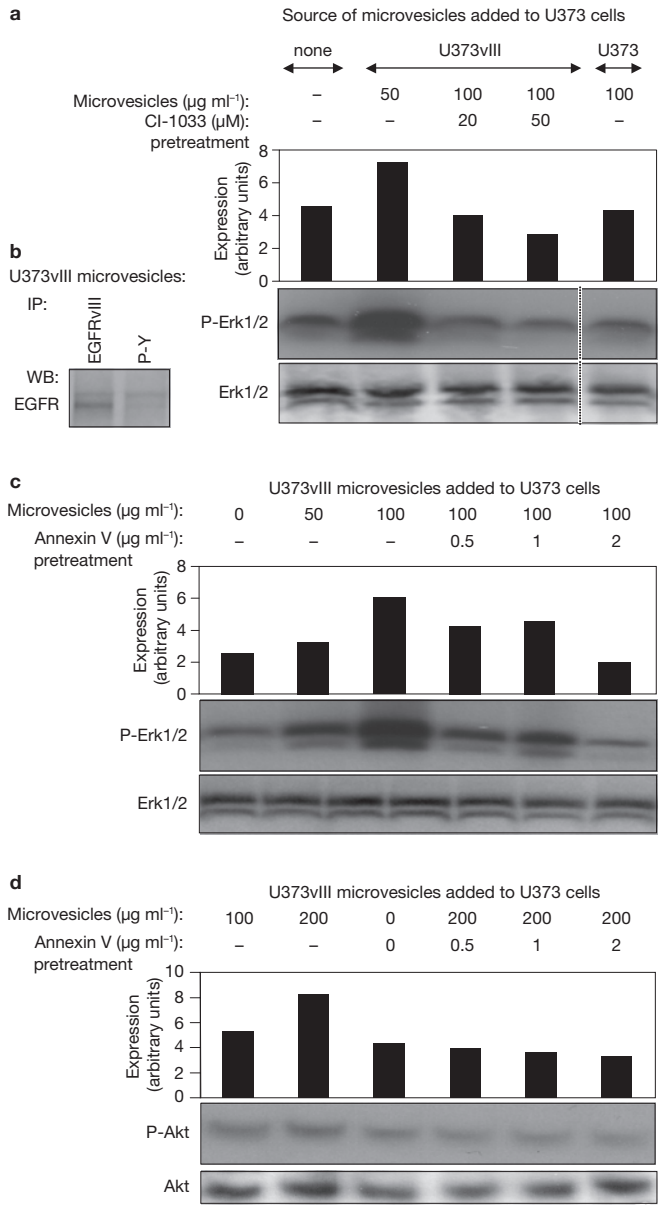


Figure 3 Activation of growth-promoting signalling pathways in cells that have acquired oncogenic EGFRvIII through microvesicle-mediated intercellular transfer. (**a**) EGFRvIII-dependent increase in Erk1/2 phosphorylation in U373 cells that had incorporated microvesicles shed by U373vIII cells. Preincubation of these microvesicles with the irreversible pan–ErbB inhibitor (CI-1033) abrogated their ability to trigger Erk1/2 phosphorylation, in a concentration-dependent manner. EGFRvIII-negative microvesicles from U373 cells were inactive in this assay. (**b**) Phosphorylation of microvesicle-associated EGFRvIII: immunoprecipitates of microvesicles (IP) with indicated antibodies were probed (WB) for EGFR. (**c**). Decreased Erk1/2 phosphorylation in U373 cells in which the uptake of EGFRvIII-containing microvesicles was blocked with annexin V. U373vIII microvesicles were pretreated with increasing concentrations of annexin V, which blocks phosphatidylserine residues required for fusion with the plasma membrane. (**d**) Increase in phosphorylation of Akt in U373 cells that had incorporated EGFRvIII-containing microvesicles. Akt phosphorylation could be prevented by blocking the microvesicle uptake with annexin V. Full scans of all relevant gels are shown in the Supplementary Information, Fig. S5.

cells exposed to U373vIII-derived microvesicles exhibited a marked (2–3-fold) increase in the production of vascular endothelial growth factor (VEGF), a potent mediator of brain tumour angiogenesis[10] and a known EGFR target[11]. EGFRvIII activity was essential for this effect, as U373-derived microvesicles (devoid of EGFRvIII), or U373vIII-derived microvesicles preincubated with CI-1033 were both unable to induce the increased release of VEGF (Fig. 4a). Under these conditions, EGFRvIII-containing microvesicles also robustly stimulated *VEGF* promoter activity and this effect was abrogated by their pretreatment with annexin V (Fig. 4b). Collectively, these observations suggest that the incorporation of U373vIII microvesicles by recipient cells triggers EGFRvIII-dependent increase in *VEGF* gene expression, probably through activation of the MAPK and Akt pathways[10].

Although VEGF upregulation often heralds the activation of oncogenic pathways, cellular transformation downstream of EGFRvIII is mediated by changes in expression of genes directly involved in cellular proliferation and survival[8]. In this regard our analysis of U373 cells treated with EGFRvIII-containing microvesicles revealed an increase in the expression of the anti-apoptotic protein Bcl-x_L and a decrease in levels of p27/Kip1 cyclin-dependent kinase inhibitor, both known EGFR targets[8] (Fig. 4c). Again, these effects were inhibited by annexin V-mediated blockade of the microvesicle uptake. We have also observed similar EGFRvIII-dependent changes in expression of other EGFRvIII target genes, for example p21/Cip (data not shown).

We reasoned that in cells that have incorporated EGFRvIII-containing microvesicles, the functional consequences of the aforementioned repertoire of molecular responses could amount to a higher degree of cellular transformation. This is suggested by more spindle-like morphology of U373 cells exposed to this material (Fig. 2b). To explore this possibility further, U373 cells were preincubated with EGFRvIII-containing microvesicles and tested for growth in semisolid medium, an assay for malignant transformation. Incorporation of microvesicle-associated EGFRvIII caused a 2-fold increase in anchorage-independent soft-agar colony formation of U373 cells, whereas exposure to the equivalent amount of microvesicles devoid of EGFRvIII was inconsequential (Fig. 4 d; Supplementary Information, Fig. S2).

It is well recognized that in human GBMs only a small subpopulation of tumour cells harbour the primary genetic alteration leading to EGFRvIII expression, though this oncogene is thought to contribute to progression of the entire tumour. Our results demonstrate that EGFRvIII expression provokes formation of cellular microvesicles, to which this transmembrane protein becomes incorporated and subsequently shed into the pericellular micromilieu (Supplementary Information, Figs S3, S4) and blood (Fig. 1f). Our experiments suggest that microvesicles containing an active oncogene (oncosomes) may serve as vehicles for rapid intercellular transfer of the transforming activity between cells populating brain tumours. This could lead to a 'horizontal' propagation of an increased proliferative, survival, motogenic and angiogenic capacity, even without enrichment in cells harbouring the respective mutation. This hitherto unappreciated form of oncosome-mediated intercellular interaction is fundamentally different from the previously postulated transfer of DNA fragments containing oncogenic sequences from apoptotic cancer cells to their non-transformed (phagocytic) counterparts[12]. Microvesicle exchange is also different from paracrine effects induced by secretion

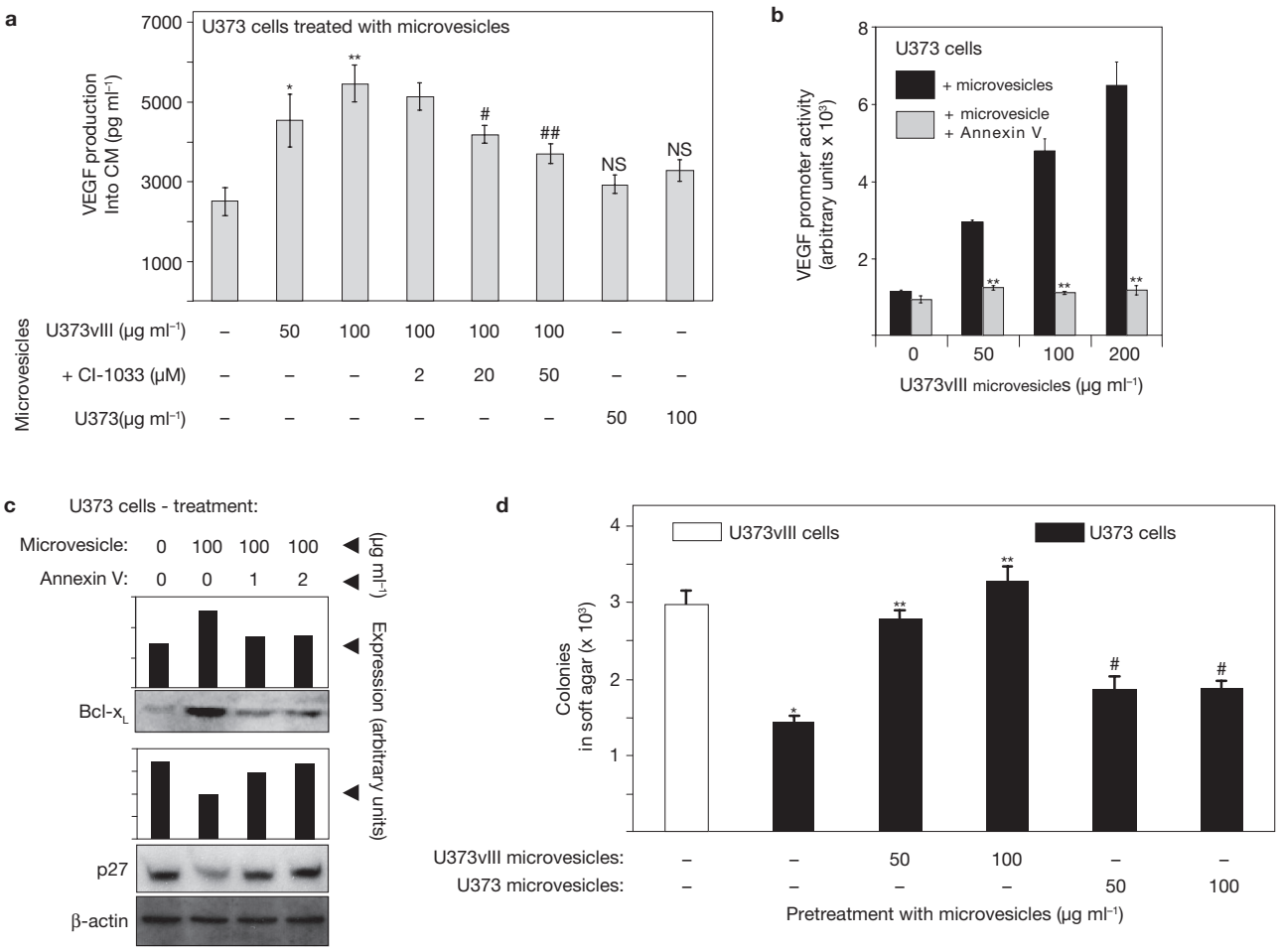


Figure 4 Induction of cellular transformation by the uptake of EGFRvIII-containing microvesicles (**a**) EGFRvIII-dependent increase in VEGF secretion by U373 cells that have incorporated U373vIII microvesicles. microvesicles pretreated with CI-1033, or originating from EGFRvIII-negative cells (U373) were unable to stimulate VEGF production (*$P < 0.05$; **$P < 0.001$; NS, $P > 0.05$, compared with untreated; #$P < 0.05$; ##$P < 0.01$, compared with U373vIII microvesicles). (**b**) Stimulation of *VEGF* promoter activity in U373 cells by incorporation of EGFRvIII-containing microvesicles can be blocked by pretreatment with annexin V. ($P < 0.0001$); (**c**) Increased expression of the pro-survival gene *Bcl-x*$_L$ and reduced expression of the cell cycle inhibitor p27 in U373 cells exposed to EGFRvIII-containing microvesicles. (**d**) Increase in soft agar colony-forming capacity of U373 cells after pretreatment with EGFRvIII containing microvesicles (*$P < 0.001$, compared with untreated U373vIII cells; **$P < 0.0001$, compared with untreated U373; #$P < 0.001$, compared with the respective microvesicles from U373 cells; *t*-test). All numbers are means ± s. d.; $n = 3$/experiment (see Supplementary Information, Methods). Full scans of all gels are shown in Supplementary Information, Fig. S5.

of tumour-stimulating soluble ligands[13], but it could amplify or modulate the latter's effects by intercellular sharing of membrane-associated (and thereby insoluble) active growth factor receptors. Although the present study focuses on EGFRvIII and human glioma cells, a similar microvesicular transfer mechanism could also involve other mutant, upregulated or otherwise activated membrane-associated oncogenic tyrosine kinases (for example, HER-2, wtEGFR, cKit or MET)[14]; such a mechanism may be operative in a variety of human tumours and propagate with blood to distant sites. It also remains to be established whether host cells (for example, endothelial cells) may also be targets of oncogenes or growth factor receptors that contain microvesicles, and what might be the consequence of such events for tumour progression, metastasis, angiogenesis and responsiveness to therapy. It is interesting to speculate that agents capable of blocking the exchange of microvesicles (for example, annexin V derivatives) may possess anticancer activities by virtue of their interference with the oncosome transfer. □

METHODS

Reagents. Antibodies against EGFR (sheep polyclonal and monoclonal), MAPK, AKT, phospho-c- Raf, phospho-PDK1, flotillin-1 and p27 were purchased from Cell Signaling Technology. Anti-EGFRvIII monoclonal antibody was from Zymed. HRP-conjugated secondary antibodies were from Cell Signaling Technology and Alexa fluor secondary antibodies were from Molecular Probes. CI-1033 was a gift from C. Marsolais and L. Levesque (Pfizer). Additional details are included in the Supplementary Information.

Cell culture and isolation of microvesicles. U373 (human astrocytoma) cells, their stable variant U373vIII expressing Tet-off regulated EGFRvIII or EGFRvIII fused at C-terminus to a GFP (pEGFPN1) cassette (U373vIII–GFP) and A431 were maintained as described previously[6] in medium containing microvesicle-depleted fetal bovine serum (FBS). HUVECs were maintained in EGM-2 (Cambrex Bioscience). Microvesicles were collected from conditioned medium or mouse plasma, as described reviously[9]. Briefly, medium was subjected to two successive centrifugations at 300*g* and 12,000*g* to eliminate cells and debris. Microvesicles were pelleted by ultracentrifugation for 2 h at 100,000*g*, quantified by protein content and analysed for EGFR or EGFRvIII. For scanning electron microscopy (SEM), the cells were grown on coverslips, fixed with 2.5% gluteraldehyde, stained

with 1% OsO4, covered with gold and observed using the JEOL 840A instrument. For *in vivo* analyses, tumours were generated by injection of 1–10 × 10^6 U373vIII or U373 cells into SCID mice (Charles River). In some cases mice were treated daily with the pan–ErbB inhibitor CI-1033 as indicated. Blood was collected from tumour-bearing, or control mice by cardiac puncture into heparinized syringes. Platelet-free plasma was used to prepare microvesicles.

Microvesicle transfer assays. U373 (acceptor) cells were treated with microvesicles for 24 h and a single-cell suspension was analysed for expression of EGFRvIII or GFP, as indicated. To detect signalling events, U373 were serum-starved before addition of microvesicles, either intact or preincubated with annexin V or CI-1033 at the concentrations indicated. The expression of microvesicle-associated molecules (EGFRvIII) and expression of total and activated MAPK and Akt, as well as other changes, were assayed by immunoblot (Bcl-x_L, p27/Kip1), ELISA (VEGF, R&D Systems) or promoter activity assays (*VEGF*), as described elsewhere[6,9] (Figs 3, 4; Supplementary Information, Methods and Fig. S5). For soft-agar colony formation assays, single-cell suspensions were prepared in 0.3% agarose from equal numbers of cells pretreated with microvesicles or control medium. Cultures were established in plates precoated with 0.5% agarose and all colonies containing more than 4 cells were counted.

Accession codes. USCD-Nature Signaling Gateway (**http://www.signaling-gateway.org**): **A000823**, **A000281**

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGEMENTS

This project was supported by grants to J. R. from the National Cancer Institute of Canada, the Canadian Cancer Society and the Terry Fox Foundations, and by a CIHR grant to A. G.; J. R. is the Jack Cole Chair in Paediatric Oncology. Infrastructure contribution came from FRSQ. We are most grateful to our families for their support.

AUTHOR CONTRIBUTIONS

K. A. N. provided conceptual input, designed and performed experiments, analysed the data and wrote the manuscript; B. M. contributed to conceptual input, performed experiments and coined the term 'oncosomes'; V. L. and L. M. performed experiments; J. M. and A. G. provided reagents and expertise (A. G. in brain tumours), and analysed data; J. R. designed experiments, provided conceptual input and supervision, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturecellbiology/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Biernat, W., Huang, H., Yokoo, H., Kleihues, P., & Ohgaki, H. Predominant expression of mutant EGFR (EGFRvIII) is rare in primary glioblastomas. *Brain Pathol.* **14,** 131–136 (2004).
2. Pilzer, D., Gasser, O., Moskovich, O., Schifferli, J. A., & Fishelson, Z. Emission of membrane vesicles: roles in complement resistance, immunity and cancer. *Springer Semin. Immunopathol.* **27,** 375–387 (2005).
3. Janowska-Wieczorek, A. *et al.* Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. *Int. J. Cancer*; **113,** 752–760 (2005).
4. Del Conde, I., Shrimpton, C. N., Thiagarajan, P., & Lopez, J. A. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. *Blood.* **106,** 1604–1611 (2005).
5. Mack, M. *et al.* Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. *Nature Med.* **6,** 769–775 (2000).
6. Yu, J. L. *et al.* Oncogenic events regulate tissue factor expression in colorectal cancer cells: implications for tumor progression and angiogenesis. *Blood* **105,** 1734–1741 (2005).
7. Yu, X., Harris, S. L., & Levine, A. J. The regulation of exosome secretion: a novel function of the p53 protein. *Cancer Res.* **66,** 4795–4801 (2006).
8. Cavenee, W. K. Genetics and new approaches to cancer therapy. *Carcinogenesis* **23,** 683–686 (2002).
9. Al-Nedawi, K., Szemraj, J., & Cierniewski, C. S. Mast cell-derived exosomes activate endothelial cells to secrete plasminogen activator inhibitor type 1. *Arterioscler. Thromb. Vasc. Biol.* **25,** 1744–1749 (2005).
10. Feldkamp, M. M., Lau, N., Rak, J., Kerbel, R. S., & Guha, A. Normoxic and hypoxic regulation of vascular endothelial growth factor (VEGF) by astrocytoma cells is mediated by Ras. *Int. J. Cancer* **81,** 118–124 (1999).
11. Viloria-Petit, A. M. *et al.* Neutralizing antibodies against EGF and ErbB-2/*neu* receptor tyrosine kinases downregulate VEGF production by tumor cells *in vitro* and *in vivo*: angiogenic implications for signal transduction therapy of solid tumors. *Am. J. Pathol.* **151,** 1523–1530 (1997).
12. Bergsmedh, A. *et al.* Horizontal transfer of oncogenes by uptake of apoptotic bodies. *Proc. Natl Acad. Sci. USA* **98,** 6407–6411 (2001).
13. Ramnarain, D. B. *et al.* Differential gene expression analysis reveals generation of an autocrine loop by a mutant epidermal growth factor receptor in glioma cells. *Cancer Res.* **66,** 867–874 (2006).
14. Blume-Jensen, P. & Hunter, T. Oncogenic kinase signalling. *Nature* **411,** 355–365 (2001).

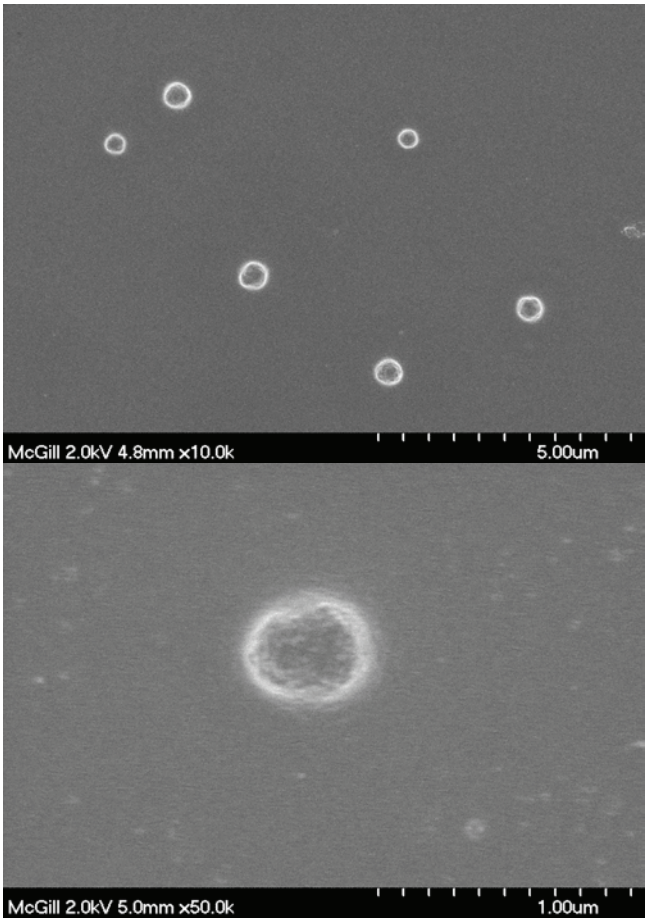


Figure S1 Microvesicles produced by U373vIII human glioma cells. Scanning Electron Micrograph of isolated microvesicles (MVs) obtained as described in Supplemental Methods reveals sizes of approximately 100-500 nm. Similar preparations were used for all treatments

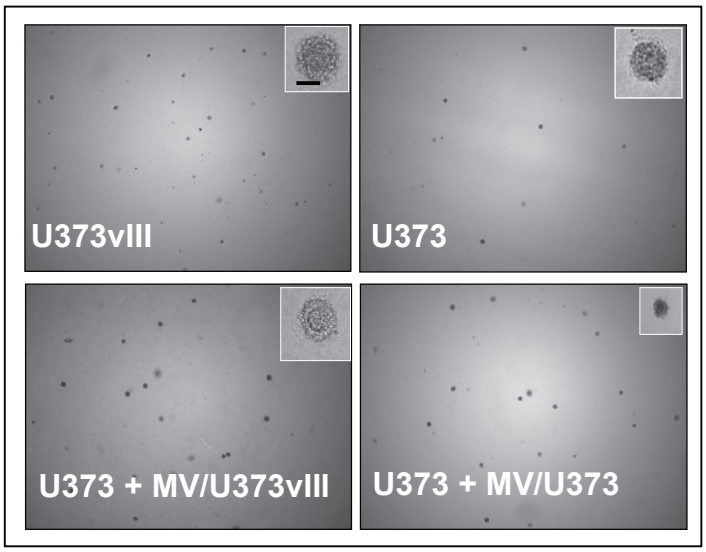


Figure S2 Morphology of U373 tumor cell colonies in soft agar after exposure to EGFRvIII-containing MVs isolated from conditioned medium of the U373vIII cell line. While the numbers of colonies differ significantly (Fig. 4D) there was no major change in sizes and appearance of the colonies (bar – 50 :m).

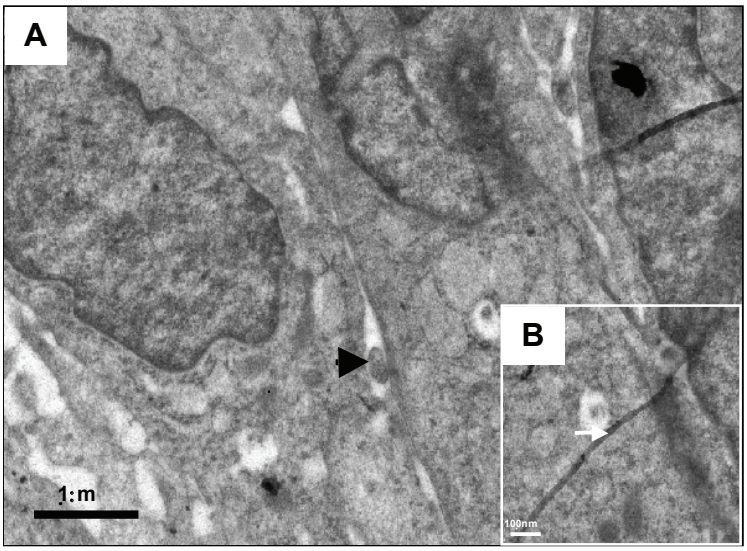


Figure S3 MV-like structures in vivo. A. Transmission Electron Micrograph of microvesicular structures present in the intercellular space between two cancer cells (black arrow) within the mixed tumor xenograft in the SCID mouse. Such tumors were generated by coinjection of U373vIII and U373-GFP cells as described in Methods (bar – 1 :m). B. Immunogold staining for EGFRvIII reveals the presence of this receptor (white arrow) in association with the MV-like structures found within mixed U373vIII/ U373-GFP tumors (bar – 100 nm).

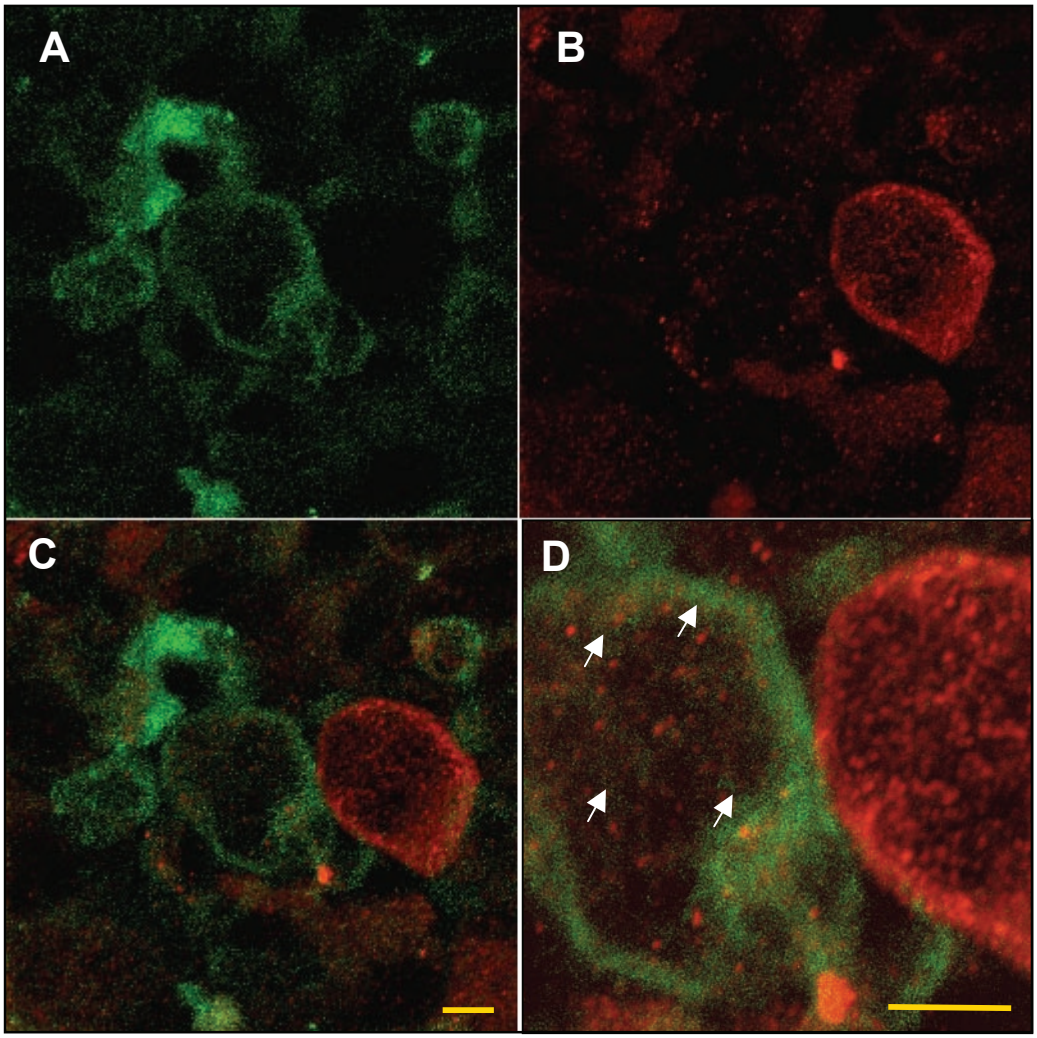
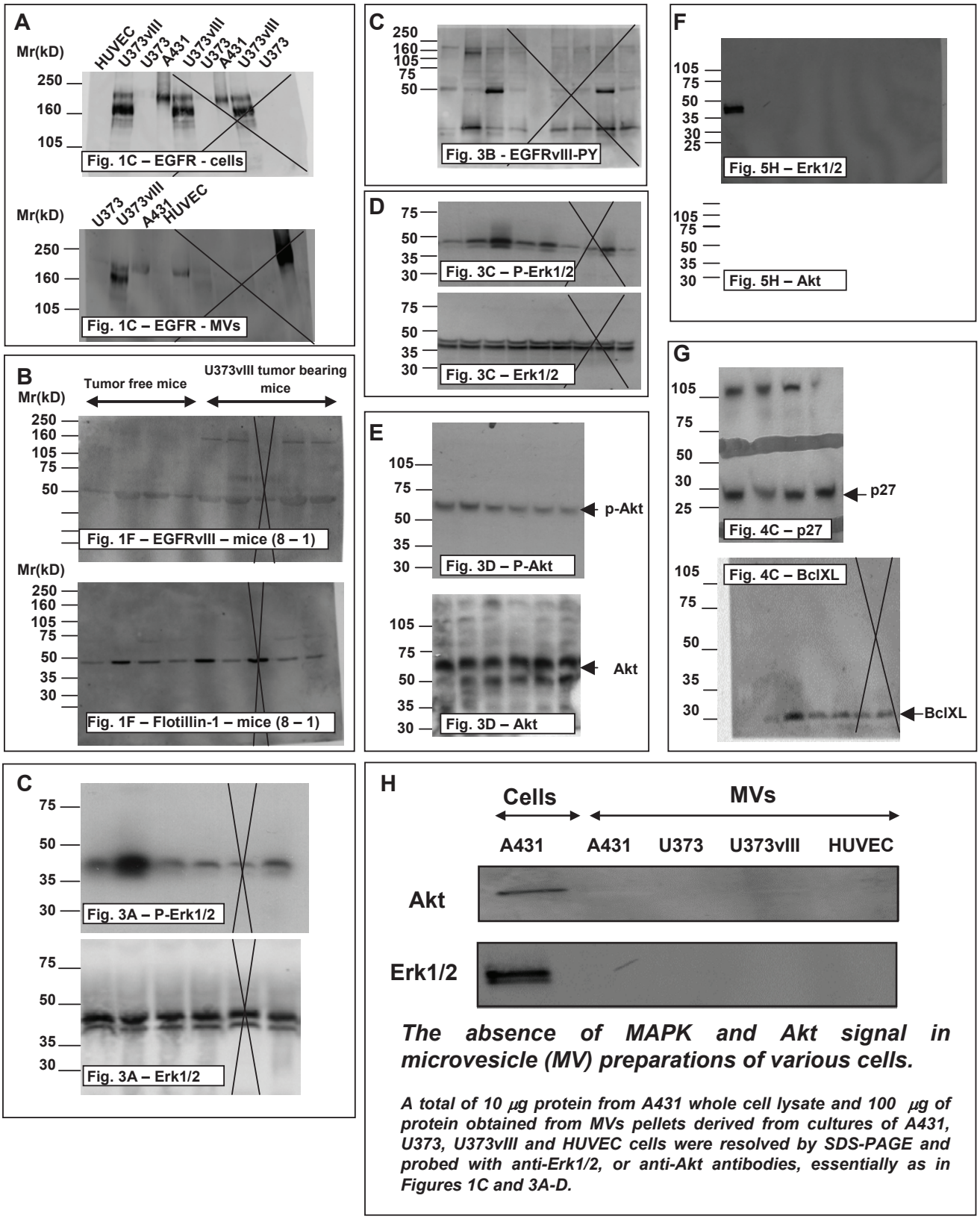


Figure S4 Emission of the FLAG/EGFRvIII-positive material from U373vIII cells in mixed tumors in vivo. Confocal microscopy of mixed tumors composed of U373-GFP (green) and U373vIII-FLAG glioma cells (red) and stained for GFP (green, panel A) and FLAG (red, panel B), respectively. U373vIII-FLAG cells express a FLAG epitope-tagged EGFRvIII oncogene (EGFRvIII-FLAG). Merged channels (C and D) reveal the presence of the FLAG/EGFRvIII-positive MV-like structures (arrows) which are associated not only with overtly FLAG/EGFRvIII-positive cells (U373vIII-FLAG, right side of panels C and D), but also with GFP-positive (U373-GFP) cells. This pattern is suggestive of the emission and intercellular exchange of EGFRvIII-containing MVs in vivo (bars – 5 :m).



The absence of MAPK and Akt signal in microvesicle (MV) preparations of various cells.

A total of 10 μg protein from A431 whole cell lysate and 100 μg of protein obtained from MVs pellets derived from cultures of A431, U373, U373vIII and HUVEC cells were resolved by SDS-PAGE and probed with anti-Erk1/2, or anti-Akt antibodies, essentially as in Figures 1C and 3A-D.

Figure S5 Display of full gels from figures, as indicated (A-G)

Supplemental Materials & Methods

Materials. Antibodies recognizing EGFR (sheep polyclonal and mouse monoclonal) as well as polyclonal antibodies against MAPK (Erk1/2), Akt, flotillin-1, BclxL, and p27 were purchased from Cell Signaling Technology (Danvers, MA). Anti-EGFRvIII-specific monoclonal and polyclonal antibodies were acquired from Zymed (San Francisco, CA) and Abcam (Cambridge, MA), respectively. All the corresponding HRP-conjugated secondary antibodies were from Cell Signaling, while the Alexa Fluor series of secondary antibodies was from Molecular Probes, (Eugene, OR). Colloidal gold-labelled secondary antibodies were from Jackson Immunoresearch laboratory(West Grove, PA).

Cell lines. U373 (human glioblastoma astrocytoma) and U373-EGFRviii (Tet off regulated EGFRvIII) were originally maintained and generated in one of our laboratories (J.M. & A.G) at the Brain Tumor Research Centre (BTC), University of Toronto. U373vIII-GFP cells expressing EGFRvIII tagged with green fluorescent protein (GFP) were generated by stable transfection of U373 cells with the expression vector containing the full length EGFRvIII sequence in frame with the cassette encoding GFP (pEGFPN1 vector). The U373 cell line harboring a FLAG-tagged EGFRvIII (U373vIII-FLAG cells) was generated at the BTC (J.M. & A.G) by expression of the EGFRvIII cDNA fused in frame with the sequence encoding the FLAG epitope (pFLAG-CMV-3 vector). A431 and HUVEC's cells were purchased from the American Type Tissue Collection (ATCC; Manassas, VA). All the cell lines used in the study were cultured in microvesicle-depleted FBS (by centrifugation overnight at 100 000g). For standard culture the cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Isolation of microvesicles from the conditioned media and plasma. Microvesicles were collected from the media of different cell lines and from mouse plasma, as previously described (Al-Nedawi, *et al*.2005). Briefly, conditioned medium was collected from cells at approximetely 80% confluence, unless indicated otherwise, and this material was subjected to two consecutive centrifugations at 300g for 5 minutes, and then at 12000g for 20 minutes to eliminate cells and debris. Finally, microvesicles were obtained after centrifugation for 2 hours at 100 000g, washed twice with a large volume of phosphate buffered saline (PBS). The amount of microvesicle proteins recovered was measured using the Bradford assay (Bio-Rad).

EGFR/EGFRvIII expression profile. The cells (U373, U373vIII, A431 and HUVEC) and their corresponding microvesicles (MVs), were lysed in the lysis buffer containing: 10 mM Tris, pH 6.8, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 2% (wt/vol) SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM Na3VO4, for 10 minutes on ice. Unless otherwise indicated the lysates were resolved by SDS-PAGE and subjected to immunobloting with sheep anti-human EGFR polyclonal antibodies (Cell Signaling). Immunodetection was accomplished using the appropriate HRP-conjugated secondary antibody and chemiluminescence plus kit (ECL kit; Amersham Pharmacia, Buckinghamshire, United Kingdom), after which the blots were scanned and protein bands quantitated using the Storm 860 scanner (GE healthcare). Both wild type EGFR and EGFRvIII bands were detected in this manner at the expected sizes. In some experiments MV lysates were immunoprecipitated using anti-EGFRvIII (ab52104), or anti-phosphotyrosine (PY20) antibodies, both from Abcam, and probed for EGFR to determine the phosphorylation state of this receptor.

Detection of signaling events downstream of activated EGFR. In order to assess the impact of MVs containing oncogenic EGFR on indolent U373 cells, the latter were plated in 100mm dishes at a density of 2 x 10^5 cells/mL, grown briefly and starved for 24 hours either in DMEM supplemented with 0.5% FBS or in serum free DMEM (the latter condition resulted in a more striking activation of cellular signaling upon MV transfer). The cultures were then stimulated overnight with different concentrations of U373vIII or U373 derived MV preparations, either intact or preincubated with Annexin-V or CI-1033. In the latter case MVs were pretreated with the indicated concentrations of the respective reagents 1h prior to their addition to the target cells and under appropriate buffer conditions. In particular for Annexin V pretreatment MVs were suspended in a minimum volume of Annexin binding buffer containing optimized levels of calcium ions (0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM $CaCl_2$). Both agents were removed from MV preparation by extensive washing prior to contact with cultured glioma cells. After MV treatment, cell lysates were prepared and analyzed for the content of signaling effectors using anti-phospho-Erk1/2 and anti-phospho-Akt antibodies (Cell Signaling), as per supplier's recommendations. The intensities of the resulting signals were quantified (Imaquant software, Amersham) and normalized to the corresponding total Erk1/2 and Akt bands. In addition, equal loading was verified by probing the membranes for beta-actin (full blots shown in Suppl. Fig. 5).

Flow cytometry. Flow cytometry (FACS) was employed to detect EGFRvIII, or EGFRvIII-GFP on the surface of viable not permeabilized cells and was carried out either with cells that expressed these receptors endogenously, or with those that have acquired such expression upon transfer of MVs. Typically, U373 cells were treated, as above, with MVs obtained from U373vIII or U373vIII-GFP cells for 24 hours. The cells were then detached using 2 mM EDTA (ethylenediaminetetraacetic acid) to obtain a single-cell suspension the aliquots of which (1.5 x 10^6/sample) were washed in phosphate-buffered saline (PBS) with 1% FBS and 0.1% sodium azide. The cells treated with U373vIII derived MVs were then stained for 30 minutes at 4°C with the monoclonal antibody against EGFRvIII (Zymed). After washing, samples were incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR) for 30 minutes at 4°C, washed with phosphate buffered saline (PBS) and analyzed. In the case of treatment with MVs derived from U373vIII-GFP cells fresh cell suspensions were directly analyzed for GFP fluorescence. The data were acquired using FACScalibur flow cytometer (BD Biosciences, Mountain View, CA).

Fluorescent imaging of the MV uptake. For in vitro analysis of the EGFR expression the cells were grown on *Cell Locate* cover slips (Sigma-Aldrich) and incubated with MVs, as described. The cultures were washed in PBS and fixed in preheated (37°C) 4% (wt/vol) paraformaldehyde (PFA) in phosphate buffered saline for 5 min, followed by three washes in PBS and anti-quenching in 50 mM NH_4Cl for 10 min at room temperature. Subsequently the cells were washed twice in PBS and incubated with BSA [1% (wt/vol) in PBS] for 30 min. Incubation with a primary antibody was carried out for 1 h and followed by washing in PBS incubation with a secondary antibody for 30 min. After staining the cover slips were mounted, using Dako fluorescent mounting medium and viewed under fluorescent and/or confocal microscope to detect the presence of MV content (EGFRvIII) on the membranes of recipient cells. In order to monitor the transfer of this receptor in vivo mixed tumors were generated by co-injection of parental or GFP-tagged U373 cells and their U373vIII counterparts harbouring EGFRvIII receptor, either intact or tagged with GFP or FLAG epitope. For instance, SCID mice were inoculated with mixtures of U373vIII-FLAG and U373-GFP cells (5x10^6 each) and allowed to

develop for only 7 days to prevent protracted hypoxia, necrosis and major shifts in subpopulation composition. In this setting, a co-localization of the FLAG epitope with GFP-positive U373 parental cells could be indicative of MV-mediated transfer of the EGFRvIII. Tumors were harvested and fixed by 4% paraformaldehyde, embedded in paraffin and sectioned (4μm thick). This was followed by double labeling with the anti-GFP (Alexa Fluor 488) and anti-FLAG (Alexa Fluor 594) antibodies and reading under the fluorescent (ZEISS Axioscope, ImagerZ1) or confocal microscope (ZEISS LSM10).

Scanning and transmission electron microscopy. To obtain a scanning electron microscopy (SEM) images of MV production by various cell types, the indicated cells were allowed to attach and grow on *Cell Locate* cover slips (Sigma-Aldrich). The adherent subconfluent monolayers were fixed with 2.5% gluteraldehyde in 0.1 M PBS, washed three times with 0.1 M PBS and then with 0.1 M cacodylate buffer, followed by staining with 1% osmium tetra oxide (OsO_4). The slides were then dehydrated with serial concentrations of ethanol, subjected to critical point drying and to fixation on a stud, after which they were covered with gold. Similar processing was implemented for visualization of isolated MVs, the pellets. The latter were diluted, attached polylysine coated cover slips and processed essentially as above. The samples were viewed and microphotographed under JEOL 840A Scanning Electron Microscope. Transmission electron microscopy (TEM) was employed to detect MVs-like structures in tumors in vivo. Thus, the aforementioned mixed tumors containing U373vIII-FLAG and U373-GFP cells were allowed to grow in SCID mice for 7 days, after which they were excised and fixed in 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The samples were maintained in fixative and then dehydrated through a graded series of ethanol, placed in a cassette and embedded in LRW media overnight. Thin sections (50nm) of these blocks were cut and stained using primary antibodies as indicated. The signal was detected using the secondary antibodies labelled with 18 nm colloidal gold particles. The outcomes were visualised by transmission electron microscope JEOL JEM-2000FX.

***In vivo* experiments.** All *in vivo* experiments were performed in 6- to 8-week-old severe combined immunodeficiency (SCID) mice (Charles River, Saint-Coustant, QC, Canada). Briefly, 1 to 10 x 10^6 of U373vIII or U373, cells were injected subcutaneously in 0.2 mL PBS. Blood was collected from mice by cardiac puncture, into heparin sodium solution. Platelet-free plasma was prepared by centrifugation at 2000*g* for 15 minutes, 2000 *g* for 5 minutes, and 16,000*g* for 5 minutes to isolate MVs.

Promoter activity assays. Semiconfluent cultures of U373 cells were established in 6-well tissue culture plates and transfected with the pGL3 plasmid containing the Firefly Luciferase reporter cassette downstream of the 2.6 kb 5' UTR region of the human *VEGF* gene (a generous gift from Dr. Deb Mukhopadhyay, Mayo Clinic). The transfection was carried out using the lipofectAMINE method, which was optimized for 4 μg of the reporter plasmid and 5 μg of pSVLacZ vector, the ß-galactosidase cassette of which enabled monitoring of the transfection efficiency and data normalization. MVs derived from U373vIII cells, either intact, or pretreated with Annexin-V, as indicated, were incubated with target U373 cells for 24 hours. The cells were thoroughly washed and harvested in the lysis buffer. The particulate matter was cleared from the lysates by microcentrifugation for 5 minutes at 4°C and the supernatants were transferred into fresh vials for reading the Luciferase activity. The bioluminescence was detected using the 1420 Multilabel Counter (Wallac, Perkin Elmer) after addition of the

commercially available substrate provided in the commercially available Luciferase Assay kit (GIBCO), as per manufacturer's instructions. For normalization purposes, the activity of ß-galactosidase was read in each protein sample using the specific Enzyme Assay System (GIBCO) and the EL340 plate reader (BIO-TEK Instruments, Inc). In parallel experiments, U373 were transfected with a promoterless Luciferase reporter vector pGL3 (Promega) and used as a negative control. Each experiment contained at least 4 independent replicates of the respective treatment conditions and several repeats were carried out.

VEGF Elisa. Secretion of VEGF into the conditioned media by U373vIII cells is at least 4-5 fold greater than that of their U373 counterparts (our unpublished observation). Thus, VEGF concentration was measured in the conditioned medium of U373 cells exposed to intact or CI-1033-pretreated MVs from U373vIII cells, or their U373 parents, essentially as indicated. Briefly, semi-confluent cultures of U373 cells were incubated with the respective MV preparations for 24 hours, washed and fed with fresh growth medium for additional 24 hours. The conditioned medium was subsequently assayed for VEGF content using Quantikine Human VEGF Immunoassay (R&D Systems), according the manufacturer's protocol. The readings were collected from multiple (n=3-4) independent samples for each experimental condition, normalized to the cell number and read at several dilutions against a standard curve.

Soft agar colony formation. U373vIII and U373 cells were trypsinized and seeded into 6 well plates at 3 X 10^5 and 4 X 10^5 cells/well, respectively. When cells were 70-80% confluent the media was removed and replaced with serum free media for 8 hours. The media was then replaced with serum free treatment mixture containing different concentrations of U373 or U373 vIII derived MVs, which were incubated with cell cultures at 37^o C overnight (15 hours). The following day, each well was trypsinized. Total cell numbers and viability were determined and the cells were diluted to achieve 1.4 x 10^5 viable cells/ml. In a new tube, 100 ul of this suspension was diluted with 6.9 ml of concentrated DMEM medium (2X) containing 10% FBS and Penicillin/Streptomycin (GIBCO), and then mixed immediately with 7.0 ml of pre-warmed (37^oC) 0.3% agarose (BioRad). Aliquots of liquid agarose containing tumor cells were layed onto 35 mm culture wells pre-coated with 0.5 % agarose in DMEM (pH=7.4). The liquid layer was allowed to solidify before 1 ml of DMEM containing 10 % FBS and 1% Penicillin/Streptomycin was added to the wells. This media was replaced twice weekly. After 3 weeks of growth the multicellular colonies were counted for the entire plate under the inverted microscope and using the 10 X lens. The average number of colonies was determined in triplicate, averaged and the experiment repeated 3 times.

Data Analysis. All experiments were reproduced at least 3 times with similar results. In experiments involving injection of cancer cells to mice between 4-8 animals were included in each assay. Thus, the data shown in Fig. 1D represent an average of 5-7 mice per group. In particular: 7 mice were used as hosts of U373vIII control tumors, 5 mice were injected with U373 cells and additional 5 mice harbouring U373vIII tumors were treated with CI-1033 in this experiment. The comparisons between U373 and U373vIII cell lines with respect to their ability to form s.c. tumors have been conducted in at least 3 independent experiments (not shown) with virtually identical results. The data shown in Fig. 1F are a compilation of 8 mice harbouring U373vIII tumors (only 4 are shown for lack of space) and 4 control, tumor-free mice. Quantification of microvesicle production was carried our using three independent cultures of each cell line (U373 and U373vIII; n=3) per experiment. From each culture the protein was quantified within the MV fraction, as described. One representative experiment carried out in

this fashion is shown in Fig. 1B out of three independent repeats. The numerical data of Elisa, promoter and colony forming assays were generated by testing between 3 to 8 independent replicate samples for each experimental condition of each separate experiment. The output of these studies was depicted as the average (mean) value for each experimental data point ± SD (standard deviation) for each of the respective experiments, of which representative ones are shown in figures. The numbers were also processed for statistical significance using a computerized two-tailed Student t-test. Differences were considered significant when the p value was below 0.05, as indicated in Figures and figure legends.

Acknowledgements. This work was supported by grants to JR from the National Cancer Institute, Canadian Cancer Society and the Terry Fox Foundation. JR is the recipient of the NCIC/CCS Scientist Award and the Cole Chair in Pediatric Oncology. We are indebted to our colleagues from Rak and Guha labs for their creative input, to Dr. Michael Stern from the McGill OTT for his support. We appreciate supply of CI-1033 from Pfizer Canada (Dr. Luc Levesque). We are thankful for the expert help we received from Jacynthe Laliberté, Confocal Microscopy Facility, Pharmacology Department, McGill University and from Jeannie Mui and Line Mongeon, FEMR (Facility of Electron Microscope Research) McGill University. Above all we are thankful for support we received from our families.