Published in final edited form as:

Nanomedicine. 2011 December ; 7(6): 896–903. doi:10.1016/j.nano.2011.05.002.

Targeted Extracellular Nanoparticles Enable Intracellular Detection of Activated Epidermal Growth Factor Receptor in Living Brain Cancer Cells

Veronica Dudu, PhD, Veronica Rotari, and Maribel Vazquez, ScD*

The City College of New York, Department of Biomedical Engineering, 160 Convent Avenue, ST-403D, New York, NY 10031

Abstract

Mechanistic study of biological processes via Quantum dots (Qdots) remain constrained by inefficient QDot delivery methods and consequent altered cell function. Here we present a rapid method to label activated receptor populations within live cancer cells derived from medulloblastoma and glioma tumors. We used Qdots to bind the extracellular domain of Epidermal Growth Factor Receptor (EGF-R) proteins and then induced receptor activation to facilitate specific detection of intracellular, activated EGF-R subpopulations. Such labeling enables rapid identification of biological markers characteristic of tumor type, grade, and chemoresistance.

Keywords

activated EGF-R; Quantum dots; brain cancer

BACKGROUND

Nanoparticles have enabled ground-breaking study of biological processes at the molecular level within live cells (reviewed in¹⁻³). Commercialization of fluorescent Quantum Dot (Qdot) probes has greatly facilitated nanoparticle usage in biomedical research due in part to Qdot surface functionalities for diverse imaging applications. Qdots have been recently used to study a variety of intra- and inter-cellular processes, including the dynamics of membrane proteins⁴⁻⁹, the motion of molecular motors in the cytoplasm¹⁰⁻¹², and the transport of nerve growth factors in neurons¹³. Yet despite their growing use, Qdot applicability to mechanistic study of biological processes remains constrained by limitations in their targeted delivery. For example, Qdot surface coatings are often ‘bulky’, consisting of amphiphilic molecules, such as polyethylene glycol (PEG), that increase nanoparticle hydrodynamic diameter (D_h) significantly to the order of ~20–40 nm^{14, 15}. Such increased size limits the applicability of most commercially-available nanoparticles to studies of intracellular molecular detection. More importantly, internalization of such large nanoparticles as non-functional aggregates in the cytoplasm¹⁶ and/or entrapment of

*CORRESPONDING AUTHOR: Maribel Vazquez. The City College of New York, Department of Biomedical Engineering, 160 Convent Avenue, #403D, New York, NY 10031. Vazquez@ccny.cuny.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

nanoparticles in the endocytic pathway¹⁷ may affect downstream signaling processes as well as generate false positives.

The labeling of dynamic intracellular protein populations via nanoparticles is becoming increasingly attractive to the field of cancer research. For example, it is now widely accepted that tumor malignancy grade can be closely correlated with a combination of specific cell markers¹⁸ that may be extracellular, intracellular, or reside within the cell membrane. Use of nanoparticles to identify multiple markers has critical potential for the rapid characterization of malignant brain tumors, which continue to present one of the lowest patient survival rates worldwide, as well as for selection of potential treatment regimens. Methodologies that enable Qdot nanoparticles to selectively target combinations of specific, intracellular markers during early-stage diagnostics will greatly aid in histotyping of brain tumors, the analysis of tumor functional states and metabolic activity, and/or resection guidance, all of which significantly extend patient lifespan.

In the present study we label and image the activated Epidermal Growth Factor Receptor (EGF-R) populations within live cells derived from medulloblastoma (MB) and glioma (GL), the most prevalent forms of pediatric and adult brain tumors, respectively. EGF-R was chosen as a model protein because of its significant role in tumor development^{24, 25}, diffusion and/or metastasis in glioblastoma^{26, 27}, head and neck cancers²⁸, breast cancer²⁹⁻³¹, human fibrosarcoma cells³², ovarian cancer^{33, 34}, colon cancer³⁵, prostate cancer^{36, 37} and lung cancer^{38, 39}. Here, we used Qdots to label extracellular EGF-R proteins and enabled imaging of activated, intracellular EGF-R populations via pathway activation to induce receptor signaling. Our results are the first to use extracellular Qdot labeling to identify activated EGF-R populations within brain tumor-derived cells. We show that intracellular Qdot detection is EGF dosage-dependent, and corresponds with activation and inhibition of the PI3 Kinase pathway. Such rapid and specific labeling of intracellular EGF-R populations not only facilitates rapid identification of biological markers characteristic of tumor type, grade, and chemo-resistance, but also opens the door to nanoparticle-based, mechanistic study of the role of activated EGF-R in the proliferation and invasiveness of brain tumors.

METHODS

Cell Culture

Medulloblastoma-derived Daoy cells #HTB-186 were purchased from ATCC, Manassas, VA. Daoy cells were cultured with EMEM (Mediatech Inc., Herndon, VA), supplemented with 2% L-Glutamine (Mediatech Inc., Herndon, VA), 1% Penicillin-Streptomycin-Amphotericin B - 100x solution (Mediatech Inc., Herndon, VA), and 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA). Glioma-derived U251 cells were a kind gift from Dr. Eric Holland (MSKCC, NY). U251 cells were cultured with DMEM (Sigma, St. Louis, MO), supplemented with 1% Penicillin-Streptomycin solution (Mediatech Inc., Herndon, VA), and 10% FBS. The cells were grown onto sterile polystyrene tissue culture flasks (BD Biosciences, Franklin Lakes, NJ).

Antibodies and Immunocytochemistry

Cells grown on coverslips were fixed with paraformaldehyde (Sigma, St. Louis, MO) and labeled with biotinylated mouse anti-external-EGF-R (eEGF-R;1:500) – recognizing an external epitope (Meridian Life Sciences, Saco, ME), mouse anti-phosphorylated EGF-R (pEGF-R;1:500) (Meridian Life Sciences, Saco, ME), rabbit anti-phosphorylated-Akt (1:200) (Cell Signaling, Danvers, MO), goat anti-mouse and anti-rabbit AlexaFluor® 488 antibodies (Invitrogen Molecular Probes, Eugene, OR). Transferrin AlexaFluor® 488 or 594

conjugates (Tf) (Invitrogen Molecular Probes, Eugene, OR) were used to label the clathrin-mediated pathway: A 20 µg/mL solution of Tf was applied and incubated with the cells for 1 hour at 37°C.

For Qdot labeling, cells were incubated with a solution containing 5 nM streptavidin-conjugated Qdot 655 (Invitrogen Molecular Probes, Eugene, OR) and 1 nM eEGF-R antibody (Qdot::eEGF-R Ab) for 10 minutes on ice, followed by activation of the EGF pathway. In the EGF-signaling activation experiments, cells were incubated for 30 minutes with 1 µg/mL EGF (Invitrogen Molecular Probes, Eugene, OR) at 37°C, to activate downstream EGF-R, prior to fixation and antibody staining. In the EGF-signaling inhibition experiments, cells were pre-incubated for 1 hour with 0.5 µM Wortmannin (EMD-Calbiochem, Gibbstown, NJ) at 37°C before being exposed to EGF to inactivate PI3K pathway downstream EGF-R, prior to fixation and staining. Cells were subsequently mounted in glycerol. As a control, non-stimulated and non-functionalized Qdot-treated cells (without eEGF-R antibody) were prepared as described above. Each experiment was performed three times. Confocal laser scanning microscopy imaging was performed using the Leica TCS SP2 instrument (Leica Microsystems, Bannockburn, IL) with 63X oil immersion objective (NA 1.4). Identical imaging conditions were used for each set of experiments. Analysis of the confocal images was performed using Matlab software (version 7.7.0.471). Co-localization of fluorescent labels was defined as the 90%-100% overlapping of data points.

MTT assay

The Vybrant MTT Cell Proliferation Assay Kit (Invitrogen Molecular Probes, Eugene, OR) was used according to protocols that determine cell growth rates. Briefly, cells incubated with or without Qdot::eEGFR Ab complexes and stimulated with multiple concentrations of EGF (0, 1, 10, 100, and 1000 ng/mL) were treated with MTT reagents, and incubated for additional 16 hours at 37°C. The absorbance of the samples was read at 570nm and normalized to that of the control (plain medium). Each experiment was done in triplicate.

Transmission Electron Microscopy (TEM)

For the analysis of intracellular Qdot distribution, Daoy cells cultured on collagen-coated plates were stimulated with EGF and labeled with Qdot::eEGF-R Ab as described above. After washing three times with PBS, the cells were fixed with 2% glutaraldehyde (Electron Microscopy Services, Hatfield, PA) for 2 h at 4°C and then post-fixed with 1% osmium tetroxide (Electron Microscopy Services, Hatfield, PA) for 2 h at 48°C. After dehydration by immersing in serially diluted aqueous ethanol solutions, the specimens were embedded in epoxy resin, sectioned 80–100 nm thick, stained with uranyl acetate, and examined via Zeiss EM 902 (Carl Zeiss, Peabody, MA). As a control, non-stimulated and non-functionalized Qdot treated cells were also imaged.

RESULTS

Our study illustrates the rapid and specific imaging of activated, intracellular EGF-R by labeling its extracellular region prior to ligand stimulation. Figure 1 depicts our methodology for live cell labeling of active, intracellular EGF-R via external Qdot. Commercial streptavidin-conjugated Qdots were functionalized with a monoclonal biotinylated antibody designed to specifically recognize extracellular EGF-R. The targeted sequence of the antibody designed to recognize the extracellular side of the EGF-R (eEGF-R Ab) was chosen such that binding of Qdots to the receptor would not affect the binding of EGF ligand. Note that the biotin-streptavidin interaction ($K_d=10^{-13}$ M) yielded a strong bond between the receptor and Qdots⁴⁰ that facilitated visualization. Upon binding of

functionalized Qdots (further referred to as Qdot::eEGF-R Ab) to cell surface receptors (Figure 1A), cell signaling was activated via EGF ligand (Figure 1B), as EGF-R is known to be particularly active in brain tumors^{24, 25}. The binding of the ligand to the receptor induced subsequent receptor activation and internalization through the endocytic pathway⁴¹, thereby enabling the imaging of intracellular, activated, Qdot-labeled EGF receptors within live samples (Figure 1C, 1D).

A first experiment illustrated that EGF-R is present in both medulloblastomas (MB) and gliomas (GL) by labeling the receptor with an antibody recognizing the extracellular domain of the receptor (eEGF-R). Note that all GL data is presented in supplementary material. (See Figure 2A for MB data and See Suppl. Figure 1A for GL data). Upon EGF stimulation of tumor cells, EGF-R became phosphorylated and was largely internalized within endosomes (MB: Figure 2B and GL: Suppl. Figure 1B). Quantification of confocal microscope images illustrated that upon EGF-R activation via ligand binding, 61% of the receptor was internalized within MB cells, compared to 23% EGF-r internalization in non-stimulated MB samples. Similarly, 74% of the activated EGF-R proteins were internalized within EGF-stimulated GL cells, versus 11% in non-stimulated GL cells.

Subsequent experiments targeted external Qdots to activated EGF-R within live MB and GL cells, as described above, and imaged the localization of the receptor via confocal microscopy and/or Transmission Electron Microscopy (TEM). As shown in Figure 2C (MB Data; GL Data: Suppl. Figure 1C), Qdot::eEGF-R Ab complexes were detected within the cytoplasm of tumor cells 30 minutes post EGF stimulation. Image quantification displayed that 59% and 70% of the Qdot-labeled, activated EGF-R was present in the endosomes of MB and GL cells, respectively, consistent with antibody immunostaining of the receptor (MB: Figure 2B and GL: Suppl. Figure 1B). Further, TEM images of EGF-stimulated MB samples labeled with Qdot::eEGF-R Ab complexes displayed the recognizable oblong shape⁴² of electron-dense Qdot cores within endocytic compartments (MB: Figure 2E). We further tested the specificity of Qdot labeling by immunostaining cells for eEGF-R to illustrate receptor co-localization with targeted Qdots at 30 minutes (Figures 2D, and Suppl. Figure 1D) and 24 hours post-labeling (data not shown). The internalized Qdots were seen to remain bound to EGF-R in both tumor cell lines, where signal from the antibody labeling co-localized with 67% of the Qdot fluorescent signal for MB samples and with 90% of the Qdot fluorescent signal for glioma. When receptors were conjugated to Qdots in the absence of EGF, no internalization of receptors was seen (MB: Figures 3C and GL: Suppl. Figure 2C). Similarly, when cells were stimulated with EGF in the presence of Qdots that were not conjugated to receptors, no internalization of QDots was recorded (MB: Figures 3D and GL: Suppl. Figure 2D).

We next tested the dosage-dependency of intracellular EGF-R labeling via external Qdot complex. Tumor cell samples were incubated with Qdot::eEGF-R complexes and stimulated with EGF concentrations of 0, 1, 10, 100, and 1000 ng/ml. Similarly, cells stimulated with identical EGF concentrations were immunostained for phosphorylated EGF-R (pEGF-R). The fluorescent intensity of pEGF-R detected at different EGF stimulations was plotted alongside Qdot fluorescent intensity, in order to compare the levels of activated receptor when labeled via Qdots versus conventional antibody staining. As shown in Figure 3A (for MB Data, GL: Suppl. Figure 2A), increasing EGF concentrations yielded increasing phosphorylation of EGF-R. Consistently, increased EGF concentration lead to increased intracellular detection of Qdot::eEGFR complexes. Furthermore, we measured no significant difference in the metabolic activity of tumor cells treated and untreated with Qdot::eEGF-R complexes and stimulated with the different EGF concentrations (Figure 3B and Suppl. Figure 2B).

Lastly, cell samples labeled with Qdot::eEGF-R complexes were stained for phosphorylated Akt (pAkt) in order to confirm activation of EGF-R downstream pathway, and also with Wortmannin to confirm inhibition of the PI3K pathway. Akt is one of the molecules activated in the PI3K signaling cascade by the stimulation of EGF-R (reviewed in ⁴³), while Wortmannin is a known inhibitor of PI3K phosphorylation ⁴⁴. As shown in Figures 4C and 4A (GL: Suppl. Figures 3C and 3A), EGF stimulation increased cellular levels of pAkt, and EGF signaling was inhibited by pre-treatment with Wortmannin (Figure 4E: MB and GL: Suppl. Figure 3E). Consistently, when activated EGF-R was detected by Qdot::eEGF-R Ab complexes, pAkt levels were high for all samples (MB: Figure 4D and GL: Suppl. Figure 3D). Furthermore, non-stimulated and inhibited cells had comparably low levels of pAkt, and low levels of activated EGF-R, as detected via Qdot fluorescence signal (MB: Figure 4B versus Figure 4F and GL: Suppl. Figure 3B versus Suppl. Figure 3F).

DISCUSSION

This study examined the highly-specific, intracellular labeling of activated EGF-R populations by labeling their extracellular domain with functionalized Qdots. EGF-R populations are significant to a variety of cancers because of the receptor over-expression, and its role in tumorigenesis and metastasis/invasion. Further, EGF-R proteins are very dynamic, as receptors cycle between the cell surface and intracellular endocytic compartments during signaling events. While covalent binding of Qdots to ligands has been achieved to evoke specific physiological responses, covalent binding of Qdots to receptors in order to examine cell responses has not been reported in the cancer literature. For example, biotinylated Nerve Growth Factor was conjugated to commercial streptavidin-Qdot surfaces and shown to retain ligand bioactivity, activate TrkA receptors, and initiate neuronal differentiation in PC12 cells (derived from neuroendocrine tumors) ⁴⁵. Similarly, Lidke and colleagues demonstrated that Qdot-conjugated EGF binds and activates the EGF receptor erbB1, being rapidly internalized into endosomes that exhibit active trafficking and extensive fusion in A431 cells (epidermal carcinoma) and CHO cells (chinese hamster ovary) ⁴⁶. However, we note that our methodology facilitated specific labeling of activated EGF-R receptor populations, not ligands. Such distinction is important, as our interest lies in dynamic intracellular populations that are used, in part, to assign malignancy grading of brain tumor samples and identify potential treatment regimens. As such, our study did not intend to achieve a 1:1 binding of Qdot to eEGF-R Ab, whose difficulty has been well-demonstrated by recent studies ⁷, but instead focused on targeting overall populations of activated receptors significant to cancer cells.

While endosomal EGF-R signaling has been reported for other cell types ⁴¹, this study is the first to use nanoparticles to confirm predominant endosomal signaling in both medulloblastoma- (61% of activated EGF-R) and glioma-(74% of activated EGF-R) derived cells (MB: Figure 2B and GL: Supplementary 1B). Internalization of the Qdot-labeled, activated EGF-R within endosomes of both tumor types was highly specific, occurring only when cells were stimulated with EGF and in a dosage-dependent manner. Two control experiments support this fact. First, Qdot uptake was highly specific (MB: Figures 3C; GL: Supplementary 2C) and required activated PI3K pathways for internalization. And second, Qdots did not get endocytosed in bulk, but instead required covalent bounding to the receptor in order to enter cells (MB: Figures 3D; GL: Supplementary 2D). Lastly, our results indicated that intracellular EGF-R detection via extracellular Qdot labeling did not affect the metabolic rates of medulloblastoma or glioma cells (MB: Figure 3B; GL:Suppl. Figure 2B), nor disrupt immediate downstream signaling pathways: Inhibition of the PI3 Kinase prevented Qdot detection, while increased Qdot signal correlated with increased phosphorylation of Akt in an EGF dosage-dependent manner (MB: Figure 4; GL: Suppl. Figure 3).

In summary, while previous studies have examined Qdot binding to EGF-R⁵⁰ and other tyrosine kinases for cell diagnostics⁴⁷⁻⁴⁹, we here present a subtle and rapid external labeling methodology that identifies the level of activated, intracellular EGF-R populations within live brain tumor cells. Such labeling will greatly facilitate tests that examine the presence and/or absence of specific biomarkers, compare activated receptor levels within different grades and types of tumors, as well as complement existing methods of tracking significant protein populations within diseased cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

RESOURCES: This work was supported by the Pediatric Brain Tumor Foundation, and the National Institutes of Health (NCI 1U54CA143798 and NCI 1R21CA118255).

References

1. Alivisatos AP, Gu W, Larabell C. Quantum dots as cellular probes. *Annu Rev Biomed Eng.* 2005; 7:55–76. [PubMed: 16004566]
2. Pinaud F, Clarke S, Sittner A, Dahan M. Probing cellular events, one quantum dot at a time. *Nat Methods.* 2010; 7(4):275–85. [PubMed: 20354518]
3. Walkey C, Sykes EA, Chan WC. Application of semiconductor and metal nanostructures in biology and medicine. *Hematology Am Soc Hematol Educ Program.* 2009:701–7. [PubMed: 20008256]
4. Bannai H, Levi S, Schweizer C, Dahan M, Triller A. Imaging the lateral diffusion of membrane molecules with quantum dots. *Nat Protoc.* 2006; 1(6):2628–34. [PubMed: 17406518]
5. Dahan M, Levi S, Luccardini C, Rostaing P, Riveau B, Triller A. Diffusion dynamics of glycine receptors revealed by single-quantum dot tracking. *Science.* 2003; 302(5644):442–5. [PubMed: 14564008]
6. Gomez-Varela D, Kohl T, Schmidt M, et al. Characterization of Eag1 channel lateral mobility in rat hippocampal cultures by single-particle-tracking with quantum dots. *PLoS One.* 2010; 5(1):e8858. [PubMed: 20111597]
7. Howarth M, Liu W, Puthenveetil S, et al. Monovalent, reduced-size quantum dots for imaging receptors on living cells. *Nat Methods.* 2008; 5(5):397–9. [PubMed: 18425138]
8. Howarth M, Takao K, Hayashi Y, Ting AY. Targeting quantum dots to surface proteins in living cells with biotin ligase. *Proc Natl Acad Sci U S A.* 2005; 102(21):7583–8. [PubMed: 15897449]
9. Kodippili GC, Spector J, Sullivan C, et al. Imaging of the diffusion of single band 3 molecules on normal and mutant erythrocytes. *Blood.* 2009; 113(24):6237–45. [PubMed: 19369229]
10. Courty S, Luccardini C, Bellaiche Y, Cappello G, Dahan M. Tracking individual kinesin motors in living cells using single quantum-dot imaging. *Nano Lett.* 2006; 6(7):1491–5. [PubMed: 16834436]
11. Pierobon P, Achouri S, Courty S, et al. Velocity, processivity, and individual steps of single myosin V molecules in live cells. *Biophys J.* 2009; 96(10):4268–75. [PubMed: 19450497]
12. Seitz A, Surrey T. Processive movement of single kinesins on crowded microtubules visualized using quantum dots. *EMBO J.* 2006; 25(2):267–77. [PubMed: 16407972]
13. Cui B, Wu C, Chen L, et al. One at a time, live tracking of NGF axonal transport using quantum dots. *Proc Natl Acad Sci U S A.* 2007; 104(34):13666–71. [PubMed: 17698956]
14. Doose S, Tsay JM, Pinaud F, Weiss S. Comparison of photophysical and colloidal properties of biocompatible semiconductor nanocrystals using fluorescence correlation spectroscopy. *Anal Chem.* 2005; 77(7):2235–42. [PubMed: 15801758]
15. Sabharwal N, Holland EC, Vazquez M. Live cell labeling of glial progenitor cells using targeted quantum dots. *Ann Biomed Eng.* 2009; 37(10):1967–73. [PubMed: 19415494]

16. Akerman ME, Chan WC, Laakkonen P, Bhatia SN, Ruoslahti E. Nanocrystal targeting in vivo. *Proc Natl Acad Sci U S A*. 2002; 99(20):12617–21. [PubMed: 12235356]
17. Duan H, Nie S. Cell-penetrating quantum dots based on multivalent and endosome-disrupting surface coatings. *J Am Chem Soc*. 2007; 129(11):3333–8. [PubMed: 17319667]
18. Cho DY, Lin SZ, Yang WK, et al. The Role of Cancer Stem Cells (CD133(+)) in Malignant Gliomas. *Cell Transplant*. 2011; 20(1):121–5. [PubMed: 20887676]
19. Sainsbury JR, Farndon JR, Needham GK, Malcolm AJ, Harris AL. Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cancer. *Lancet*. 1987; 1(8547):1398–402. [PubMed: 2884496]
20. Hall WA, Merrill MJ, Walbridge S, Youle RJ. Epidermal growth factor receptors on ependymomas and other brain tumors. *J Neurosurg*. 1990; 72(4):641–6. [PubMed: 2319323]
21. Werner MH, Humphrey PA, Bigner DD, Bigner SH. Growth effects of epidermal growth factor (EGF) and a monoclonal antibody against the EGF receptor on four glioma cell lines. *Acta Neuropathol*. 1988; 77(2):196–201. [PubMed: 3265834]
22. Lemoine NR, Hughes CM, Barton CM, et al. The epidermal growth factor receptor in human pancreatic cancer. *J Pathol*. 1992; 166(1):7–12. [PubMed: 1538276]
23. Veale D, Ashcroft T, Marsh C, Gibson GJ, Harris AL. Epidermal growth factor receptors in non-small cell lung cancer. *Br J Cancer*. 1987; 55(5):513–6. [PubMed: 3038157]
24. Kang CS, Zhang ZY, Jia ZF, et al. Suppression of EGFR expression by antisense or small interference RNA inhibits U251 glioma cell growth in vitro and in vivo. *Cancer Gene Ther*. 2006; 13(5):530–8. [PubMed: 16410821]
25. Meco D, Servidei T, Riccardi A, et al. Antitumor effect in medulloblastoma cells by gefitinib: Ectopic HER2 overexpression enhances gefitinib effects in vivo. *Neuro Oncol*. 2009; 11(3):250–9. [PubMed: 19033425]
26. Brockmann MA, Ulbricht U, Gruner K, Fillbrandt R, Westphal M, Lamszus K. Glioblastoma and cerebral microvascular endothelial cell migration in response to tumor-associated growth factors. *Neurosurgery*. 2003; 52(6):1391–9. discussion 99. [PubMed: 12762884]
27. Katakowski M, Zheng X, Jiang F, Rogers T, Szalad A, Chopp M. MiR-146b-5p suppresses EGFR expression and reduces in vitro migration and invasion of glioma. *Cancer Invest*. 2010; 28(10):1024–30. [PubMed: 20874002]
28. Wang X, Schneider A. HIF-2 α -mediated activation of the epidermal growth factor receptor potentiates head and neck cancer cell migration in response to hypoxia. *Carcinogenesis*. 2010; 31(7):1202–10. [PubMed: 20395290]
29. Howlin J, Rosenkvist J, Andersson T. TNK2 preserves epidermal growth factor receptor expression on the cell surface and enhances migration and invasion of human breast cancer cells. *Breast Cancer Res*. 2008; 10(2):R36. [PubMed: 18435854]
30. Kimura F, Iwaya K, Kawaguchi T, et al. Epidermal growth factor-dependent enhancement of invasiveness of squamous cell carcinoma of the breast. *Cancer Sci*. 2010; 101(5):1133–40. [PubMed: 20219074]
31. Rodenhiser DI, Andrews J, Kennette W, et al. Epigenetic mapping and functional analysis in a breast cancer metastasis model using whole-genome promoter tiling microarrays. *Breast Cancer Res*. 2008; 10(4):R62. [PubMed: 18638373]
32. Hwang YP, Yun HJ, Choi JH, et al. Suppression of EGF-induced tumor cell migration and matrix metalloproteinase-9 expression by capsaicin via the inhibition of EGFR-mediated FAK/Akt, PKC/Raf/ERK, p38 MAPK, and AP-1 signaling. *Mol Nutr Food Res*. 2011
33. Ji C, Cao C, Lu S, et al. Curcumin attenuates EGF-induced AQP3 up-regulation and cell migration in human ovarian cancer cells. *Cancer Chemother Pharmacol*. 2008; 62(5):857–65. [PubMed: 18214481]
34. Moss NM, Liu Y, Johnson JJ, et al. Epidermal growth factor receptor-mediated membrane type 1 matrix metalloproteinase endocytosis regulates the transition between invasive versus expansive growth of ovarian carcinoma cells in three-dimensional collagen. *Mol Cancer Res*. 2009; 7(6):809–20. [PubMed: 19509114]
35. Grondin V, Seksik P, Dumont S, et al. Regulation of colon cancer cell proliferation and migration by MD-2 activity. *Innate Immun*. 2010

36. Gan Y, Shi C, Inge L, Hibner M, Balducci J, Huang Y. Differential roles of ERK and Akt pathways in regulation of EGFR-mediated signaling and motility in prostate cancer cells. *Oncogene*. 2010; 29(35):4947–58. [PubMed: 20562913]
37. Zhong M, Boseman ML, Millena AC, Khan SA. Oxytocin induces the migration of prostate cancer cells: involvement of the Gi-coupled signaling pathway. *Mol Cancer Res*. 2010; 8(8):1164–72. [PubMed: 20663860]
38. Rho JK, Choi YJ, Lee JK, et al. Epithelial to mesenchymal transition derived from repeated exposure to gefitinib determines the sensitivity to EGFR inhibitors in A549, a non-small cell lung cancer cell line. *Lung Cancer*. 2009; 63(2):219–26. [PubMed: 18599154]
39. Yan X, Han J, Zhang Z, et al. Lung cancer A549 cells migrate directionally in DC electric fields with polarized and activated EGFRs. *Bioelectromagnetics*. 2009; 30(1):29–35. [PubMed: 18618607]
40. Piran U, Riordan WJ. Dissociation rate constant of the biotin-streptavidin complex. *J Immunol Methods*. 1990; 133(1):141–3. [PubMed: 2212686]
41. Le Roy C, Wrana JL. Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. *Nat Rev Mol Cell Biol*. 2005; 6(2):112–26. [PubMed: 15687999]
42. Nisman R, Dellaire G, Ren Y, Li R, Bazett-Jones DP. Application of quantum dots as probes for correlative fluorescence, conventional, and energy-filtered transmission electron microscopy. *J Histochem Cytochem*. 2004; 52(1):13–8. [PubMed: 14688213]
43. Takeuchi K, Ito F. EGF receptor in relation to tumor development: molecular basis of responsiveness of cancer cells to EGFR-targeting tyrosine kinase inhibitors. *FEBS J*. 2010; 277(2): 316–26. [PubMed: 19922467]
44. Wymann MP, Bulgarelli-Leva G, Zvelebil MJ, et al. Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol Cell Biol*. 1996; 16(4):1722–33. [PubMed: 8657148]
45. Vu TQ, Maddipati R, Blute TA, Nehilla BJ, Nusblat L, Desai TA. Peptide-conjugated quantum dots activate neuronal receptors and initiate downstream signaling of neurite growth. *Nano Lett*. 2005; 5(4):603–7. [PubMed: 15826094]
46. Lidke DS, Nagy P, Heintzmann R, et al. Quantum dot ligands provide new insights into erbB/HER receptor-mediated signal transduction. *Nat Biotechnol*. 2004; 22(2):198–203. [PubMed: 14704683]
47. Arndt-Jovin DJ, Kantelhardt SR, Caarls W, de Vries AH, Giese A, Jovin Ast TM. Tumor-targeted quantum dots can help surgeons find tumor boundaries. *IEEE Trans Nanobioscience*. 2009; 8(1): 65–71. [PubMed: 19304503]
48. Huang DH, Su L, Peng XH, et al. Quantum dot-based quantification revealed differences in subcellular localization of EGFR and E-cadherin between EGFR-TKI sensitive and insensitive cancer cells. *Nanotechnology*. 2009; 20(22):225102. [PubMed: 19433879]
49. Lee J, Choi Y, Kim K, et al. Characterization and cancer cell specific binding properties of anti-EGFR antibody conjugated quantum dots. *Bioconjug Chem*. 2010; 21(5):940–6. [PubMed: 20420360]
50. Kawashima N, Nakayama K, Itoh K, Itoh T, Ishikawa M, Biju V. Reversible dimerization of EGFR revealed by single-molecule fluorescence imaging using quantum dots. *Chemistry*. 2010; 16(4):1186–92. [PubMed: 20024999]

Figure 1.

Schematics of direct labeling of active EGF-R with Qdots. **A.** Qdots functionalized with biotinylated anti-eEGF-R antibodies are incubated with the cells on ice, allowing the binding of the functionalized Qdots to the cell-surface EGF-R; **B.** Cells are stimulated with 1 mg/ml EGF at 37°C; **C.** EGF activates EGF-R, inducing the intra-cellular internalization of the labeled active receptors; **D.** Qdot::eEGF-R Ab complexes bound to the active receptors are internalized in endosomes.

Figure 2.

Labeling of EGF-R in medulloblastoma-derived cells. **A.** Labeling for EGF-R with anti-eEGF-R antibody (A1) and for the endocytic pathway with Transferrin (Tf) (A2) in unstimulated cells. Merged image (A3); **B.** Labeling of EGF-stimulated cells for EGF-R stained with anti-eEGF-R antibody (B1) and for Tf (B2). Merged image (B3); **C.** Activated EGF-R labeled directly with Qdots::eEGF-R Ab (C1), and the endocytic pathway labeled by Tf (C2). Merged image (C3); **D.** Activated EGF-R labeled directly with Qdots::eEGF-R Ab (B1), and immunostaining for eEGF-R (D2). Merged image (D3); (A-D) Nuclei are indicated by the letter “N”. Scale bar equals 25 μm; **E.** TEM of Qdots::eEGF-R Ab-labeled EGF-R in EGF-stimulated cells. (E1) Arrows point to endoplasmic reticulum (ER) and mitochondria. Scale bar equals 500 nm; (E2) High magnification of the area labeled in E1:

arrow points to Qdots labeling activated EGF-R internalized in endocytic compartment.
Scale bar equals 100 nm.

Figure 3.

Characterization of Qdot labeled EGF-R in medulloblastoma-derived cells. **A.** Increasing EGF concentration lead to increasing phosphorylation of the EGF-R, as shown by standard immunofluorescence (green), as well as by Qdot labeling (purple) (n=3); **B.** Qdot labeling of the receptor has no effect on the metabolic rate of the cells, at any of the EGF concentration tested (n=6); **C.** Non-stimulated cells: Qdots::eEGF-R Ab (C1), and Tf (C2). Merged image (C3); **D.** Cells stimulated with EGF: non-functionized Qdots (D1), and endocytic pathway labeled by Tf (D2). Merged image (D3). Nuclei are indicated by the letter “N”. Scale bars equal 25 μ m.

Figure 4.

Activation of PI3K pathway upon EGF-R activation in medulloblastoma-derived cells. **A.** Labeling of pAkt in non-stimulated cells. **B.** Labeling of non-stimulated cells for pAkt (B1), and for EGF-R labeled directly with Qdots::eEGF-R Ab (B2). Merged image (B3). **C.** Labeling of pAkt in stimulated cells. **D.** Labeling of stimulated cells for pAkt (D1), and for EGF-R labeled directly with Qdots::eEGF-R Ab (D2). Merged image (D3). **E.** Labeling of pAkt in cells inhibited with Wortmannin. **F.** Labeling of cells inhibited with Wortmannin for pAkt (F1), and for EGF-R labeled directly with Qdots::eEGF-R Ab (F2). Merged image (F3). Nuclei are indicated by the letter “N”. Scale bars equal 25 μm.