Changes in membrane morphology and membrane protein dynamics based on its fluidity are critical for cancer metastasis. However, this subject has remained unclear, because the spatial precision of previous in vivo imaging has been limited to the micrometer level and single molecule imaging is impossible. Here, we have imaged the membrane dynamics of tumor cells in mice with a spatial precision of 7–9 nm under a confocal microscope. A metastasis-promoting factor on the cell membrane, protease-activated receptor 1 (PAR1), was labeled with quantum dots conjugated with an anti-PAR1 antibody. Movements of cancer cells and PAR1 during metastasis were clearly observed in vivo. Images used to assess PAR1 dynamics were taken of representative cells for four stages of metastasis; i.e. cancer cells far from blood vessels in tumor, near the vessel, in the bloodstream, and adherent to the inner vascular surface in the normal tissues near tumor were photographed. The diffusion constant of PAR1 in static cells far from tumor blood vessels was smaller than in moving cells near the vessels and in the bloodstream. The diffusion constant of cells adhering to the inner vascular surface in the normal tissues was also very small. Cells formed membrane protrusion during migration. The PAR1 diffusion constant on these pseudopodia was greater than in other membrane regions in the same cell. Thus, the dynamics of PAR1 movement showed that membrane fluidity increases during intravasation, reaches a peak in the vessel, decreases during extravasation, and is also higher at locally formed pseudopodia.

During metastasis, cancer cells detach from the parent tumor, invade surrounding connective tissue and blood vessels, are transported in the bloodstream, and invade other organs after extravasation (1, 2). Membrane dynamics are significantly altered in metastatic cancer cells (3). Many studies using cultured cells have suggested that metastatic cancer cells form pseudopodia termed filopodia, lamellipodia, and invadopodia; this process is driven by actin polymerization in the direction of cellular migration and invasion (4–7). Additionally, greater membrane fluidity is thought to enhance the malignancy of cultured cancer cells (8, 9). High membrane fluidity is coupled to increased diffusion speed of membrane proteins. Greater diffusion speed accelerates the reaction rate between receptors and their ligands or adhesion proteins and their extracellular substratums. In this way, the metastatic ability of cancer cells is activated (9). Thus, to elucidate the mechanisms of cancer metastasis, analysis of membrane protein dynamics during metastasis is crucial. In living tumors in vivo, there are blood vessels and three-dimensional communication systems between cells, unlike in cultures in vitro. It is therefore essential that in vivo membrane morphology and fluidity based on membrane protein dynamics are clarified.

Previous studies used imaging of GFP- or luciferase-expressing cancer cells in vivo to examine the behavior of metastatic cancer cells (10–14). However, because the spatial precision of such imaging is limited to the micrometer level and single molecule imaging is impossible, the details of in vivo dynamics of individual membrane proteins remain unknown. We have been developing single molecule imaging using fluorescent molecules and quantum dots (QDs) with 1 nm precision in vitro and have elucidated the molecular mechanisms of motor proteins, myosin, kinesin, and dynein (15–18). By applying this imaging method in vivo, our previous studies (19, 20) succeeded in tracking an anti-HER2 (human epidermal growth factor receptor 2) monoclonal antibody conjugated with bright QDs (21, 22) in vivo with a spatial precision of 30 nm. However, the size of a typical protein ranges from several nanometers to 20 nm. Therefore, 30 nm precision is not suitable to understand the molecular function-associated dynamics of proteins.

Here, we have further developed a method to image a tumor cell membrane protein with antibody-conjugated QDs. We examined the membrane dynamics of PAR1 in living metastatic tumor cells using quantum dots (QDs) with 1 nm precision in vivo. The spatial precision of previous imaging has been limited to the micrometer level and single molecule imaging is impossible. However, the spatial precision of such imaging is limited to the micrometer level and single molecule imaging is impossible, the details of in vivo dynamics of individual membrane proteins remain unknown.
used this technique to visualize the details of membrane fluidity and morphology during metastasis in living mice with a spatial precision of 7–9 nm under a Nipkow disk confocal microscope. This new nanotechnology would enable us to understand the functional dynamics of proteins and nanometer-scale anticancer agents in vivo.

EXPERIMENTAL PROCEDURES

PAR1 Antibody—The oligopeptide CNATLDPRSFLL, including a sequence from Asn to Leu of PAR1, was cross-linked with keyhole limpet hemocyanine, and the cross-linked oligopeptide was used as an antigen to generate an anti-human PAR1 monoclonal antibody. Epitope mapping for the antibody was performed by enzyme-linked immunosorbent assay utilizing the culture supernatant of hybridoma cells for the primary antibody and a horseradish peroxidase-conjugated anti-mouse IgG antibody (Upstate) as the secondary antibody. The absorbance was read at 492 nm to detect substrate reactivity by the horseradish peroxidase-conjugated antibody. To obtain high purity anti-PAR1 antibody, the cloned hybridoma cells were injected into the abdominal cavity of a severe combined immunodeficient (SCID) mouse (Charles River), which is an immunodeficient mouse without immunoglobulins, and ascites were prepared from the mouse. Anti-PAR1 antibody was purified from the ascites using protein G-Sepharose (Amersham Biosciences), and the purified antibody was applied to a preparation of anti-PAR1 antibody-conjugated QDs (anti-PAR1-QDs) using a Qdot® 705 Antibody Conjugation Kit (Invitrogen), where the number indicates the emission wavelength.

DNA Constructs—To make a construct of the PAR1-GFP gene that would be stably expressed in cultured cells, the open reading frame of human PAR1 cDNA was cloned into the HindIII and BamHI sites of the pEGFP vector (Clontech). The PAR1-GFP gene was excised from the plasmid at the HindIII and NotI sites and cloned into the HindIII and NotI sites of the pLNCX2 retroviral vector (BD Bioscience). The sequences of these DNA constructs were checked according to the ABI DNA sequencing system protocol.

Cell Culture—The human KPL-4 (KPL) breast cancer cell line (23), non-metastatic in SCID mice, was kindly provided by Dr. J. Kurebayashi (Kawasaki Medical School, Japan). The KPL cells were transfected into the metastatic cancer cell PAR1-GFP-expressing KPL (PAR1-KPL) by transduction with the pLNCX2 retroviral vector system containing the PAR1-GFP gene as the insert, and the cells were then cloned. KPL and PAR1-KPL cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum. PAR1-KPL cells were grown in the presence of 400 μg/ml G418.

Optical System with Confocal Microscope—The optical system for observation of the fluorescence of GFP or QDs consisted primarily of an epifluorescence microscope (IX-71, Olympus) with modifications, a Nipkow disk-type confocal unit (CSU10, Yokogawa), and an electron multiplier type charge-coupled device camera (EM-CCD, Ixon DV887, Andor Technology). A UPlanFL N (×100, 1.30 numerical aperture, Olympus) objective lens was used for in vivo imaging and a PlanApo (×60, 1.40 numerical aperture, Olympus) objective lens was used for in vitro imaging. GFP was illuminated by a blue laser (488 nm wavelength, Furukawa Electric), and QDs were illuminated by a green laser (532 nm wavelength, CrystaLaser). The laser-excited fluorescence was filtered with a 500–550 nm bandpass filter for GFP, a 685–725 nm bandpass filter for QDs, and a >580 nm long-pass filter for imaging QDs and autofluorescence of red blood cells. Images were taken at a rate of 5–10 frames per second. For in vivo imaging, to remove the oscillation of heartbeat and respiration in observations, an aluminum stage was developed for this study and attached to the above microscopy system.

In Vitro Imaging—To investigate the specificity of the PAR1 antibody, KPL and PAR1-KPL cells were mixed with 40 nM anti-PAR1-QDs in serum-free L-15 medium (Invitrogen) for 30 min at 37 °C. After washing with L-15 medium, these cells were incubated with L-15 containing 0.5% fetal bovine serum in a glass-bottomed dish and then observed. The captured images are shown in Figure 1.
were converted to autovideo interleaving files, and fluorescence intensities of QDs in the files were calculated as gray values using ImageJ software. To track PAR1 movements, PAR1-KPL cells were mixed with 2.5 nM anti-PAR1-QDs in serum-free L-15 medium for 30 min at 37 °C. After washing with L-15, these cells were incubated with L-15 containing 0.5% fetal bovine serum in a glass-bottomed dish and then observed. The position of QDs on the cell membrane was tracked using a previously described single molecule tracking method (18).

In Vivo Imaging—PAR1-KPL cells (1 × 10⁶) were suspended in 100 μl of L-15 medium containing 10% fetal bovine serum and transplanted subcutaneously into the skin of female SCID mice at 5–7 weeks of age. 5–10 weeks after transplantation, anti-PAR1-QDs were injected into the tail vein of the mice. The probe concentration in the blood was 5 nM. This concentration does not inhibit migration and invasion of PAR1-KPL cells in vitro. The mice labeled with the probe were placed under anesthesia with ketamine and xylazine, and the anesthetized condi-
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Development of in Vivo Nano-imaging Method—To image the membrane dynamics of metastatic cancer cells with a high spatial precision, we improved three points in our previous imaging method: the targeted protein, the type of cancer cell, and the method of fixing tumors on a microscope stage. First, a metastasis-promoting factor on the cell membrane, protease-activated receptor 1 (PAR1), was targeted. PAR1 is a G-protein-coupled receptor that plays a critical role in metastatic processes of various cancers of the breast, colon, lung, pancreas, and prostate (26–28). PAR1 expression correlated strongly with cellular metastatic capability in breast cancer (29), whereas HER2 did not. Matrix metalloprotease 1 activates PAR1 by cleaving its exodomain at the Arg31–Ser42 peptide bond, and this activation promotes cell migration and invasion. We prepared anti-PAR1 antibody that specifically binds to a human PAR1 sequence, \(^{35}\text{NATLDPRSFLL}^{45}\) (Fig. 1, A–C), which differs from mouse PAR1 by three amino acids (letters in italic), \(^{35}\text{DATYVNPRSFLL}^{45}\) (30).

In our previous imaging studies, we prepared tumor-bearing mice by transplantation of human KPL-4 (KPL) breast cancer cells (23), which express HER2 at a high level but PAR1 at low levels. Thus, KPL cells are non-metastatic in mice. To examine the metastatic process, KPL cells were transformed into a PAR1-GFP-expressing KPL (PAR1-KPL) cell line by transduction of a PAR1-GFP gene (Fig. 2A). PAR1 expression induced metastasis of KPL cells (supplemental Fig. S1). To examine the specificity of anti-PAR1 antibodies, we performed immunostaining with anti-PAR1 antibody-conjugated QDs (anti-PAR1-QDs) in vitro. Anti-PAR1-QDs specifically reacted to human PAR1 on the cell membranes of PAR1-KPL cells, whereas its fluorescence intensity on KPL cells was only 1/17 of that on PAR1-KPL (Fig. 2, B and C). PAR1 antibody at a concentration of 10 nM did not prevent the endocytosis of PAR1 (supplemental Fig. S2) and the metastatic activity of PAR1-KPL.
cells activated by matrix metalloprotease 1. At a high concentration of 10 μM, antibody decreased the activity to ∼40% (data not shown). These results demonstrate that the anti-PAR1 antibody could function as a specific tracer against PAR1-expressing metastatic cancer cells and an effective anticancer agent.

Exposed mouse tumors (supplemental Fig. S3, A–D) were bonded on a polystyrene plate containing a small window using Aron alpha instant adhesive, which features low osmolarity and fluidity, without damaging tumor cells (Fig. 2D). Aron alpha minimized oscillations derived from heartbeat and respiration, in contrast to larger oscillations observed in the previous method using thread (20). The plate was fixed tightly to a handmade aluminum stage using screws (Fig. 2D). After injection of QDs into the tail vein of tumor-bearing mice, QDs bound to the tumor stroma were imaged under a confocal microscope (Fig. 2D). The center of the QD image was calculated by fitting the fluorescence intensity profiles of QDs to two-dimensional Gaussian curves (18). We measured values of ∼7 nm for the X-axis and ∼9 nm for the Y-axis (Fig. 2E), indicating that we can track the movement of PAR1 labeled with anti-PAR1-QDs with a spatial precision of 7–9 nm.

To confirm whether the QDs bound to the surface of targeted tumor cells, two methods were employed after injection of anti-PAR1-QDs into tumor-bearing SCID mice. First, the fluorescence positions of anti-PAR1-QDs were compared with those of PAR1-GFP on tumor cell membranes. We analyzed the QDs that colocalized with PAR1-GFP on cell membrane (Fig. 2F). The second method involved superimposed imaging of anti-PAR1-QD fluorescence over 40 frames, showing the position of PAR1 on the cell membrane. Membrane proteins move randomly along the cell membrane, and the traces of QDs show the outline of the membrane (Fig. 2G).

We confirmed that anti-PAR1-QDs labeled targeted PAR1-KPL cells but not the blood cells in SCID mice. We injected anti-PAR1-QDs into the tail vein of mice that were not transplanted with PAR1-KPL cells and examined the QDs bound to blood cells. Most of the cells (>99.9% for whole blood cells and >99.9% for leukocytes) were labeled with three or fewer QDs (Fig. 2, H and I), but many anti-PAR1-QDs (typically >10 QDs) bound to PAR1-KPL cells (Figs. 3 and 4). The probability of a cell being bound by more than ten QDs is extremely low. Therefore, in tumor-bearing mice, cells labeled with dozens of QDs represent metastatic cancer cells.

**Membrane Dynamics in Metastatic Cancer Cells Outside of Vessels**—We observed the in vivo membrane dynamics of metastatic cancer cells in four regions: far from the blood vessel in tumors, near the vessel, in the bloodstream, and adherent to the inner vascular surface in normal tissues near tumors. These locations represent the process of cancer metastasis (Fig. 5).
First, the cancer cells outside vessels, cells far from tumor blood vessels, and cells near the vessels were visualized. Anti-PAR1-QDs were bound to the surface of cells in colonies far from tumor blood vessels (Fig. 3A and supplemental video 1). In these cells, mobile edges, such as pseudopodia, were not seen, suggesting that the cells were static. In contrast, cancer cells near tumor blood vessels formed membrane protrusions oriented toward the vessel (Fig. 3B–D, and supplemental video 2), although actual migration of the cells was not observed. To analyze the diffusional movement of anti-PAR1-QDs quantitatively, the MSD of the QD position was analyzed (24, 25). The MSD plots of anti-PAR1-QDs for cell membranes of cancer cells outside of vessels were fitted to linear functions representing the diffusion coefficient for random diffusion (Fig. 3F). The diffusion constant of PAR1 on cells far from tumor vessels (blue squares in Fig. 3A) was low, at 72 nm²/s (Fig. 3E and F). This low diffusion constant was not due to systematic noise, because the spatial precision of imaging was ~7 nm for the x-axis and ~9 nm for the y-axis at an exposure time of 0.2 s (Fig. 2E), and their calculated diffusion constant, 10.4 nm²/s, was much smaller than that of PAR1. In cells near blood vessels, the QDs on the cell edge facing the vessel (green symbols in Fig. 3, C and E) moved farther along the membrane than those in the edge on the opposite side of the cell (red symbols in Fig. 3, C and E). The
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**DISCUSSION**

PAR1 diffusion at the mobile edge was greater than at the immobile edge *in vivo* (Fig. 5). As membrane protein movement is coupled with actin filament density underneath the cell membrane (24, 32), membrane fluidity and actin dynamics might synergistically activate local pseudopodial formation. In mice, cancer cells in colonies far from tumor blood vessels did not show active membrane dynamics (Fig. 5A). This might have been due to low local concentrations of signaling factors derived from blood components (31). The cells near blood vessels are likely attracted to the vessels by signaling factors, resulting in the formation of membrane protrusions (Fig. 5B). The concentration gradient of such signaling factors through the extracellular matrix might also result in increased membrane fluidity and actin dynamics within individual cells on sides facing the blood vessel (31); such directionality might permit the cells to extend membrane protrusion and migrate in the direction of the vessel. These protrusions might be a kind of invadopodia. In the bloodstream, the membrane fluidity of cells was 1100-fold higher than that of cells far from vessels (Fig. 5C). The high membrane fluidity was observed similarly in both wide and narrow vessels. This increased diffusion was likely caused by the existence of fewer actin filaments underneath the cell membrane due to a lack of adhesion between cells and the extracellular substratum (33, 34). This idea is supported by the observation that these cells can use their membrane flexibility to change their shape to pass through narrow vessels (Fig. 5C′) (35). Additionally, greater diffusion of membrane protein in vessels might enhance cancer metastasis to another organ by accelerating the reaction rate between receptors and their ligands or adhesion proteins and their extracellular substrata. After adhering to the inner vascular surface, diffusion decreased (Fig. 5D). The reformation of the actin cytoskeleton underneath the cell membrane likely slowed the diffusion of PAR1. Cells subsequently moved slowly and directionally on the inner vascular surface (Fig. 5E). The network of actin filaments might have become more developed to permit directional cellular migration (3, 4, 6). The membrane fluidity of the lamellipodia-like structure was 2-fold higher than that of other regions (Fig. 5E). Similar results were also obtained in cultured cells. The diffusion constant of QDs on mobile lamellipodia (1.2 × 10^5 nm^2/s) *in vitro* was 5-fold greater than that on immobile cell edges (2.2 × 10^4 nm^2/s) (supplemental Fig. S5, A–D). These results suggest that the difference in membrane fluidity between the mobile and immobile edge is important for directional migration. Additionally, it was previously reported that the diffusion constant of transferrin receptor without GFP
fusion on the cultured cell membrane was $\sim10^5$ nm$^2$/s (36), which is very similar to the diffusion constant of PAR1-GFP, suggesting that GFP does not interfere the diffusion of PAR1.

In conclusion, the membrane fluidity of metastasizing tumor cells increases at intravasation, peaks in blood vessels, decreases at extravasation, and is higher in locally formed pseudopodia. Such dramatic changes in membrane fluidity and morphology can enable cancer cells to metastasize. This new method of imaging protein dynamics with high spatial precision could make useful contributions to understanding mechanisms of cancer progression as well as the antitumor effects of nanometer-scale anticancer agents in vivo (37, 38).

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