Microvesicle Cargo and Function Changes upon Induction of Cellular Transformation*

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Extracellular vesicles (EVs), including exosomes and microvesicles (MVs), have emerged as a major form of intercellular communication, playing important roles in several physiological processes and diseases, including cancer. EVs generated by cancer cells contain a variety of proteins and RNA species that can be transferred between cancer cells as well as between cancer and non-transformed (normal) cells, thereby impacting a number of aspects of cancer progression. Here we show how oncogenic transformation influences the biogenesis and function of EVs using a mouse embryonic fibroblast (MEF) cell line that can be induced to express an oncogenic form of diffuse B cell lymphoma (Dbl). Although MEFs induced to express oncodoBBL generated a similar amount of MVs as uninduced control cells, we found that MVs isolated from onco-DbI-transformed cells contain a unique signaling protein, the ubiquitously expressed non-receptor tyrosine kinase focal adhesion kinase. The addition of MVs isolated from MEFs expressing onco-DbI to cultures of fibroblasts strongly promoted their survival and induced their ability to grow under anchorage-independent conditions, outcomes that could be reversed by knocking down focal adhesion kinase and depleting it from the MVs or by inhibiting its kinase activity using a specific inhibitor. We then showed the same to be true for MVs isolated from aggressive MDAMB231 breast cancer cells. Together, these findings demonstrate that the induction of oncogenic transformation gives rise to MVs, which uniquely contain a signaling protein kinase that helps propagate the transformed phenotype and thus may offer a specific diagnostic marker of malignant disease.

Classical intercellular signaling involves the secretion of growth factors, pro-inflammatory cytokines, and extracellular matrix proteins by a cell into its local environment (1, 2). These soluble factors then bind to their corresponding receptors expressed in a neighboring cell and induce the activation of intracellular signaling events that determine whether a cell grows, differentiates, migrates, or dies (3). These types of paracrine signaling activities are required throughout development and for tissue homeostasis, whereas deregulation of these events often leads to developmental abnormalities and the onset of diseases.

The generation of EVs by cells has quickly become appreciated as another major form of intercellular communication with important consequences in biology (4–7). Cells generate two distinct types of EVs: MVs and exosomes. MVs typically range in size from 0.2–2.0 μm in diameter and are formed via the budding and release (shedding) of membrane-enclosed packages from plasma membranes. Exosomes represent the second major class of EVs. They are significantly smaller than MVs, averaging only 0.03–0.1 μm in size, and are formed through a distinct mechanism. Specifically, exosomes are generated as a result of the endosomal sorting complex required for transport- and Rab-dependent rerouting of multivesicular bodies containing endosomes from the lysosome, where they would be degraded, to the cell surface. The multivesicular bodies then fuse with the plasma membrane and release their contents (i.e. endosomes) into the extracellular environment, at which point they are referred to as exosomes.

One of the main reasons why MVs and exosomes have been attracting a good deal of attention has to do with the cargo they contain, which includes cell surface receptors, cytosolic signaling proteins, metabolic enzymes, and even nuclear proteins, as well as RNA transcripts and microRNAs (1, 2, 6, 7). When released from a cell, EVs can function in a paracrine or endocrine manner through the transfer of their cargo to a recipient cell (3, 4). This cargo is then used by the cell to elicit specific cellular processes or outcomes.

Although it is beginning to be appreciated that both normal cell types and cancer cells generate MVs and exosomes, EVs have been most often studied in the context of cancer, where they have been shown to play important roles in the progression of the disease (4–9). For example, MVs generated by highly aggressive human cancer cells are capable of stimulating angiogenesis, reorganizing the stroma to establish the tumor microenvironment, as well as promote tumor growth and chemoresistance (10–12). The role of MVs in cancer progression was exemplified in a study showing that a highly oncogenic form of the epidermal growth factor receptor (EGFR), known as EGFR variant type III (EGFRvIII), is present in MVs generated by glioma cells engineered to express this truncated EGFR. When MVs from these glioma cells were isolated and then

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§ The abbreviations used are: EV, extracellular vesicle; MV, microvesicle; EGFR, EGF receptor; EGFRvIII, EGFR variant type III; MEF, mouse embryonic fibroblast; Dbl, diffuse B cell lymphoma; FAK, focal adhesion kinase; TEM, transmission electron microscopy; WCL, whole cell lysate; NTA, nanoparticle tracking analysis; KD, kinase-dead.
added to EGFRvIII-negative glioma cells, EGFRvIII was transferred from the MVs to the cells, where it triggered oncogenic signaling events that promoted cell growth and survival (8).

Increasing evidence suggests that cancer cell-derived MVs also impact the behavior of normal cell types that can be found bordering a tumor (13, 14). For example, our laboratory has shown that MVs generated by the highly aggressive MDAMB231 breast cancer cell line are capable of conferring a transformed-like phenotype onto normal mammary epithelial cells and fibroblasts, including the ability to grow under serum-limiting or anchorage-independent conditions. We further showed that an important aspect of the mechanism underlying the ability of MVs to mediate such phenotypic changes involved the cross-linking of the extracellular matrix protein fibronectin, which is associated with MVs, through the acyl transferase activity of another MV-associated protein, tissue transglutaminase. This enabled the MVs to dock onto normal epithelial cells and fibroblasts through the binding of the MV-associated cross-linked fibronectin to integrins on the surfaces of these cells (6).

In addition to EVs acting locally to promote tumor growth, they can also impact cells at distant sites through their ability to enter the bloodstream and circulate throughout the body. Thus, the isolation of EVs from blood samples is being actively pursued as a potential source of diagnostic information (15). Many lines of evidence have shown that high-grade/highly aggressive cancer cells shed considerably more EVs than lower-grade cancer cells and normal cells (16). In one such study, patients with malignant melanoma were found to have nearly twice the amount of EVs in their blood serum compared with normal patients (17). Moreover, a study conducted on glioblastoma patients found that the amount of EVs in the circulation increased proportionally to tumor volume (18). Collectively, these findings suggest that the levels of circulating EVs, and/or the cancer-specific cargo contained within these vesicles, could be used as potential diagnostic indicators.

Given the importance of EVs in cancer progression, we set out to better understand the key differences between MVs generated by normal and transformed cells, as this information would shed additional light on how cancer cell-derived MVs impact recipient cells, as well as further examine their potential as diagnostic markers. Here, using an inducible model of cellular transformation, we show that the amount of MVs shed by non-transformed MEFs is comparable with that generated by MEFs transformed by inducing the expression of onco-Dbl (19), a truncated guanine nucleotide exchange factor that constitutively activates members of the Rho family of small GTPases. However, we found that MVs from transformed MEFs were specifically enriched in the non-receptor tyrosine kinase FAK, despite this kinase being expressed at similar levels in both the non-transformed and transformed MEFs. We went on to determine that FAK is also preferentially expressed in MVs derived from several highly aggressive breast cancer cell lines. Moreover, we found that the presence of FAK in MVs from transformed MEFs as well as highly aggressive MDAMB231 breast cancer cells has important functional consequences; it enables MVs to promote cell survival and anchorage-independent growth. Overall, the findings reported here highlight that specific cargo is recruited to MVs upon oncogenic transformation, thus conferring these vesicles with unique functional capabilities.

Results

Onco-Dbl Expression in MEFs Induces Cellular Transformation—The formation and shedding of MVs by cells is now appreciated as a form of intercellular communication with important roles in human cancer progression. These vesicles are also being aggressively pursued as a novel source of diagnostic information. However, there still remains a gap in our understanding of how cancer cell-derived MVs mediate their effects and how we might best utilize their unique properties for diagnostic purposes. Therefore, we took advantage of a well-defined, inducible system of oncogenic transformation to compare the cargo and functional properties of MVs isolated from transformed cells and their non-transformed counterparts.

Specifically, this system involves MEFs that stably express a doxycycline-regulated form of onco-Dbl, a truncated guanine nucleotide exchange factor that activates the small GTPases Rho and Cdc42 (20–22).

When cultured in the presence of doxycycline, the expression of HA-tagged onco-Dbl in MEFs is inhibited (Fig. 1A, top panel, first lane). However, 24 h after placing the MEFs in medium lacking doxycycline, HA-tagged onco-Dbl is readily detected in lysates derived from these cells (Fig. 1A, compare the first and third lanes), with maximal expression occurring within 48 h (Fig. 1A, compare the third, fourth, and fifth lanes). Thus, for all the experiments where MEFs expressing onco-Dbl are used, we first induced its expression for 48 h prior to beginning the assays.

As reported previously (19), marked actin cytoskeletal rearrangements accompanied the expression of onco-Dbl and its activation of the small GTPases Cdc42 and Rho. Fig. 1B shows an example of an uninduced control MEF stained with an HA antibody and rhodamine-conjugated phalloidin to label F-actin. The uninduced control MEFs, which lacked onco-Dbl expression (Fig. 1B, top left panel), showed a typical fibroblast-like morphology (Fig. 1B, top right panel). However, upon induction of HA-tagged onco-Dbl expression (Fig. 1B, bottom left panel), there was a significant increase in the number and size of F-actin stress fibers (Fig. 1B, bottom right panel).

We then subjected the uninduced control and onco-Dbl-expressing MEFs to soft agar and cell death assays. Soft agar assays determine the ability of cells to grow and form colonies under anchorage-independent conditions, an *in vitro* measure of tumorigenicity. Fig. 1C, top panel, shows that, after 14 days in culture, the control MEFs remained as single cells. In contrast, the induction of HA-tagged onco-Dbl expression resulted in the formation of large colonies of cells (Fig. 1, C, bottom panel, and D). To examine how onco-Dbl expression influenced MEF viability, we then cultured uninduced control MEFs as well as MEFs induced to express HA-tagged onco-Dbl in medium lacking or containing serum for 48 h. The cells were then collected, stained with DAPI to label their nuclei, and analyzed by fluorescent microscopy for the appearance of condensed or blebbed nuclei, a hallmark of apoptosis. There was a nearly 18-fold increase in the number of control MEFs that died when
MVs Generated by MEFs Expressing Onco-Dbl Promote the Survival and Anchorage-independent Growth of Cells—We then set out to determine whether MVs generated by non-transformed MEFs, versus MVs from MEFs transformed by onco-Dbl, exhibit distinct functional capabilities. Thus, MVs generated by each of these cell types were isolated, quantified, and analyzed. The results showed that MVs generated by non-transformed MEFs are smaller in size and contain less cargo than those generated by MEFs transformed by onco-Dbl. These findings underscore the importance of considering the cellular context in interpreting the biological significance of MVs.
using NTA, and normalized based on MV number. The MVs were then added to cultures of serum-starved NIH-3T3 cells for 48 h (Fig. 3A), at which point the amount of cell death for each condition was determined by staining the cells with DAPI to identify condensed or blebbled (apoptotic) nuclei. Approximately 60–70% of the NIH-3T3 cells cultured in serum-free medium underwent cell death (Fig. 3B, first column). When NIH-3T3 cells were cultured in serum-free medium supplemented with MVs derived from uninduced control MEFs, a modest reduction (~30%) in cell death was observed (Fig. 3B, compare the first and third columns). Doubling the amount of these MVs did not reduce the extent of cell death further (Fig. 3B, compare the third and fourth columns), suggesting that a maximal MV dose for conferring a survival advantage had been achieved in these experiments. However, when NIH-3T3 cells were cultured in serum-free medium supplemented with MVs from MEFs expressing onco-Dbl, the apoptotic rate was dramatically reduced and approached the extent of protection from cell death that was provided when culturing cells in 2% serum (Fig. 3B, compare the second and fifth columns).

The ability of MVs derived from MEFs expressing onco-Dbl to induce the anchorage-independent growth (i.e. colony formation in soft agar) of the non-transformed NIH-3T3 cell line was also determined (Fig. 3A). As expected, NIH-3T3 cells either left untreated (no MVs) or treated with MVs from the uninduced control MEFs for 14 days remained primarily as single cells (Fig. 3C, left and center panels). However, when the same cells were treated with MVs derived from the transformed MEFs they began to form colonies (Fig. 3, C, right panel, and D, compare the first and second columns with the third column), similar to what was observed with MVs isolated from highly aggressive brain and breast cancer cells (6).

These findings suggest that MVs generated by transformed cells may contain distinct cargo that enables them to promote the anchorage-independent growth and survival of cells to a much greater degree compared with MVs isolated from non-transformed MEFs. To identify such cargo, lysates prepared from control and transformed cells, as well as the lysates of the MVs shed by these cells, were subjected to Western blotting analysis to detect proteins linked to cell survival. We examined MV lysates for the presence of a number of signaling proteins linked to cell survival. One that stood out was FAK, a non-receptor tyrosine kinase that is overexpressed and/or hyperactive in many different cancer cell types, where it has been shown to participate in the maintenance of the transformed phenotype, including the evasion of apoptosis and promotion of anchorage-independent growth (23–27). In fact, targeting FAK for inactivation is currently being used as a strategy to treat several different types of human cancer (28, 29). Fig. 4A, top panel, WCL, shows that FAK is expressed at similar levels in control MEFs and MEFs expressing onco-Dbl. However, although FAK was only barely detectable in the MVs derived from control MEFs, a significantly greater amount of FAK was present in MVs derived from cells induced to express onco-Dbl (Fig. 4A, top panel, MVs). Quantifying the levels of FAK in MVs isolated from control MEFs versus MVs obtained from MEFs expressing onco-Dbl indicated that there was ~12-fold more FAK in MVs generated by transformed cells (Fig. 4B).

We then determined whether the non-receptor tyrosine kinase c-Src and the protein scaffold paxillin, two proteins that cooperate with FAK and mediate cell growth and survival (30, 31), are also enriched in MVs generated by transformed MEFs. Although similar amounts of c-Src were detected in MVs from uninduced control MEFs and MEFs expressing onco-Dbl (Fig.
A second panel, MVs), paxillin was absent from both of these MV preparations (Fig. 4A, third panel, MVs). These findings further underscore that FAK is uniquely enriched in MVs generated by transformed MEFs.

To determine whether FAK was present in the smaller class of EVs referred to as exosomes, the total EVs shed by non-transformed and onco-Dbl-transformed MEFs were separated by collecting the MVs on a 0.22-μm filter and comparing their protein contents to those of exosomes collected from the flow-through following centrifugation at 100,000 x g. Fig. 4C, second panel, shows that the exosome preparation contains the specific exosomal marker CD-63, whereas the MV fraction does not (compare Exosomes and MVs). It should be noted that FAK is only present in WCLs and lysates collected from MVs derived from transformed MEFs (Fig. 4C, top panel).

We then examined whether the levels of FAK were similarly increased in MVs generated by human breast cancer cells. The non-malignant mammary epithelial MCF-10A cell line, together with several highly aggressive triple-negative breast cancer cell lines, including MDA-MB231, BT-549, and Hs-578T cells, were cultured, and the MVs shed by each of these cell lines were isolated. Fig. 4D, top panel, WCL, shows that each of these cell lines expressed comparable levels of FAK. However, although FAK was absent in MVs isolated from the normal mammary MCF-10A cell line, its expression was readily detectable in the MVs derived from each of the breast cancer cell lines (Fig. 4D, top panel, compare the MV lanes).

FAK Plays an Important Role in the Ability of MVs from Transformed Cells to Promote Cell Growth and Survival—We set out to determine whether FAK in the MVs from the MEFs expressing onco-Dbl was necessary for their strong cell survival and anchorage-independent growth-promoting capabilities. As a first step toward addressing this question, we collected MVs generated by control MEFs and MEFs expressing onco-Dbl and confirmed that FAK is preferentially loaded in MVs isolated from cells expressing onco-Dbl (Fig. 5A, second panel, compare the MV lanes). Interestingly, we found that activated FAK, as well as the activated form of its major binding partner, c-Src (32), were both present in these MVs, as detected by Western blotting MV lysates with antibodies that detect FAK phosphorylated at tyrosine 397 (P-FAK Tyr397) and c-Src phosphorylated at tyrosine 416 (P-Src Tyr416) (Fig. 5A, first and second panels, fourth lane). These findings raise the interesting possibility that FAK and c-Src may form a complex in MVs that is capable of strongly activating downstream signaling events (33, 34).
To demonstrate that the FAK present in the MVs can be transferred to recipient cells, we took advantage of the fact that transformed MEFs transiently transfected with an HA-tagged form of FAK (Fig. 5B, top panel, WCL), generate MVs that contain the ectopically expressed protein (Fig. 5B, top panel, MVs). We then prepared MVs derived from onco-Dbl-expressing MEFs that had been either mock-transfected or transfected with HA-tagged FAK and incubated these preparations with cultures of NIH-3T3 fibroblasts for 2 h, at which time the cells were washed extensively, fixed, and then stained with an HA antibody and rhodamine-conjugated phalloidin to label F-actin. 

FIGURE 4. MVs generated by transformed MEFs and aggressive breast cancer cells contain FAK. A, Western blotting analysis using FAK, Src, Paxillin, IκBα, and flotillin antibodies was performed on control MEFs and MEFs expressing onco-Dbl (WCL) as well as on the MVs these cells generated (MV). Note that FAK is uniquely enriched in MVs derived from MEFs expressing onco-Dbl. B, the relative amounts of FAK detected in MVs generated by control (Uninduced) MEFs and MEFs expressing onco-Dbl (Onco-Dbl Induced) was determined across several experiments. The data represents the mean ± S.D. Student’s t tests were performed. ***, p < 0.001. C, Western blotting analysis using FAK, IκBα, and flotillin antibodies was performed on uninduced MEFs and MEFs expressing onco-Dbl (WCL) as well as on the exosomes (Exosomes) and MVs (MV) these cells generated. The blots were also probed with a CD-63 antibody, an exosome-specific marker. The vertical lines placed through the blots indicate where a small portion of each blot was deleted. Note that FAK is present only in the MVs from MEFs expressing onco-Dbl. D, Western blotting analysis using FAK, IκBα, HA, and flotillin antibodies was performed on normal MCF-10A mammary epithelial cells and MDAMB231, BT-549, and Hs-578T breast cancer cells (WCL) as well as on the MVs these various cell lines generated (MV). The experiments in A–D were performed a minimum of three separate times, and molecular weight markers (in kilodaltons) are included along the right side of the blots in A, C, and D.

We then examined whether the activated FAK present in MVs can induce signaling events in recipient cells. MVs collected from either control MEFs or MEFs expressing onco-Dbl were added to cultures of serum-starved NIH-3T3 fibroblasts for increasing lengths of time. Fig. 5D, top panel, Treatment with MVs from onco-Dbl induced MEFs, shows that, within 0.5 h of treating cells with MVs from MEFs expressing onco-Dbl, an increase in the level of phosphorylated FAK could be detected, with maximal phosphorylation occurring at 1 h. This increase in FAK phosphorylation was not observed in NIH-3T3 cells treated with MVs isolated from control MEFs (Fig. 5D, top panel, Treatment with MVs from uninduced MEFs). Because the HA-tagged FAK transferred by MVs appears to remain primarily along the surface of recipient cells for up to 2 h, we suspect that the FAK associated with MVs generated by transformed cells is most likely sending signals from the cell surface. We found that the phosphorylation level of AKT, a downstream effector of FAK (35), is preferentially activated in a time-dependent fashion by MVs from transformed cells (Fig. 5D, third panel). It is worth mentioning that this appears to be a specific signaling outcome, as the MVs isolated from both uninduced and onco-Dbl-expressing MEFs were able to stimulate ERK activation to similar extents (Fig. 5D, fifth panel).

We then set out to build upon this finding by directly assaying whether the MV-mediated transfer of FAK to recipient cells accounted for the ability of MVs from MEFs expressing onco-Dbl to strongly promote cell survival by performing two sets of experiments. In the first set, we obtained conditioned medium from cultures of control MEFs or MEFs expressing onco-Dbl and treated them without or with the FAK III inhibitor, a small molecule that undergoes a covalent interaction with FAK and blocks its activation (36). MVs were then isolated from the con-
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FIGURE 5. The FAK associated with MVs can be transferred to recipient cells and activate intracellular signaling events. A, lysates of control MEFs (uninduced) and MEFs induced to express onco-Dbl (WCL) as well as lysates of the MVs these cells generated (MV) were subjected to Western blotting analysis using an antibody that recognizes FAK when it is phosphorylated on tyrosine 397 (P-FAK Tyr397). The blot was also probed with FAK, P-c-Src Tyr416, IxBα, and flotillin antibodies. B and C, multiple sets of MEFs expressing onco-Dbl were either mock-transfected or transfected with an HA-tagged form of FAK (HA-FAK). B, one set of the transfectants (WCL) and the MVs they generated (MV) were subjected to Western blotting analysis using HA and flotillin antibodies. C, the MVs generated by another set of transfectants were collected and then incubated with NIH-3T3 fibroblasts for 2 h, at which point immunofluorescence using an HA antibody was carried out on the cells. The cells were also stained with phalloidin to label F-actin. Note that the HA-FAK can be detected as puncta on the surface of the cells treated with MVs expressing HA-FAK (arrows). D, serum-starved cultures of NIH-3T3 fibroblasts were treated without (time 0) or with an equivalent amount of MVs isolated from either control MEFs (Treatment with MVs from uninduced MEFs) or MEFs expressing onco-Dbl (Treatment with MVs from onco-Dbl induced MEFs) for the indicated length of time and lysed. Western blotting analysis was performed on the cell lysates using P-FAK Tyr397, FAK, P-AKT, AKT, P-ERK 1/2, and ERK 1/2 antibodies. The experiments in A–D were performed a minimum of three separate times, with each experiment yielding similar results. Molecular weight markers (in kilodaltons) are included along the right side of each of the blots in A, B, C, and D.

ditioned medium using a 0.22-μm filter to remove any unbound (freely soluble) inhibitor. A portion of the isolated MVs was lysed and Western blotted using phospho-FAK Tyr397 and total FAK antibodies. Although the MVs from the transformed MEFs treated with the FAK III inhibitor still contained FAK (Fig. 6A, center panel, compare the fifth and sixth lanes), the extent of its phosphorylation was greatly diminished (Fig. 6A, top panel, compare the fifth and sixth lanes). These MVs were then added to cultures of NIH-3T3 cells that were being serum-starved. Two days later, the cells were collected, and the amount of cell death for each condition was determined. Fig. 6B shows that the relative ability of MVs from transformed cells to promote cell survival is completely lost under conditions where the activity of FAK associated with the MVs was inhibited prior to adding the MVs to the serum-starved cells (compare the third and fourth columns). As a control, we verified that the relative extent of cell death for NIH-3T3 cells incubated with MVs from control MEFs was unaffected by the FAK inhibitor (Fig. 6B, compare the first and second columns). Moreover, the ability of the MVs isolated from onco-Dbl-expressing MEFs to induce the anchorage-independent growth of NIH-3T3 cells was also blocked by inhibiting the FAK activity associated with these MVs (using a FAK inhibitor) prior to adding them to the NIH-3T3 cells (Fig. 6C, compare the second and third columns).

As a complimentary approach, we knocked down FAK in control and onco-Dbl-expressing MEFs. The FAK-targeting siRNA reduced endogenous FAK expression by at least 85% in control MEFs and ~70% in MEFs expressing onco-Dbl (Fig. 7A, top panel, WCL). Knocking down FAK in the transformed MEFs also effectively decreased FAK levels in their MVs (Fig. 7A, top panel, MVs). Using NTA to determine the concentration of MVs in each of the various MV preparations, we normalized them and then compared the ability of an equivalent number of MVs isolated from control MEFs or MEFs expressing onco-Dbl that had been transfected with either control or FAK-targeting siRNAs to promote cell survival. Fig. 7B shows again that MVs from the MEFs expressing onco-Dbl are capable of promoting the survival of serum-starved NIH-3T3 cells (compare the first and third columns). However, this advantage was lost when FAK levels in the MVs were reduced as a result of depleting FAK expression in the cells (Fig. 7B, compare the third and fourth columns).

These experiments were expanded to determine whether FAK expressed in MVs derived from the highly aggressive MDMB231 breast cancer cell line also promoted cell survival. Cultures of MDMB231 cells were transfected with either control or FAK-targeting siRNA, and the levels of FAK in these cells or in the MVs generated by the MDMB231 cells were determined. Fig. 7C shows that the FAK-targeting siRNA decreased FAK expression in the cells and also in the MVs from these cells by ~75%. We then performed NTA on the MVs collected from MDMB231 cells to normalize the MV preparations to assess
The remaining portion of the isolated MVs were incubated with NIH-3T3 fibroblasts for 2 h and lysed. Western blotting analysis of these NIH-3T3 cell lysates revealed that the ectopically expressed HA-tagged forms of WT FAK and FAK KD could be detected in the cells (Fig. 8A, Recipient Fibroblasts).

Survival assays were then carried out on serum-starved NIH-3T3 fibroblasts that were treated without (just serum-starved) or with MVs derived from onco-Dbl-transformed MEFs that had been mock-transfected or transfected with either HA-FAK WT or HA-FAK KD. The ability of the ectopically expressed HA-FAK WT to become incorporated into MVs derived from the transformed MEFs did not change their ability to promote cell survival compared with MVs from the mock-transfected cells (Fig. 8B, compare the second and third bars). On the other hand, MVs from the transformed MEFs containing the kinase-dead form of FAK significantly reduced their survival-promoting capabilities (compare the second and fourth columns). These findings, combined with those showing that transiently expressing HA-tagged FAK WT, but not FAK KD in NIH-3T3 fibroblasts (Fig. 8C, top panel), is sufficient to promote cell survival (Fig. 8D), support the conclusion that activated FAK contained in MVs derived from transformed or cancer cells plays a key role in mediating their survival-promoting capabilities.

**Discussion**

Only a short time ago, EVs were considered to be nothing more than cell debris. Now, however, EVs are recognized as a genuine form of cell-cell communication that has far-reaching implications in physiological processes and disease progression (4, 38, 39). This has been perhaps best studied in the context of human cancer, where EVs have been linked to a number of stages of cancer progression, including the shaping of the tumor microenvironment, angiogenesis, and the creation of the premetastatic niche at secondary sites of tumor colony formation (10, 40, 41). The group of larger EVs, MVs, has been especially linked to changes in the tumor microenvironment, as demonstrated in studies showing that MVs isolated from the highly aggressive MDAMB231 breast cancer cell line and the U87 glioblastoma cell line caused normal (non-transformed) cell lineages to acquire the ability to grow under serum-limiting condition and survive apoptosis-inducing stresses (6).

MVs have also caught the attention of clinical researchers and pharmaceutical companies, largely because of the realization that cancer cell-derived EVs can be isolated from blood samples of patients suffering from a number of types of cancer, including brain, breast, prostate, kidney, and pancreatic cancer (16, 42, 43). This has raised the exciting possibility that EVs, which, given their size, might be the more readily detectable EVs in plasma and tissue fluids, offer a novel and non-invasive source of diagnostic information. However, to realize such a possibility, it will be important to establish that MVs shed by transformed/cancer cells in fact contain unique cargo that is not present within their normal cellular counterparts.

Here we set out to establish whether there might be differences both in the number of MVs generated by transformed/cancer cells versus their non-transformed (non-cancerous) counterparts as well as in the protein cargo that they contain. We started by using a well defined system that allows for the
inducible expression of the oncogenic Dbl protein in MEFs, resulting in the activation of Rho GTPases and giving rise to a number of transformed phenotypes. Somewhat surprisingly, in light of the suggestions from some reports that the amount of EVs present in the bloodstream of a cancer patient can be correlated with the aggressiveness and/or grade of a particular tumor, we found that the amount of MVs generated by these cells did not increase upon induction of oncogenic transformation. However, importantly, we did detect a specific change in the MV cargo from transformed cells. Specifically, we found that only MVs generated by transformed cells contained the non-receptor tyrosine kinase FAK. Not only was FAK missing from MVs generated by non-transformed (normal) cells, it was also absent from exosomes isolated from either transformed or non-transformed MEFs. We then went on to demonstrate that the same is true in a number of human breast cancer cell lines; i.e., they shed MVs that specifically contained this important signaling kinase.

The presence of FAK within MVs derived from transformed/cancer cells had important functional consequences. Indeed, we found that the ability of these MVs to specifically impart increased survival and anchorage-independent growth capabilities upon non-transformed cells was absolutely dependent upon the vesicles containing FAK. A particularly interesting aspect of these findings concerns how the MV-mediated transfer of FAK to recipient cells that already contain endogenous levels of this protein kinase is able to elicit a significant functional outcome. The answer may lie in our findings that the FAK in the MVs from transformed/cancer cells is already activated and that these microvesicles also contain the major FAK signaling partner c-Src. Thus, it is plausible that the MV-mediated transfer of just activated FAK or an activated FAK-c-Src signaling complex to recipient cells may provide a significant boost to the necessary signaling events within the cells to provide growth and survival benefits.

It now appears clear that MVs derived from transformed or cancer cells can drive the recipient cells they target to undergo marked phenotypic changes as an outcome of the specific protein cargo they contain and independent of any changes in total vesicle numbers. The finding that FAK is a unique cargo of these MVs is particularly significant because of the major role this tyrosine kinase plays in cell migration, metastasis, and inhibition of apoptosis. In particular, FAK is able to activate downstream signaling pathways that ultimately prevent apoptosis by inhibiting the caspase-3 cascade (25). Cancer cells take advantage of this capability, often by overexpressing FAK (44), and therefore the ability of their MVs to transfer this important signaling kinase to surrounding recipient cells can obviously have important consequences for the microenvironment and tumorigenesis.

Among the important questions for the future will be to better understand how MVs from transformed/cancer cells are...
Experimental Procedures

Cell Culture and Transfections—The tetracycline-off inducible onco-Dbl MEF cell line was generated as described previously (19). The cells were maintained in DMEM supplemented with 10% Tet system-approved FBS (Clontech), 100 μg/ml G418 (Gibco), and 1 μg/ml doxycycline (Sigma). To induce onco-Dbl expression, the cells were trypsinized and then plated in doxycycline-free culturing medium, where any residual doxycycline remaining in the cultures was removed by replacing the medium 6 h after plating the cells. NIH-3T3 cells were maintained in DMEM supplemented with 10% calf serum (Clontech), whereas the normal mammary MCF10A epithelial cell line as well as the MDAMB231, BT-549, and Hs-578T breast cancer cell lines were grown in RPMI 1640 medium supplemented with 10% FBS. Expression constructs encoding HA-tagged forms of either wild-type FAK (HA-FAK WT) or a kinase-dead form of FAK (HA-FAK KD) that was generated by mutating lysine 454 to arginine (37) were transfected into cells expressing onco-Dbl and HA-tagged FAK WT and then cultured as indicated. The cells were fixed with 3.7% formaldehyde, permeabilized with PBS containing 0.1% Triton X-100, and blocked with 10% bovine serum albumin diluted in PBS. The cells were stained with an HA rabbit polyclonal antibody (Covance) at a dilution of 1:500, followed by incubation with an Oregon green 488-conjugated secondary antibody (Molecular Probes). Rhodamine-conjugated phalloidin (Life Technologies) and DAPI (Sigma) were used to label actin filaments and nuclei, respectively. To detect MVs on the surfaces of cells, cultures of NIH-3T3 or onco-Dbl MEFs were treated with MVs isolated from transformed MEFs. MVs were collected from NIH-3T3 cells that had been mock-transfected or transfected with either HA-tagged forms of either FAK WT or FAK KD. Approximately 48 h later, the cells were stained with DAPI to identify the condensed/blebbed (apoptotic) nuclei.

Fluorescent Microscopy—Cells grown on glass coverslips were either transfected without or with the indicated expression constructs and siRNAs or treated with MVs isolated from MEFs expressing onco-Dbl and HA-tagged FAK WT and then cultured as indicated. The cells were fixed with 3.7% formaldehyde, permeabilized with PBS containing 0.1% Triton X-100, and blocked with 10% bovine serum albumin diluted in PBS. The cells were stained with an HA rabbit polyclonal antibody (Covance) at a dilution of 1:500, followed by incubation with an Oregon green 488-conjugated secondary antibody (Molecular Probes). Rhodamine-conjugated phalloidin (Life Technologies) and DAPI (Sigma) were used to label actin filaments and nuclei, respectively. To detect MVs on the surfaces of cells, cultures of cells were incubated with the fluorescent membrane dye FM1–43FX (Invitrogen) diluted to 5 μg/ml in PBS for 1 min, and fixed on ice for 20 min. All cells were visualized using a Zeiss Axio-
Pelleted exosomes were then lysed with 200 μl of lysis buffer (25 mM Tris, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM NaVO₄, 1 mM β-glycerol phosphate, and 1 μg/ml each aprotinin and leupeptin). WCLs were prepared by rinsing dishes of cells with PBS, adding 1 ml of lysis buffer, and scraping the cells off the plate. Exosomes were isolated from the conditioned medium that flowed through the 0.22 μm Steriflip filter (i.e. vesicles smaller than 220 nm) by centrifugation at 100,000 rpm for 10 min, and then the supernatants were generated to lysates, the MVs retained by the filter were lysed using 200 μl of lysis buffer (25 mM Tris, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM NaVO₄, 1 mM β-glycerol phosphate, and 1 μg/ml each aprotinin and leupeptin). The resulting MV, exosome, and cell lysates were centrifuged at 13,000 rpm for 10 min, and then the supernatants were analyzed.

**Immunoblot Analysis**—The protein concentrations of the cell lysates as well as the MV and exosome lysates were determined using the Bio-Rad DC protein assay. The lysates were normalized by protein concentration and resolved by SDS-PAGE, and then the proteins were transferred to PVDF membranes. The membranes were incubated with various primary antibodies, including β-actin (Sigma, 5316), FAK (Cell Signaling Technology, 32855), flotillin-2 (Cell Signaling Technology, 2123P), paxillin (BD Biosciences, 5635), and CD63 (Thermo Fisher, 10628D), diluted 1:1000 in 20 mM Tris, 135 mM NaCl, and 0.02% Tween 20 buffer. The primary antibodies were detected with HRP-conjugated secondary antibodies (Cell Signaling Technology), followed by exposure to ECL reagent (Pierce).

**Anchorage-independent Growth Assay**—Control MEFs, MEFs expressing onco-Dbl, or parental NIH-3T3 cells were plated at a density of 8 × 10³ cells/ml in growth medium containing 0.3% agarose, with or without MVs as indicated, onto a base layer composed of growth medium containing 0.6% agarose in 6-well dishes. The soft agar cultures were refed every third day, including the addition of freshly prepared MVs where indicated. After 14 days, the number of colonies that formed for each condition was counted. Each of the assays was performed a minimum of three times, and the results were averaged.

**Cell Death Assays**—Parental NIH-3T3 cells or NIH-3T3 cells transfected with the indicated constructs were plated in each well of a 6-well dish and then cultured in medium containing 2% calf serum or serum-free medium supplemented without or with 1 × 10⁵ MVs derived from control MEFs, MEFs expressing onco-Dbl, or MDAMB231 cells. Twenty-four hours later, the cells were treated with an additional dose of freshly prepared MVs. Forty-eight hours from the start of the assay, the cells were stained with DAPI for viewing by fluorescence microscopy. Cells undergoing apoptosis were identified by nuclear condensation or blebbing, and the percentage of cell death was determined by calculating the ratio of apoptotic cells to total cells for each condition examined.

**NTA**—The sizes and concentrations of EVs in a given sample were determined using a NanoSight NS300 (Malvern). The samples were diluted in PBS made from ultrapure water and passed through the beam path to detect EVs as points of diffracted light moving rapidly under Brownian motion. Five 60-s digital videos of each sample were taken and analyzed to determine the concentration and size of the individual EVs based on their movement, and then results were averaged together.

**TEM**—5 μl of an MV preparation resuspended in PBS was added to a carbon-coated, 300-mesh copper grid and then stained with 1.75% uranyl acetate. When dry, the samples were imaged using the FEI T12 Spirit 120-kV field emission TEM at the Cornell Center for Materials Research.

**Author Contributions**—B. T. K., A. L. D., and K. S. G. performed the experiments. B. T. K., M. A. A., and R. A. C. designed the study and wrote the manuscript.

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