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EMMPRIN/CD147-encriched membrane vesicles released from malignant human testicular germ cells increase MMP production through tumor–stroma interaction ☆

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ABSTRACT

Background: Elevated levels of EMMPRIN/CD147 in cancer tissues have been correlated with tumor progression but the regulation of its expression is not yet understood. Here, the regulation of EMMPRIN expression was investigated in testicular germ cell tumor (TGCTs) cell lines.
Methods: EMMPRIN expression in seminoma JKT-1 and embryonal carcinoma NT2/D1 cell lines was determined by Western blot, immunofluorescence and qRT-PCR. Membrane vesicles (MVs) secreted from these cells, treated or not with EMMPRIN siRNA, were isolated by differential centrifugations of their conditioned medium. MMP-2 was analyzed by zymography and qRT-PCR.
Results: The more aggressive embryonic carcinoma NT2/D1 cells expressed more EMMPRIN mRNA than the seminoma JKT-1 cells, but surprisingly contained less EMMPRIN protein, as determined by immunoblotting and immunostaining. The protein/mRNA discrepancy was not due to accelerated protein degradation in NT2/D1 cells, but by the secretion of EMMPRIN within MVs, as the vesicles released from NT2/D1 contained considerably more EMMPRIN than those released from JKT-1. EMMPRIN-containing MVs obtained from NT2/D1, but not from EMMPRIN-siRNA treated NT2/D1, increased MMP-2 production in fibroblasts to a greater extent than those from JKT-1 cells.
Conclusion and general significance: The data presented show that the more aggressive embryonic carcinoma cells synthesize more EMMPRIN than seminoma cells, but which they preferentially target to secreted MVs, unlike seminoma cells which retain EMMPRIN within the cell membrane. This cellular event points to a mechanism by which EMMPRIN expressed by malignant testicular cells can exert its MMP inducing effect on distant cells within the tumor microenvironment to promote tumor invasion. This article is part of a Special Issue entitled Matrix-mediated cell behaviour and properties.

1. Introduction

Testicular germ cell tumors (TGCTs), although relatively rare are the most frequent malignancies found in men between 15 and 45 year old and represent 95% of testicular neoplasias [1]. Although TGCTs have become one of the most curable solid neoplasms, their incidence has been increasing over the past decades [2,3] and the prognosis of highly advanced cases with metastatic lesions is still generally poor. Histologically, the TGCTs can be classified as seminomas germ cell tumors, which originate from undifferentiated germ cells and are fairly homogenous with a poorly invasive phenotype, and nonseminomatous (NSGCTs), which arise from undifferentiated (embryonal carcinoma) and differentiated multipotent cells and have a more aggressive phenotype with a heterogeneous histology [4]. It is currently thought that carcinoma in situ (CIS) is the common precursor of both seminomas and non seminomas [5,6] and approximately 90% of these tumors were shown to contain CIS [7]. It was furthermore suggested that CIS may give rise to seminomas as an early stage in the development of non-seminomas [8].

The factors that trigger the transition from CIS to invasive germ cell tumors are not fully understood, although microenviromental factors are thought to be involved in the origin and progression of these types of TGCT [9]. Indeed, tumor microenvironment is known to play an important role in tumor progression. We have previously demonstrated significant alterations in the composition of matrix-secreted proteoglycans in

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testicular tumors which correlated with the metastatic potential of the tumors [10]. Matrix metalloproteinases (MMPs) are crucial mediators of the alterations observed in the microenvironment during cancer progression and are highly expressed by aggressive testicular germ cell tumor cell lines [11]. They regulate a variety of physiological processes and signaling events, like extracellular matrix degradation, cell migration, invasion, proliferation and angiogenesis and are generally known to be involved in tumor progression [12–18].

The high levels of MMPs found in cancer tissues are thought to originate primarily from the stroma surrounding the tumor through the interaction of the tumor cells with the stromal cells [19,20]. EMMPRIN (extracellular matrix metalloproteinase inducer), also termed CD147, is a membrane glycoprotein greatly enriched on the surface of tumor cells, which was shown to stimulate the synthesis of MMPs in stromal cells, such as fibroblasts and endothelial cells [21–25] and may account for the increased MMPs expression in most cancer tissues. EMMPRIN has been implicated in tumor invasion and its elevated levels have been correlated with tumor progression in numerous malignant tumor models [26,27] although its expression in TGCT has not yet been reported. In this study we compared the expression of EMMPRIN in a progression model of two TGCTs cell lines of representing testicular tumors with high (embryonic carcinoma) and low (seminoma) aggressive properties [11].

As EMMPRIN is a membrane-spanning molecule, its role in mediating interaction between tumor and stromal cells would only be compatible with the cells being in close proximity to allow direct cell-cell contact between them. However, the release from tumor cells of membranous vesicles into the extracellular environment may represent a mechanism that allows EMMPRIN-mediated communication at a distance and would represent an advantage to the tumor cells as they can benefit from the cooperation of non-adjacent cells in the tumor microenvironment. Different types of shed membrane vesicles have been described depending on the mechanism of their formation and release from the cells and in this report we will collectively refer to them as membrane vesicles (MVs). By transmitting paracrine information to the environment, tumor derived MV are able to modulate the molecular makeup and behaviour of the recipient cells [28] and are increasingly considered as mediators of the tumor microenvironment. EMMPRIN has already been shown to be contained within MV released from human uterine epithelial cells [29] or from ovarian carcinoma cells [30]. In this report we present evidence that the more aggressive embryonic carcinoma release more EMMPRIN-containing MVs which can than further increase their malignant properties by increasing cooperation with the surrounding stroma.

2. Materials and methods

2.1. Cell culture

The human seminoma cell line JKT-1, a generous gift from Prof P Fenichel (Inserm, UMR U895, Nice, France), was established as previously described and was cultured for up to 40 passages to avoid a drift [31]. The molecular signature of JKT-1 cells used in our study was similar to that described previously [31] concerning the expression of seminoma markers: placenta alkaline phosphatase (PLAP), NANOG, OCT3/4, AP2γ and HIWI.

The human embryonal carcinoma NTERA2/D1 cell line (non-seminomatous) was purchased from American Type Culture Collection (ATCC, Manassas, VA). The NTERA2/D1 cell line was cultured in DMEM supplemented with 1 mM sodium pyruvate and 10% heat inactivated fetal calf serum (HI-FCS), while JKT-1 cell line was cultured in DMEM supplemented with 2 mM sodium pyruvate and 10% HI-FCS. All culture media contained 100 UI/mL penicillin and 100 UI/ml streptomycin. Cells were also treated with the proteasome inhibitor MG-132 (1 to 10 μM) and the lysosome inhibitor, chloroquine diphospate salt (1 to 100 μM) for 6 h in medium containing 1% serum at 37 °C. The concentrations of the inhibitors used were based on preliminary experiments performed to establish the highest amount that can be applied without an effect on cell viability. Culture medium was collected and protein concentration was determined.

Telomerase-immortalized fibroblasts (HTK cell line), established from human cornea [32], were kindly donated by James V. Jester (University of California at Irvine, USA). They were cultured in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Les Mureaux, France).

2.2. Small interfering RNA (siRNA) transfection

EMMPRIN siRNA oligos (Ambion, siRNA IDs: 10372 and 215973, Applied Biosystems) or scramble siRNA oligos (BLOCK-iT fluorescent oligo, Invitrogen), siScramble, (33 nmol/L) were transfected into the cells using oligofectamine transfection reagent in Opti-MEM I (Invitrogen) in the absence of FCS and antibiotics. After 6 h incubation, an equal volume of DMEM/20% FCS was added to the transfection mixture, which was then cultured for a further 18 h. The cells were then washed and cultured in the presence of DMEM/10% FCS prior to incubation for MV collection and further experiments.

2.3. Isolation of MV and incubation with fibroblasts

MVs were were isolated as previously described [33]. Subconfluent (approximately 80% confluence) JKT-2 or NT2/D1 cells were incubated in serum-free medium) for 24 hr. Supernatants were collected and centrifuged at 800 ×g for 10 min and then at 12,000 ×g for 30 min to sediment suspended cells. The resulting supernatants were ultracentrifuged at 100,000 ×g for 1 h at 4 °C to pellet the MV. Pellets were washed in PBS, centrifuged again at 100,000 ×g and resuspended in 100 μl serum free DMEM. The supernatants (MV-deficient conditioned medium) were concentrated to achieve the same volume as the MV suspension using 10 K Amicon Ultracentrifugal Filter Units (Milipore, France). All steps were carried out under sterile conditions using sterilized centrifuge tubes. Aliquots from pellet suspensions and from concentrated supernatants were evaluated for the presence of EMMPRIN protein by Western blot analysis. The rest of the MV preparations were added to subconfluent (about 80% confluence) HTK fibroblasts cultures and incubated for 24 h in serum free medium. The effect of MV on MMPs expression in fibroblasts was evaluated by both zymorgraphy analysis of the CM and by qRT-PCR on cells lyzed in Trizol.

2.4. RNA isolation and qRT-PCR

Total RNA was extracted from the cells by Trizol Reagent (Invitrogen, Cergy Pontoise, France). The quality of the RNA was analyzed by electrophoresis on the agarose gels stained with ethidium bromide. Reverse transcription of 1 μg of RNA was performed using M-MLV reverse transcriptase and random hexamers according to the manufacturer's protocol (Invitrogen, Cergy Pontoise, France). To evaluate the expression levels of EMMPRIN, MMP-2 and MMP-1, normalized to the housekeeping β2 microglobulin (β2M) gene transcript, real-time quantitative PCR was performed using Perfect Master Mix Probe (AnyGenes, Paris, France) on LightCycler 2.0 (Roche Diagnostics, Meylan, France). Selected sets of primers and labeled probes were purchased from Eurogentec, Biosense, Italy.

2.5. Fluorescence immunocytochemistry

Cells were seeded on glass coverslips and grown for 24 h in complete medium. Then cells were washed with PBS and fixed for 10 min with 4% paraformaldeyde, containing 0.5% glutaraldeyde. After fixation, blocking was performed with 3% BSA in PBS. Cells were incubated with EMMPRIN mAbs, anti-CD147 clone HIM6 against the extracellular domain of EMMPRIN, diluted 1:200 (Becton Dickinson France, Le Pont de

Claix, France) for 2 h at room temperature, followed by two washes with PBS and incubation for 1 h at room temperature with the anti-mouse IgG Alexa 488 antibody and anti-rabbit IgG Alexa 594 (Invitrogen; Molecular Probes). Cells were washed with PBS and incubated with DAPI (Molecular Probes Inc., Eugene, OR, USA; Invitrogen) for 5 min. Stained cells were mounted under coverslips in Mowiol buffer (Mowiol 4.88, Calbiochem).

Confocal fluorescence images were acquired using an IX81 inverted Olympus microscope equipped with a DSU spinning disk confocal system (Olympus; Rungis, France), coupled to an Orca R2 CCD camera (Hamamatsu Corporation; Japan).

2.6. Western blot analysis

JKT-2 or NT2/D1 cells were lysed in RIPA buffer, incubated on ice for 5 min, and then scraped with a rubber policeman. The lysates were clarified by centrifugation at 13,000 g for 5 min at 4 °C, and 20 μg samples were analyzed by Laemmli SDS-PAGE (Bio-Rad, Marne la Coquette, France) on 10% gels. Aliquots of MV suspensions obtained from an equal number of cells (JKT-2 or NT2/D1) were mixed with Laemmli sample buffer prior to the electrophoretic run. The gels were transferred to Immobilon-P PVDF membrane (Millipore, Guyancourt, France). Membranes were immunoblotted with either anti-EMMPRIN mAb (clone HIM6, Becton Dickinson France), or anti-caveolin-1 rabbit pAb (Transduction Laboratories, Lexington, KY), for 2 h at room temperature or with GAPDH mAb (clone 6C5, Applied Biosystems, Courtabouef, France) for 1 h. This was followed by 1 h incubation with horseradish-peroxidase (HRP)-conjugated anti-mouse antibody or anti-rabbit antibodies (Jackson ImmunoResearch, Immunotech, Marseille, France), and visualized with BM chemiluminescence substrate. Protein loading was verified by ponceau red staining and by comparing with the GAPDH intensity of bands.

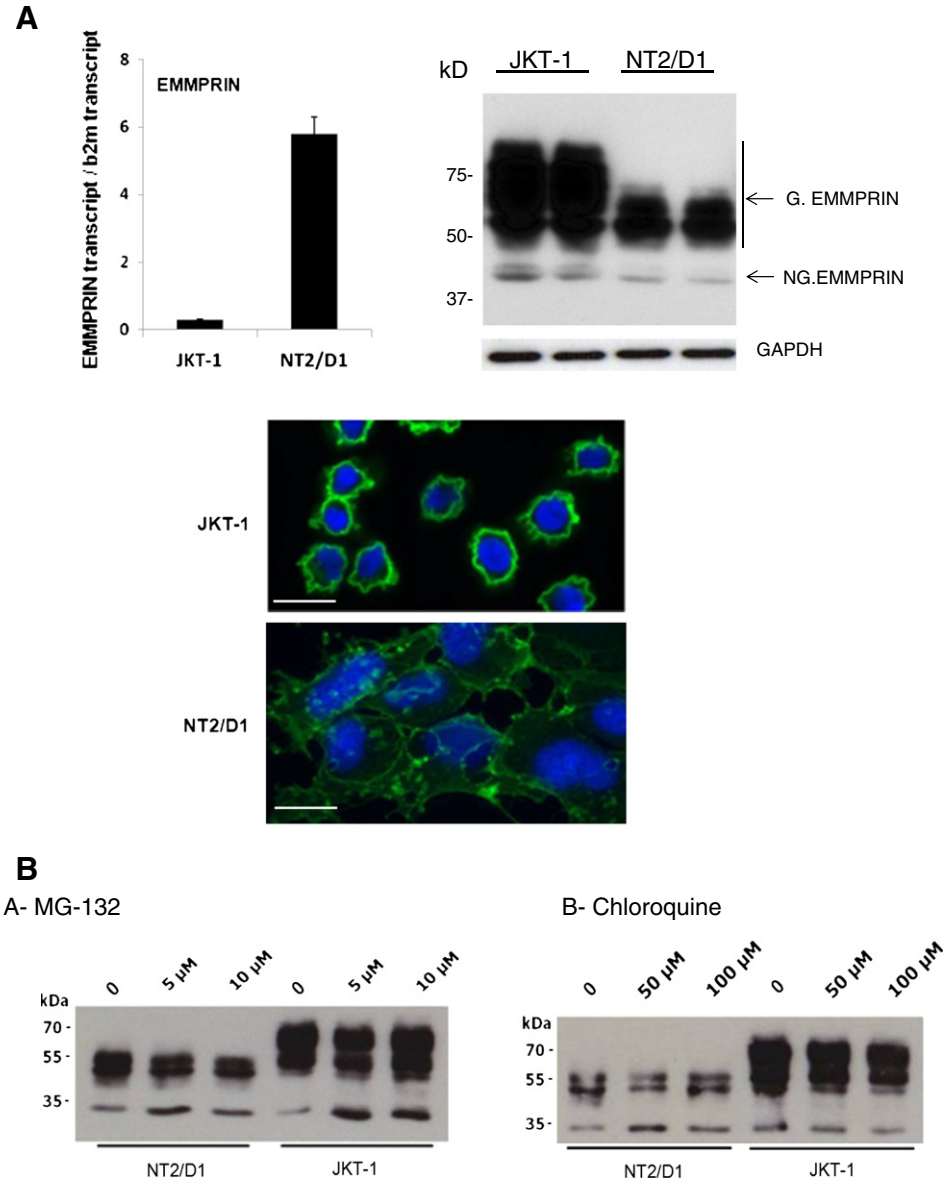

Fig. 1. EMMPRIN expression in human seminoma (JKT-1) and embryonic carcinoma (NT2/D1) cells. EMMPRIN mRNA expression was measured by qRT-PCR, western blot and Immunostaining. EMMPRIN transcripts were quantified using quantitative RT-PCR in JKT-1 and NT2/D1 cells. The columns shown represent average values from at least three independent experiments. EMMPRIN protein was evaluated by western blot analysis of JKT-1 and NT2/D1 cell lysates. NG and G denote non-glycosylated and glycosylated EMMPRIN forms. Representative blot is shown. Immunofluorescence staining of EMMPRIN is shown in green and counterstaining with DAPI in blue (bar scale 20 μm). **A. Effect of proteasome and lysosome inhibitors on EMMPRIN cellular levels in TCGC cells.** JKT-1 and NT2/D1 cells at near confluence were treated with 5 and 10 μM of the proteasome inhibitor MG-132 and 50 and 100 μM of the lysosome inhibitor chloroquine diphosphate salt for 6 h. Cell toxicity test by tryptan blue exclusion showed less than 10% of cell death in either cell type. Cell lyzates were then analyzed by western blot. Representative blots of at least three experiments are shown.

2.7. Zymographic analyses

Gelatin zymography was performed essentially as described [34]. Serum-free conditioned media corresponding to the same cell number and/or containing equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide SDS gels, containing 0.1% (w/v) gelatin. After electrophoresis, the gels were washed with 2.5% Triton-X-100 to remove SDS and then incubated at 37 °C for 20 h in 10 mM Tris–HCl, pH 7.5, containing 150 mM NaCl and 5 mM $CaCl_2$. The gels were stained with 0.25% (w/v) Coommassie blue G-250 and then distained in 20% methanol containing 10% acetic acid. Areas of protease activity were detected as clear bands against the blue gelatin background.

2.8. Extraction of Triton-X100 insoluble Proteins

Triton-X100 insoluble fractions were obtained as previously described with minor modifications [35]. Briefly, cells were homogenized in ice-cold lysis buffer, containing 25 mM Tris–HCl pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 0.25 M sodium orthovanadate, 1% of Triton X100 with protease inhibitor (Cocktail Set V, EDTA free, Calbiochem), sonicated three times for 10 sec and shaken for 30 min at 4 °C. Samples were then centrifuged twice at 10000 ×g at 4 °C for 10 minutes to obtain a pellet, the Triton-X100 insoluble fraction, and a supernatant, the Triton-X100 soluble fraction. The pellets were then resuspended in Laemmli sample buffer, sonicated three times for 10 sec and boiled prior to analyze.

2.9. Statistical analysis

Data are expressed as mean ± SD. Mann–Whitney and Student's *t* test were used to compare differences between groups in various experiments.

3. Results

3.1. Expression of EMMPRIN in TGCT cells as a function of malignant phenotype

The association between EMMPRIN levels and testicular germ tumor cell malignancy was evaluated by measuring the expression of EMMPRIN mRNA and protein in two testicular cell lines representing high and low aggressive state (embryonal carcinoma NT2/D1 and seminoma JKT-1, respectively).

These two cell models presented a differential expression of EMMPRIN (Fig. 1A), with NT2/D1 showing higher transcript levels but surprisingly, lower protein levels of EMMPRIN compared to JKT-1 cells. The reversed pattern of EMMPRIN protein level was observed by both immunoblotting and by immunohistochemisty (Fig. 1A). The different bands observed by Western blot correspond to differently glycosylated forms of EMMPRIN with the lowest MW representing the non-glycosylated molecule of 28 kDa. Similar results were obtained whether the cells were cultured on plastic, collagen type I or matrigel (not shown). EMMPRIN immunostaining was homogenous and strong on JKT-1 cell membrane while appeared as patches in NT2/D1.

The possibility that EMMPRIN is degraded immediately after transcription in the NT2/D1 cells was examined by treating the two cell lines with either the proteasome inhibitor MG-132, (5 and 10 μM) or the lysosome inhibitor, chloroquine diphospate salt (50 and 100 μM) (Fig. 1B). Neither of these inhibitors, even at the highest concentrations which could be applied without associated cell toxicity, had any apparent effect on EMMPRIN protein levels and therefore cannot explain the loss of EMMPRIN protein in NT2/D1 and the inverse RNA to protein ratio between the two cell lines.

3.2. EMMPRIN-containing membrane vesicles release in TGCT cells

As EMMPRIN was previously shown to be contained within tumor MVs which are released from the cells into the extracellular environment, we questioned whether an accumulation of EMMPRIN in released MVs may decrease its cell-associated levels in NT2/D1 cells. To this end, MVs were isolated by differential centrifugation of 24 h serum-free media, conditioned by equal number of cells of each cell line. After centrifugation, the resuspended pellet, containing the MVs, and the concentrated MV-deficient supernatant were adjusted to an equal volume and analyzed by immunoblotting. EMMPRIN was not detected in the MV-deficient conditioned media (Fig. 2A) suggesting that it was essentially of vesicular origin. The yield of MV obtained from NT2/D1, as assessed by protein content, was approximately 1.23 ± 0.3 times greater than from JKT-1. Fig. 2A shows that EMMPRIN levels in MVs released from the more aggressive NT2/D1 cells was much higher than in those released from an equal number of the seminoma JKT-1 cells (over 9 times greater, as estimated by density measurement of EMMPRIN Western blot bands), suggesting a much greater EMMPRIN content in the MVs derived from NT2/D1 cells than those derived from JKT-1. This implies that NT2/D1 cells secrete EMMPRIN to a greater extent in its released MVs. Noteworthy is the fact that, although the level of EMMPRIN in the MVs vary greatly in the two cell lines, its molecular weight was comparable in the MVs of the two cell types (approx. 45 kDa), unlike their profile in total cell lysate, suggesting that the molecular forms in the region of 45 kDa may be preferentially taken up in the vesicles.

It was previously reported that secreted MVs membrane resembles typical detergent-resistant membranes from the cell plasma membrane, having a similar cholesterol-phospholipid ratio [36]. In order to examine whether EMMPRIN distribution pattern observed in the MVs fractions of the 2 cell types reflects that in detergent-insoluble membranes, we analyzed EMMPRIN levels in Triton-X100 insoluble fractions prepared from JKT-1 and NT2/D1 cells. Interestingly, a different and opposing distribution of EMMPRIN was observed in Triton-X100 insoluble compared with that in MVs, with much greater EMMPRIN level observed in the Triton-insoluble fractions of the JKT-1 seminoma cells than of NT2/D1, observations that evoke the possibility that localization in these domains is incompatible with MV secretion (Fig. 2B). The caveolae marker protein, caveolin 1 had a similar distribution in these fractions as that of EMMPRIN, which may suggest that EMMPRIN is retained within caveolae in JKT1 cells while it is secreted within MVs in the more aggressive NT2/D1 cells (Fig. 2B). This is nicely shown in the immunofluoresent staining where in JKT-1 both EMMPRIN and caveolin-1 are localized on the cell surface, while in NT2/D1 cells caveolin-1 is much less abundant and is seen mainly intracellular while EMMPRIN is typically on the cell surface. Hence, these results suggest that in NT2/D1, EMMPRIN is targeted to the MVs, while in JKT-1 it is preferentially addressed to lipid rafts domains (Fig. 2C).

3.3. EMMPRIN contained within MVs is functional

To check whether EMMPRIN contained within these MV is active and able to increase MMPs production in host cells of the tumor microenvironment, we incubated subconfluent fibroblasts (approx 80% confluence) with MVs prepared under sterile conditions from equal number of NT2/D1 and JKT cells. Zymographic analysis of the serum-free conditioned medium (CM) of fibroblasts incubated for 24 h with the MVs show that incubation with NT2/D1 MV caused a greater stimulation of MMP-2 production in fibroblasts than with those obtained from the same number of JKT cells (Fig. 3A). Note that the MVs do not contain detectable MMP-2, as seen by the 3rd lane where the vesicles were incubated in the absence of fibroblasts, which suggests that the MMP-2 detected in fibroblasts conditioned medium was derived solely from the fibroblasts.

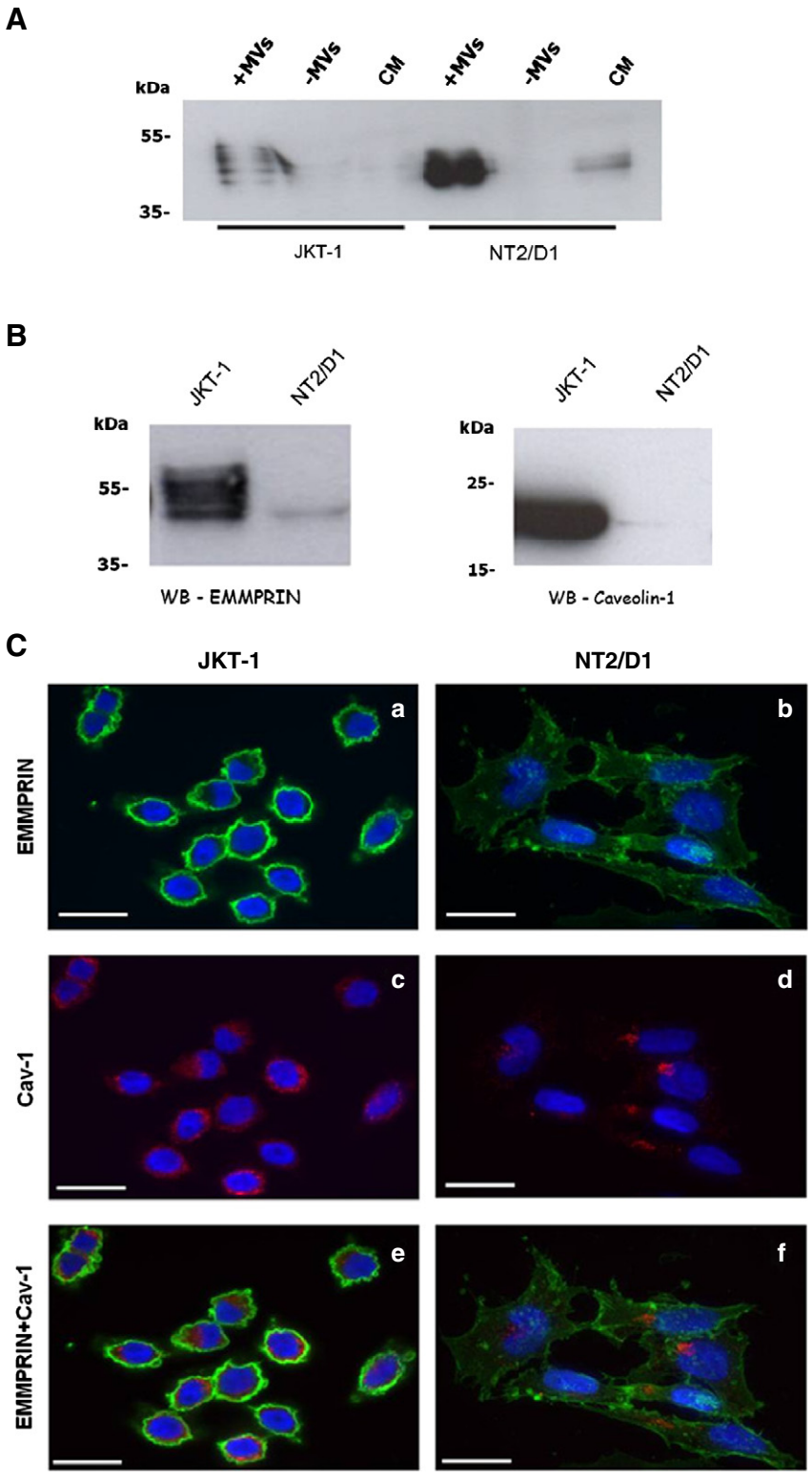

Fig. 2. EMMPRIN content in MVs and Triton-insoluble membrane domains isolated from TCGC cells. A- Membrane vesicles (+MV), prepared by differential centrifugation of serum free medium which was conditioned for 24 h by identical number of JKT-1 and NT2/D1 cells, were analyzed by western blot. Blots also show the MVs deficient concentrated medium (−MV) and the conditioned medium prior to centrifugation (CM). Note that for comparison purpose, the supernatant of MV deficient medium was concentrated to the same volume as that of the MV fraction. Representative blots of at least three experiments are shown. **B-** EMMPRIN levels in Triton-X100 resistant membrane fraction. Insoluble fractions obtained after Triton-X100 treatment of identical number of JKT-1 and NT2/D1 cells were analyzed for EMMPRIN and for caveolin-1 by western blot analysis. Representative blot is shown. **C–** Immunofluorescence co-staining for EMMPRIN and caveolin-1 in JKT-1 and NT2/D1 cells. EMMPRIN is shown in green, caveolin-1 in red and the nucleus is stained in blue by DAPI counterstaining (bar scale 20 μm).

To determine whether the MMP-2 increase was due to EMMPRIN's effect, EMMPRIN expression was inhibited by siRNA strategy in both NT2/D1 and JKT-1 cells prior to MVs isolation. The confirmation for EMMPRIN RNA and protein inhibition by siRNA is shown in Fig. 3B. EMMPRIN inhibition was also demonstrated in the MVs. MMP-2 induction in fibroblasts incubated with the MVs obtained from EMMPRIN-

siRNA-treated NT2/D1 was more notably decreased reaching similar levels for both cell lines (Fig. 3C). These zymographic results were also confirmed by qRT-PCR performed on fibroblasts after incubation with MVs showing a greater increase in MMP-2 induction, and its greater inhibition by EMMPRIN siRNA, after incubation with NT2/D1 MVs (Fig. 3C). Similar results were obtained for MMP-1 expression (not shown). These results suggest that EMMPRIN present within MVs is able to signal to cells in the environment to increase proteolysis.

4. Discussion

MVs have been attracting increasing attention for their possible role in tumor progression as they are thought to mediate interactions between tumor cells and the stromal cells in the microenvironment, without the need for a direct cell-cell contact or even cell proximity. These membrane vesicles have already been shown to contain EMMPRIN [37–39], a member of the immunoglobulin superfamily of adhesion molecules, enriched on tumor cell membranes and is known to promote tumor cell invasion through the induction of MMPs [22]. Using a testicular tumor cells model, we demonstrate in this study that in the more aggressive embryonic carcinoma cells, EMMPRIN is preferentially addressed to the MVs, whereas in seminoma cells, EMMPRIN seems to be confined to the specialized membrane microdomains or rafts. Accordingly, EMMPRIN cellular content retained in JKT-1 seminoma cells is higher than in the carcinoma NT2/D1 cells, despite the lower levels of its transcript level. Furthermore, these JKT-1 cells display an immunoblot profile with more glycosylated species suggesting that EMMPRIN has a longer lifespan in these cells which allows further glycosylation. Although the mechanisms underlying the sorting of these proteins to the specific membrane domains or membrane vesicles are yet to be unravelled, our results suggest that the embryonic carcinoma cells release MVs which accumulate more EMMPRIN and therefore are better equipped to modulate cells in the microenvironment. This is substantiated by the demonstration that EMMPRIN-contained within MVs retains its MMPs inducing activity as it was able to increase expression of MMP-1 and MMP-2 in fibroblasts, an increase which was inhibited when EMMPRIN was knocked down by siRNA. Hence, embryonic carcinoma cells can increase their invasive potential by shedding more abundantly EMMPRIN-containing MVs, allowing them to communicate with surrounding stromal fibroblasts and to signal them to increase MMPs production.

As a membrane-spanning molecule, EMMPRIN depends on a direct contact with the target cell membrane in order to exert its role in mediating interaction between tumor and stromal cells. Previous studies have suggested that tumoral EMMPRIN may also be able to communicate with more distant cells through the generation of soluble EMMPRIN by MT1-MMP mediated photeolytic cleavage [40,41]. In our study however, EMMPRIN was only found within the MV-containing pellet, obtained after ultracentrifugation of the conditioned medium, and was not detected in the concentrated MV-deficient supernatant, suggesting that cleaved solubilised EMMPRIN would only be, if any, a very minor component in our cell system and that most released EMMPRIN is vesicular. However, it was not possible to determine the nature of the EMMPRIN-containing membrane vesicles isolated in the present study, whether they represent exosomes from multivesicular bodies undergoing exocytosis [30], or microvesicles shed off from the surface [38], or both. These are generally defined on the basis of size and markers proposed to be specific for each type of vesicles. Identifying the type of vesicles would have to await the development of more discriminate purification techniques, since vesicle preparations currently used contain different subtypes of vesicles, even when the most accurate protocols of purification are used [42]. However, from observation of the cells in the immunofluoresent pictures (Fig. 1), we are tempted to speculate at this stage that the cells secrete mostly microvesicles budding of the cell surface membrane. Whatever the type of vesicles involved, their release from tumor cells into the extracellular environment represents a mechanism that would allow EMMPRIN-mediated communication at a distance, which results in an increase in MMPs in the tumoral microenvironment. Such mechanism represents an advantage to the tumor cells as they can benefit from the cooperation of non adjacent cells in the tumor microenvironment.

EMMPRIN protein level has been largely associated with tumor progression and worse prognosis in many types of cancers [20,22]. However, the RNA/protein discrepancy observed in these two cell lines cannot be extrapolated to cancer tissues in situ analysis which represents EMMPRIN staining in both cells and the associated microvesicles which are trapped within the tissue, while EMMPRIN protein level observed in this study only concern the cellular content excluding microvesicular EMMPRIN.

The demonstration that EMMPRIN protein level does not correspond with the extent of its transcription in our in vitro tumor model should be taken into account when studying EMMPRIN regulation. Indeed, the cause of RNA/protein discrepancy does not reflect accelerated degradation of the protein, which would have for effect to suppress its function. To the contrary, lower EMMPRIN protein level may in some cases signify its release within membrane vesicles, which would in fact boost

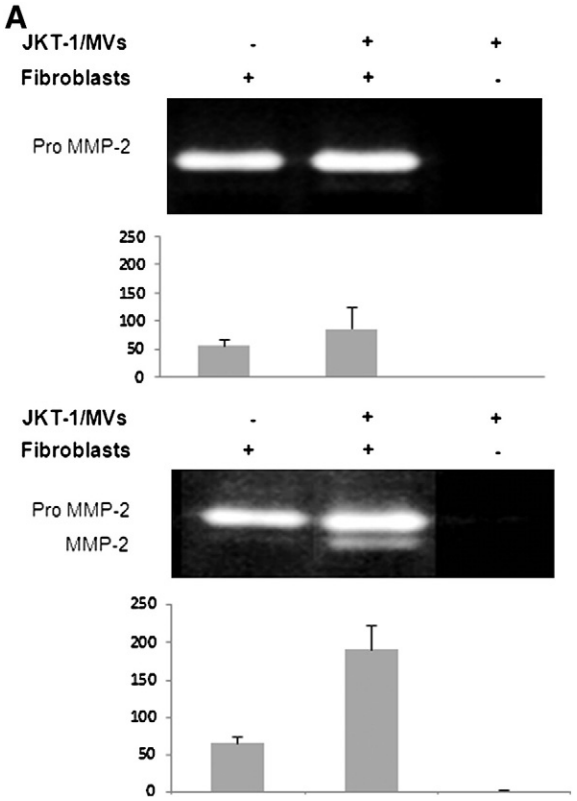

Fig. 3. Regulation of fibroblasts-derived MMP-2 by EMMPRIN contained within MVs. **A-** Zymography analysis of CM obtained from fibroblasts treated with MVs. Fibroblasts were incubated for 24 h with MVs derived from equal number of JKT-1 and NT2/D1 cells in serum-free medium and the CM was analyzed by gelatin zymography. Note that MVs do not contain detectable gelatinase activity (3rd lane). Representative gels are shown. Graphs represent densitometric quantification of the zymograms. **B-** Confirmation of EMMPRIN inhibition by siRNA. Both transcript quantification by qRT-PCR, and protein analysis by western blot, illustrate EMMPRIN siRNA inhibition in both cell lines compared to control cells transfected with scrambled siRNA. Moreover, western blot of MVs demonstrate that siRNA inhibition had also an impact on EMMPRIN contained within the vesicles as it was markedly decreased in NT2/D1. The columns shown represent average values from at least three independent experiments. * denotes significant difference with $p < 0.05$. **C–** Differential inhibition of MMP-2 induction in fibroblasts mediated by NT2/D1 and JKT-1 derived MVs upon EMMPRIN knockdown. qRT-PCR and zymography analyses of MMP-2 expression in fibroblasts treated with MVs derived from JKT-1 and NT2/D1 cells transfected with EMMPRIN or scrambled siRNA. The columns shown represent average values from at least three independent experiments. Zymographic bands were quantified by densitometry measurement. *denotes significant difference with $p < 0.05$.

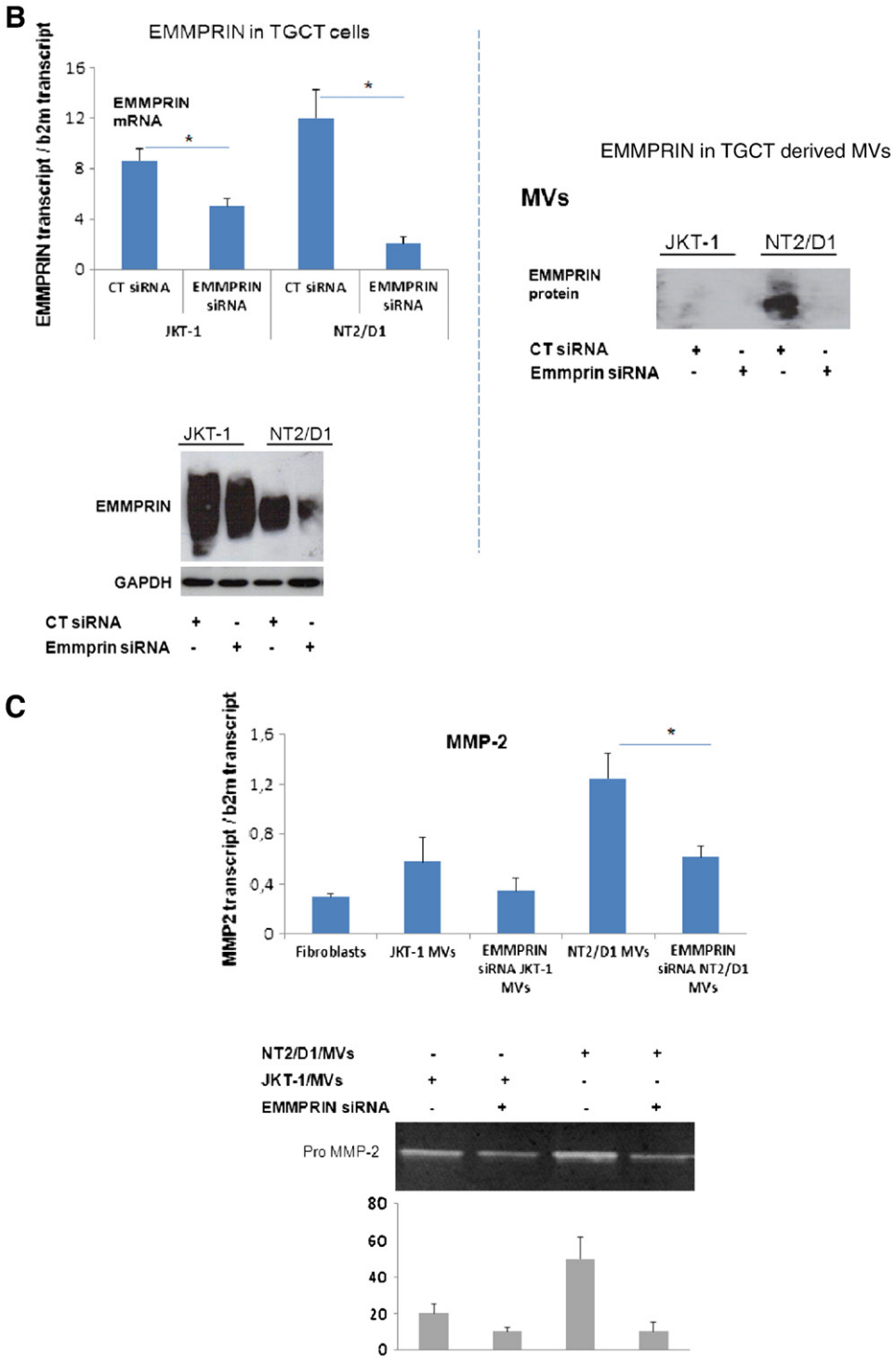

Fig. 3 (*continued*).

EMMPRIN's effect as it becomes available to act on greater number of distant cells and by that modify the microenvironment for the benefit of the tumor cell.

Acknowledgments

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