

## Short Communication

## Novel interactions between erythroblast macrophage protein and cell migration

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## ABSTRACT

Erythroblast macrophage protein is a novel protein known to mediate attachment of erythroid cells to macrophages to form erythroblastic islands in bone marrow during erythropoiesis. Emp-null macrophages are small with round morphologies, and lack cytoplasmic projections which imply immature structure. The role of Emp in macrophage development and function is not fully elucidated. Macrophages perform varied functions (e.g. homeostasis, erythropoiesis), and are implicated in numerous pathophysiological conditions such as cellular malignancy. The objective of the current study is to investigate the interaction of Emp with cytoskeletal- and cell migration-associated proteins involved in macrophage functions. A short hairpin RNA lentiviral system was used to down-regulate the expression of Emp in macrophage cells. A cell migration assay revealed that the relocation of macrophages was significantly inhibited when Emp expression was decreased. To further analyze changes in gene expression related to cell motility, PCR array was performed by down-regulating Emp expression. The results indicated that expression of mitogen-activated protein kinase 1 and thymoma viral proto-oncogene 1 were significantly higher when Emp was down-regulated. The results implicate Emp in abnormal cell motility, thus, warrants to assess its role in cancer where tumor cell motility is required for invasion and metastasis.

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## 1. Introduction

Cell migration forms the basis of essential functions of living systems such as wound healing, tissue remodeling, and embryogenesis. Also, cell migration plays a vital role in tumor cell motility in metastasis [1]. Similarly, migration of macrophages is crucial for a broad array of functions such as response to infection, and regeneration [2]. Conventionally, macrophages are considered to be the key regulators of inflammatory responses, but recently their role has been defined in many pathophysiological conditions such as atherosclerosis [3], rheumatoid arthritis [4], systemic lupus erythematosus [5], and cancer [6].

Erythroblast macrophage protein was first identified via culture of erythroid progenitors and is present on both erythroblast and

macrophage cells. Moreover, it mediates homophilic interactions between erythroblast and macrophage cells and facilitates enucleation of erythroblasts [7]. Subunits of Emp include a relatively small, extracellular amino terminus involved in cell-to-cell contact, a single transmembrane domain, and a large cytoplasmic domain involved in downstream signaling [8].

Previous studies demonstrated that Emp-null mice were not viable and had impaired development of both erythroid and macrophage lineages [9]. Furthermore, Emp-null macrophages were small, round, and had condensed, with less organized actin filaments resulting in the lack of cytoplasmic projections, and demonstrate immature macrophage morphology. Emp expression is intense in the nuclei of immature macrophages, and increases at the plasma membrane and all along the microspikes in mature macrophages [10,11]. However, how the differential expression of Emp is correlated to its functions is unknown. Therefore, the present study addresses the role of differential expression of Emp in macrophages in relation to its pathophysiology to mimic the malignancy.

In the present study, we utilized the GIPZ lentiviral shRNA gene silencing system to down-regulate expression of Emp and evaluated its role in macrophage cell motility. Since little is known about how

**Abbreviations:** ABPs, actin binding proteins; Akt 1, thymoma viral proto-oncogene 1; CSF-1R, colony stimulating factor-1 receptor; ECIS, electric cell-substrate impedance sensing; Emp, erythroblast macrophage protein; *Ezr*, *ezrin*; *MAPK1*, mitogen associated protein kinase 1; PGE2, prostaglandin E2; shRNA, short hairpin RNA; *Tln1*, *talin*.

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**Table 1**

The shRNA lentiviral constructs utilized were: V2LMM\_70753, V2LMM\_62031, V3LMM\_420136, named as Emp#7, Emp#6, Emp#4, respectively.

The mature anti-sense sequences of Emp GIPZ lentivirus are as follows:	
V2LMM_70853	TATCATATCGAAACTGCTG
V2LMM_62031	TTAGGCAGCATCATGGGTG
V3LMM_420136	ATCATATCGAAACTGCTGG
The non-silencing control hairpin sequence is as follows:	
TGCTGTTGACAGTGAGCGATCTCGCTTGGGCGAGAGTAAGTAGTGAAGCCACAGATGTACTTACTCTCGCCCAAGCGAGAGTGCCTACTGCCTCGGA	
22mer sense	ATCTCGCTTGGGCGAGAGTAAG
22mer antisense	CTTACTCTCGCCCAAGCGAGAG

dysregulated Emp is involved in the mechanism of cell motility, our studies will provide important clues that are highly relevant to cancer.

## 2. Materials and methods

### 2.1. Lentivirus-mediated shRNA targeting of Emp

The shRNA-mediated down-regulation of Emp was performed in RAW 264.7 cells (mouse monocyte macrophages) (ATCC, Manassas, VA) using GIPZ shRNA lentiviral particles (Dharmacon-GE health care, USA) according to the manufacturer's instructions. The shRNA lentiviral constructs utilized were: V2LMM\_70753, V2LMM\_62031, V3LMM\_420136, named as Emp#7, Emp#6, Emp#4, respectively (Table 1). Non-silencing shRNA was referred to as the negative control. The efficiency of down regulation of Emp was determined by immunoblotting and densitometry analyses.

### 2.2. Cell migration assay

Cell migration assay was performed using automated electric cell-substrate impedance sensing (ECIS) system (Applied BioPhysics Inc.). Wells containing  $10 \times 10^4$  cells/mL,  $15 \times 10^4$  cells/mL,  $20 \times 10^4$  cells/mL, and  $30 \times 10^4$  cells/mL were plated on sterile 8w10E electrode arrays, incubated at 37 °C and 5% CO<sub>2</sub>, and attached to recorder device. Cell monolayers were evaluated by electric pulse and readings were measured every 5 min for 40 h.

### 2.3. PCR array analysis

Total RNA was isolated from RAW 264.7 cells and Emp#4 cells using RNeasy Mini Kit (Qiagen) according to the manufacturer's instruction. cDNA was synthesized using RT<sup>2</sup> First Strand kit (Qiagen). Cell motility-related gene expression profile was assessed using mouse RT<sup>2</sup> Profiler PCR arrays (SA Biosciences). Data was analyzed using the SA Biosciences Software (<http://www.sabiosciences.com/pcr/arrayanalysis.php>).

## 3. Results and discussion

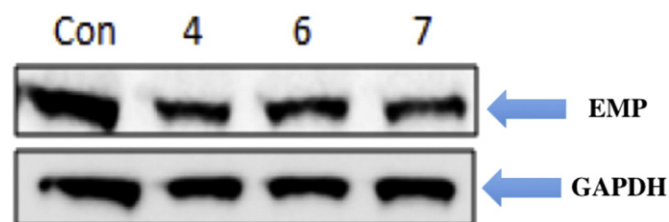
Western blot analysis demonstrated that all three lentivirus-mediated shRNA transduction resulted in efficient down regulation of Emp. Results were very distinct in Emp#4 and Emp#6 shRNA lentivirus transduced cells (Fig. 1). Next, the effect of Emp down-regulation on cell motility was investigated. Quantification of cellular movements every 5 min up to 40 h showed that cell migration of Emp#4, Emp#6, and Emp#7 shRNA-lentivirus transduced cells were severely suppressed as compared to the control. Moreover, as the number of cells increased, results were more evident with time (Fig. 2). These results imply that cellular migration was significantly impaired following down regulation of Emp. Therefore, Emp can be considered as a novel molecule in cell migration. It can be a structural component of the

actin cytoskeletal-adhesome complex or may be a crucial piece of signaling pathway machinery.

To the best of the authors' knowledge, this study is the first that includes comprehensive analysis of transcriptional responses related to cell motility following down regulation of Emp gene expression in macrophages. PCR array analysis revealed significant changes in the gene expression profile associated with cell motility when Emp expression was down regulated (Fig. 3). Of the differentially expressed genes, only three transcripts were increased by greater than 2 folds, whereas other transcripts were down regulated. Transcripts that were increased were thymoma viral protooncogene 1 and mitogen associated protein kinase 1 (28.5 and 7.0 folds up regulated, respectively). Comparatively, Rho family GTPase 3 (Rnd3) was 12.05 times overexpressed (Fig. 4).

Cell migration is a dynamic, well-coordinated process which orchestrates focal adhesion, actin cytoskeletal rearrangements, physical interactions of adaptor protein, and various signaling pathways [12]. Components of MAPK pathways such as ERK 1 and ERK 2 play essential roles in cell migration through several mechanisms such as regulation by IQGAP1 [13] and prostaglandin E2/colony stimulating factor-1 receptor [14]. Up-regulation of *MAPK1* and *Akt1* suggests that Emp may be a part of Erk1/Erk2 or PI3/Akt pathways or may be parallel/alternative events to the pathway involving Emp regulating cell motility. *MAPK1* up-regulation has been implicated in many types of cancers [15] such as myeloid leukemia [16]; thus, findings of the present study necessitate further exploration.

The expressions of mRNA encoding 56 cell motility genes were decreased compared to the control. Also, cell adhesion and cell polarity genes such as actin genes, *Actn3* (162.3 folds) and *Actn4* (13.3 folds), were significantly under expressed. Calpain genes, *Capn1* and *Capn2*, were also reduced 5.1 and 9.2 folds, respectively. Other cell adhesion genes and integrin-mediated signaling genes (i.e., *Itgb1*, *Itgb2*) and matrix metalloproteases (*Mmp14*) were also substantially reduced in expression. Genes related to cellular projections such as *Cdc42*, *ezrin* (*Ezr*) and *talin* (*Tln1*) had decreased expression when Emp was down-regulated. These results revealed that although genes related to chemotaxis, polarization, and proteolysis were significantly up-regulated, genes related to adhesion and cellular projections were reduced. Since the preliminary results suggested that actin localization pattern changes in the absence of Emp, we conclude that down-regulated Emp



**Fig. 1.** Western blot analysis of RAW 264.7 control cells and cells transduced with Emp shRNA lentivirus. Densitometric analysis of immunoblot assay.

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