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# EMR3: A Potential Mediator of Invasive Phenotypic Variation in Glioblastoma and Novel Therapeutic Target

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## Abstract

EGF module containing, mucin-like hormone receptor-3 (EMR-3) is a G-protein coupled receptor with unknown ligand and cellular function. Upregulation of EMR-3 in glioblastoma multiforme is associated with poor survival. We investigated expression patterns and functional significance of EMR3 in GBM using immunohistochemistry, western blot, RT-PCR, and siRNA knockdown in proliferation and invasion assays. EMR-3 is variably expressed in primary human GBM tissues and cell lines. Knocking down EMR-3 has no impact on cellular proliferation, but decreases cellular invasion by greater than 3-fold. EMR-3 is a potential mediator of cellular invasion in GBM. Given the poor survival associated with high levels of EMR-3 expression in glioma patients our results provide impetus to explore EMR-3 as a potential therapeutic target.

### Keywords

EMR3; glioma; invasion

## Introduction

Surgical resection with negative margins is the cornerstone of effective multimodality therapy for most solid malignancies. Surgery for high-grade glial neoplasms is inherently limited by the propensity of these tumors to widely infiltrate the normal brain, making total tumor removal an unrealized ideal [1]. While post-operative recurrence is universal, glioblastomas exhibit a wide range of clinical behaviors, with many tumors recurring as a well localized mass in the previous resection cavity, while others present initially or at recurrence with extensive white matter, transcallosal, or subependymal spread [2]. The exact molecular mechanisms that underlie glioma cell invasion of surrounding tissue remain to be elucidated, and as such, little data exist to explain the clinical heterogeneity of glioblastoma, or more specifically, what mechanisms drive the numerous pathoanatomic phenotypes observed in this disease.

EGF module containing, mucin-like hormone receptor-3 (EMR-3) is a member of the epidermal growth factor-seven transmembrane (EGF-TM7) family of proteins. Its ligand and cellular function are entirely unknown [3], and most of what is known about this protein

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comes from functional genomics, which suggest that it is a G-protein coupled receptor [4]. Previous work has shown its expression localized to mature granulocytes [3,4].

We first became interested in EMR-3 while analyzing microarray survival data for glioblastoma on The Cancer Genome Atlas (TCGA) website [5]. A Kaplan-Meier analysis performed using this database on September 13, 2009 demonstrated that a significant survival benefit for patients with tumor specimens demonstrating EMR-3 downregulation ($P<0.001$) (figure 1). Given the known limitations of microarray analysis, these results lead us to further study EMR-3 to clarify whether EMR-3 was actually expressed in glioblastoma, whether this molecule's expression demonstrated inter-individual variability, and whether altering the EMR-3 expression in EMR-3 expressing glioma cells, caused alterations of cellular function that could be linked to clinical heterogeneity of invasive phenotypes.

## Methods

### Cell lines and cell culture techniques

Four human glioblastoma cell lines: U87 and U251 (American Type Culture Collection), SF767 (University of California San Francisco Brain Tumor Research Center), and G55 (kindly donated by Dr. David James) were used. Cell lines were maintained at 37°C, 95% $O_2$, and 5% $CO_2$, in DMEM with 10% fetal bovine serum (FBS) and 5% penicillin-streptomycin (100 units/mL penicillin, and 100 μg/mL streptomycin).

### Immunohistochemistry

Formalin-fixed paraffin-embedded tumor tissue from eight human patients was obtained with informed consent through the University of California San Francisco (UCSF) Brain Tumor Research Center with approval from the Committee on Human Research (protocol H41175-26125-02). All immunohistochemistry was performed on a Benchmark XT Ventana (Ventana Medical Systems). Slides were deparaffinized, rinsed, and blocked before EMR3 (Sigma-Aldrich) (1:25) antibody incubation at 37°C for 2 hours. EMR3 antibody was detected with Ultraveiw Universal DAB Detection Kit (Ventana Medical Systems) according to the manufacturer's protocol. Sections were analyzed and scored by a neuropathologist with an Olympus U-D03 microscope (Olympus) for EMR3 expression. A score of 0 denoted no positive tumor cells; a score of 1 denoted that 1–5% of the tumor cells stained positive; 2 for 6–25% of the tumor cells; and 3 for >25% of the tumor cells.

### RT-PCR Analysis

RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse transcribed using Superscript III kit (Invitrogen) according to the manufacturer's protocol. Gene expression was detected by PCR using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) with the following primers: EMR3 (5′-CTGGC-CTCAC-CTTTA-TGGAA/GGTGA-AGAGG-TAGGC-CATGA-3′) and HPRT (5′-GACCAG-TCAACA-GGGGAC-AT/CCTGAC-CAAGGA-AAGCAA-AG-3′). PCR products were run on a 2% agarose gel in 1x TAE buffer stained with Ethidium Bromide and visualized under UV light.

### Western blotting

Cells were lysed in lysis buffer (Cell Signaling) with protease inhibitor (Roche), centrifuged, and supernatant collected, concentrations normalized, and samples denatured in NuPage LDS sample buffer (Invitrogen). 20μg was electrophoresed through 4–20% Tris-Glycine gels (Invitrogen), and electrotransferred to Immun-Blot PVDF membrane (BioRad). Membranes were probed for EMR3 antibody (Sigma-Aldrich) (1:250) or GAPDH antibody (Cell Signaling) (1:2500) in TBS containing 1% milk and 0.05% Tween-20 overnight at 4

°C. Secondary antibody was HRP-goat anti-rabbit IgG (Cell Signaling) (1:2500) incubated for1 hour at room temperature. Blots were developed with Amersham ECL Western Blotting Detection reagent (GE Healthcare).

### siRNA knockdown

SF767 cells were transfected with siRNA targeting EMR3 (*Silencer* Select siRNA, sense 5′-CCUCCUCAAAGACAACCGAtt-3′) (Ambion) or a scramble control (*Silencer* Select Negative Control #1 siRNA) (Ambion) using the siPORT *NeoFX* transfection agent (Ambion) per manufacturer's protocol to achieve a final siRNA concentration of 5nM.

### ATP chemiluminescence cellular proliferation assay

Proliferation was determined using a previously validated ATP chemiluminescence assay, the ATPlite 1-Step Assay (Perkin Elmer) and the experiment was carried out as previously described [6].

### In vitro cellular migration assay

SF767 cells underwent siRNA knockdown with control or EMR3 targeted transfection for 36 hours. $2 \times 10^5$ cells were loaded in triplicate into a matrigel invasion chamber (BD Biosciences) and allowed to migrate toward 10% FBS in D-MEM for 24 hours. Knockdown was verified using western blot. The non-migratory cells were scrapped off and the invading cells were fixed with 4% paraformaldehyde and stained with hematoxylin. Three fields of invading cells were visualized and counted at 20x for each repetition in order to determine the invasive index for the two conditions.

### Statistical analysis

Between group comparisons of continuous data were performed using student's t-test. The *p-value* was considered significant below the 5% level (i.e. $p<0.05$).

## Results

### Human glioblastoma cells variably express EMR-3 protein

Immunohistochemistry for EMR-3 on eight primary glioblastoma tissue samples demonstrated robust expression (>25% of tumor cells) in 3/8, weak expression (1–5% of tumor cells) in 1/8, and no evidence expression in 4/8 (figure 2). Given the marked differences in EMR-3 expression, these data suggest that the variability in EMR-3 transcripts seen in microarrays might represent real differences at the protein expression level in human patients.

Western blot and RT-PCR demonstrated EMR-3 is variably expressed in different glioma cell types, and there was a strong correlation between protein and transcript levels. The SF-767 cell line demonstrated evidence of high levels of EMR-3 mRNA transcript and protein, while U87 showed low levels, and U251 and G55 showed no expression (figure 3). Given these results, we utilized the SF767 cell line to further determine the phenotypic impact of EMR-3 expression on glioblastoma cells *in vitro.*

### EMR-3 mediates glioblastoma cell invasion in vitro

EMR-3 is thought to represent an adhesion G-protein coupled receptor, and other members of this family (EGF-TM7) are known to mediate cellular migration and invasion in leukocytes [3,7]. We utilized siRNA targeting to knockdown the expression of EMR-3 in the SF767 to determine if suppression of EMR-3 altered the invasion potential of these cells. As demonstrated in figure 4, knockdown of EMR-3 in SF767 cells had a greater than 3-fold

reduction in invasion compared to controls (3.42 vs 1, $p<0.05$). These data suggest that EMR-3 is involved in mediating migration and invasion in EMR-3 expressing glioblastoma cells.

### EMR-3 does not influence cell proliferation in vitro

We utilized siRNA to suppress the expression of EMR-3, which we found achieved significant knockdown of the EMR-3 protein for 72 hours (figure 5). The ATP based chemiluminescence assay at 24, 48, and 72 hours demonstrated no significant differences in growth rates between control and EMR3 siRNA knockdown (figure 5).

## Discussion

This study provides the first evidence linking EMR-3 protein expression to cellular behavior in a human disease. More specifically, we found that EMR-3 protein is variably expressed in human glioblastoma tissues and cell lines, and that by suppressing this expression in a high EMR-3 expressing cell line, we could significantly alter the invasive behavior of these cells in an *in vitro* assay.

The exact function of EMR-3 is entirely unknown, and no ligands or downstream signaling targets have clearly been linked to EMR-3 [8], although some evidence suggests that it is involved in cell-cell interactions of activated leukocytes [8]. Based on our migration data, combined with the lack of anti-proliferative effect of EMR-3 suppression, we hypothesize that EMR-3 is a molecule which is involved in the mediation of cellular transmigration, and tissue invasion in normal cells, which in the case of glioma cells might be inappropriately expressed and permit these cells to invade surrounding tissue. Supporting this hypothesis are observations of the function of other members of the EGF-TM7 family, which seem to be involved in transmigration, and with which EMR-3 shares significant homology. For example, EMR-2 is a member of EGF-TM7 family which shares significant protein homology with EMR-3, and in fact EMR-3 was first identified as an unintentional co-amplification product of an attempt to clone the EMR-2 gene [8]. EMR-2 has been demonstrated to bind chondroitin sulfate glycosaminoglycans, which has been suggested to mediate myeloid lineage cell interaction with the extracellular matrix [9]. Subsequent work demonstrated that EMR-2 is a critical mediator of neutrophil migration, adhesion, and invasion [7,10]. More pertinent to the present study Mustafa and colleagues demonstrated a correlation between tumor cell expression levels of the EGF-TM7 protein CD97 and the presence of metastasis of oral cancers [11]. Thus, when combined with observations regarding the overall function of this family of largely conserved proteins, our data suggests that EMR-3 might mediate invasion functions, which are inappropriately activated in some glioblastomas.

These observations raise a number of interesting questions which deserve future investigation. Most notably, the expression of EMR-3 has previously been thought to be restricted to myeloid lineage cells [3], however our data suggest that this EMR-3 can be expressed by human cancers. The mechanisms by which oncogenic mutations and alterations in signaling pathways lead the EMR-3 protein to be expressed in cancer cells represent an important question not only for oncology, but also for better understanding EMR-3 function. The downstream effects of EMR-3 are similarly unknown, but likely important. In addition, future in vivo and clinical efforts will be needed to further clarify a number of important questions: Does EMR-3 protein expression mediate variability in tumor behavior between different glioblastoma patients? Does EMR-3 protein expression predict worsened clinical outcome? Does the loss of EMR-3 in some cell lines explain the lack of infiltrative behavior in some xenograft cell lines? These ambitious questions lie outside the scope of the present report.

## Conclusion

In summary, we provide evidence for the first time that that the adhesion G-protein coupled receptor EMR-3 is expressed in some human glioblastomas, and may mediate invasive behavior in these cells. These data serve to identify an interesting potential target for future investigations, and to expand our understanding of EMR-3 function.

## Acknowledgments

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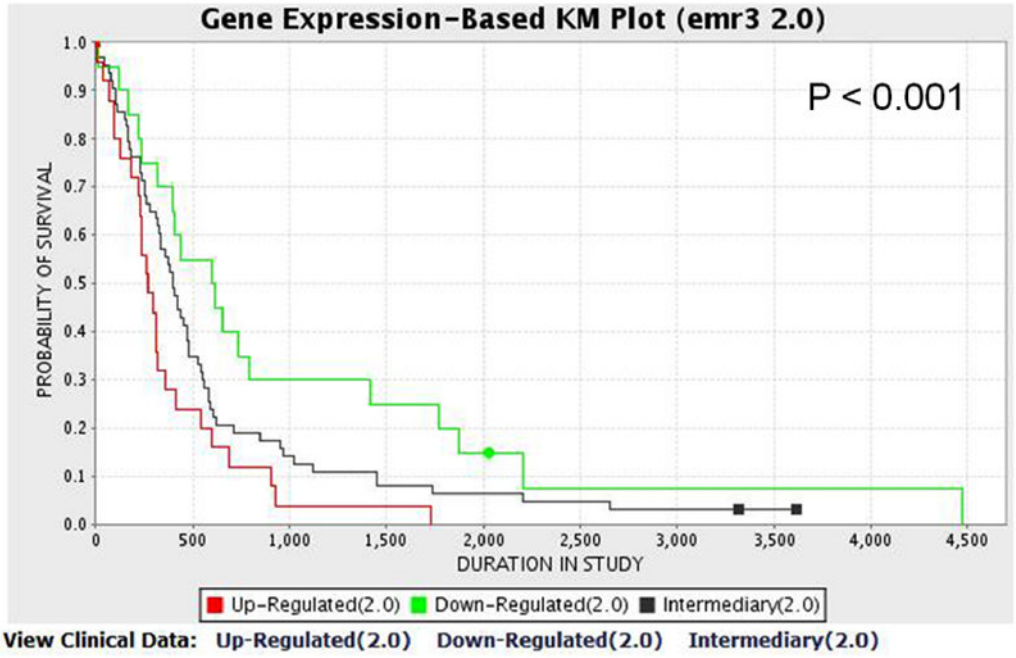


**Figure 1.**
Kaplan-Meier analysis comparing post-diagnosis survival of patients with high (red), intermediate (black), and low (green) EMR-3 expressing glioblastomas. These data represent microarray expression data from the TCGA database queried in September 2009.

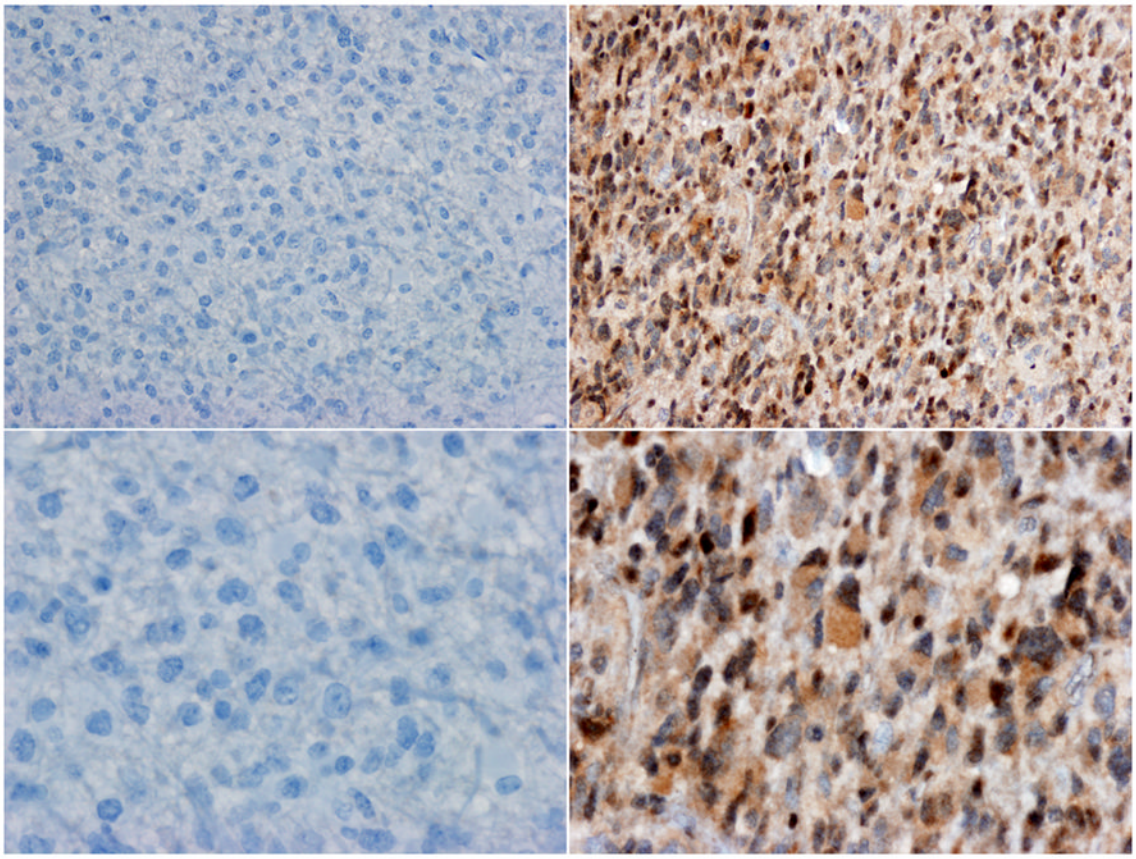

**Figure 2.**
Immunohistochemistry of glioblastoma patients stained for EMR-3. Low EMR-3 expressing (left) and high EMR-3 expressing (right) samples, are demonstrated at 20X (top images), and 40X (bottom images) power.

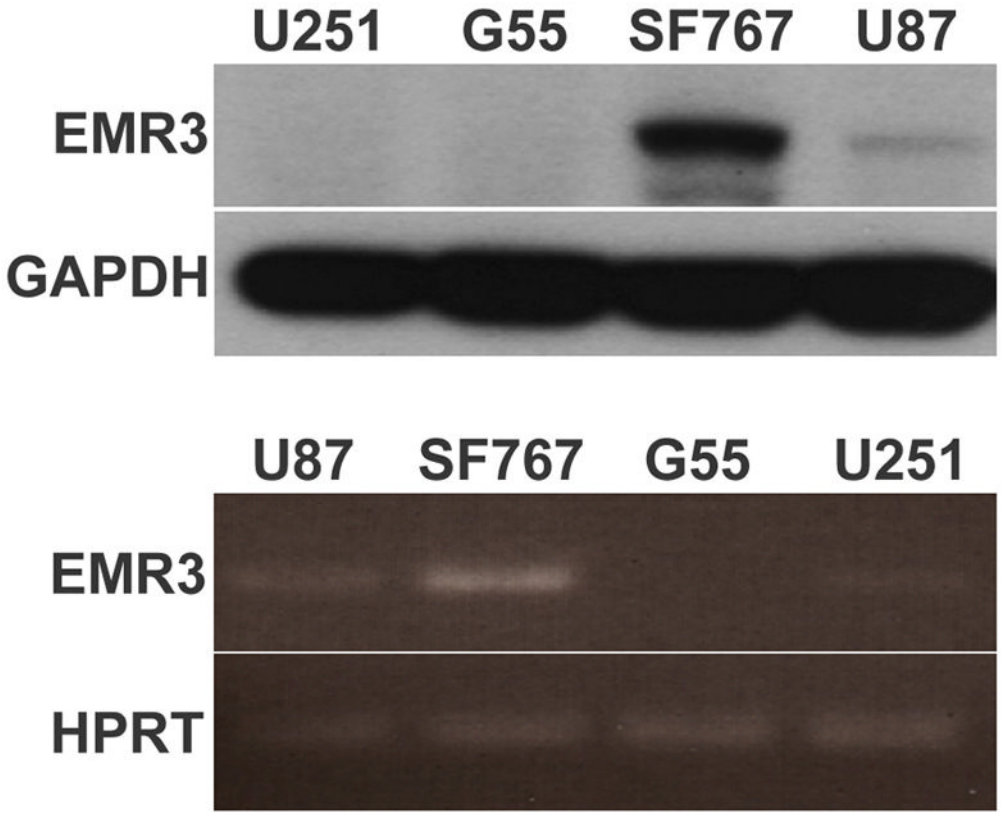


**Figure 3.**

Expression of EMR-3 in human glioblastoma cell lines. Western blot (top) and RT-PCR blot (bottom) for the EMR-3 mRNA and protein, respectively, in human glioblastoma cell lines (U87, U251, SF767) and one human glioblastoma xenograft cell line (G55). Rows two and four represent loading controls GAPDH for western blot and HPRT for RT-PCR.

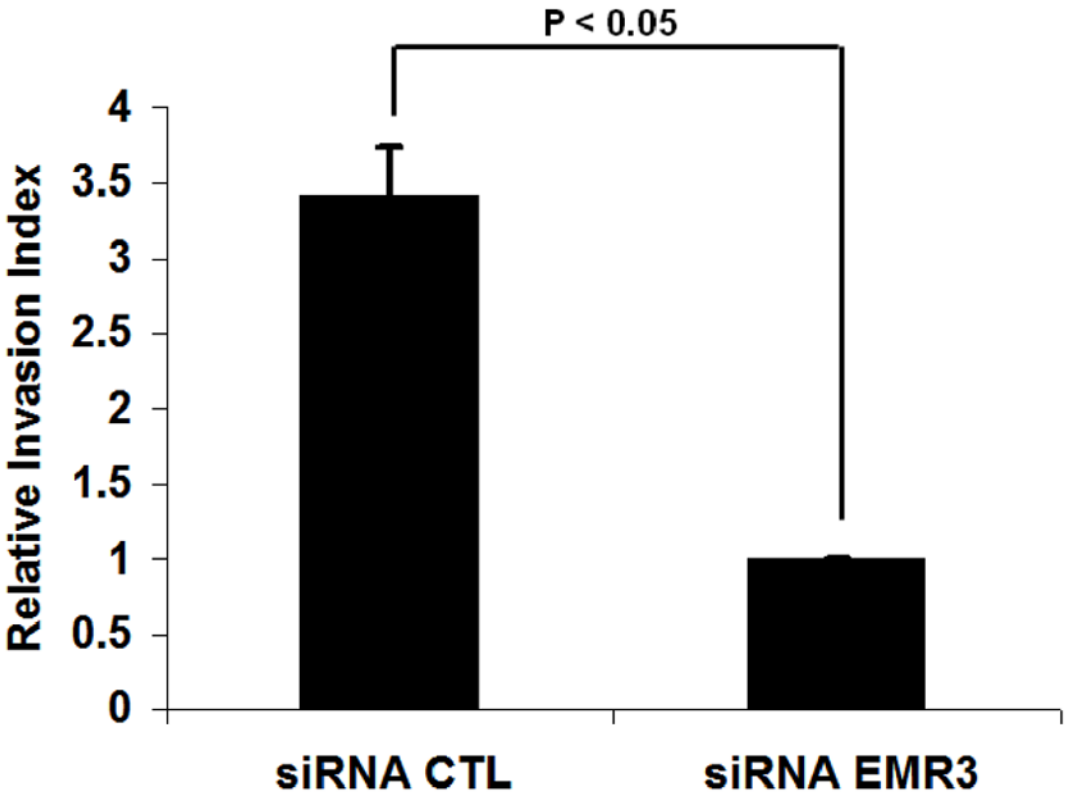


**Figure 4.**
Results of the cellular invasion assay in SF767 cells treated with either siRNA targeting EMR-3 (right bar) or a non-targeted construct (left bar). Invasion index was normalized to the less invasive knock-down to demonstrate fold-decrease in invasiveness by the knockdown.

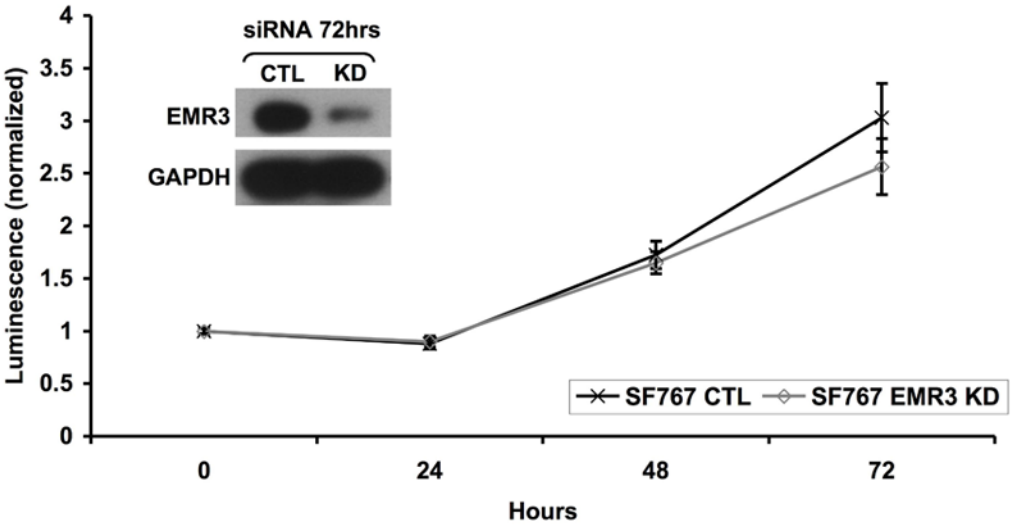


**Figure 5.**

Results of the cellular proliferation assay at 24, 48, and 72 hours in SF767 cells treated with either siRNA targeting EMR-3 (lighter bar) or a non-targeted construct (darker bar). Western blot inset demonstrates significant knockdown of EMR-3 at 72 hours with loading control shown below.