Emerin Represses STAT3 Signaling Through Nuclear Membrane-Based Spatial Control

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Running Title: Inhibition of STAT3 signaling by emerin

Abbreviations:

STAT3, Signal transducer and activators of transcription3; JAK, Janus-kinase; LEM, LAP2-emerin-MAN1; TM, Transmembrane; PM, Plasma membrane; NE, Nuclear envelope; EDMD, Emery-Dreifuss muscular dystrophy

ABSTRACT

Emerin is the inner nuclear membrane protein involved in maintaining the mechanical integrity of the nuclear membrane. Mutations in EMD encoding emerin cause Emery–Dreifuss muscular dystrophy (EDMD). There has been accumulating evidence that emerin regulation of specific gene expression is associated with this disease, but the exact function of emerin has still less revealing. Here, we have shown that emerin downregulates Signal transducer and activators of transcription 3 (STAT3) signaling, activated exclusively by Janus-kinase (JAK). Deletion mutation experiments showed that the lamin-binding domain of emerin is essential for the inhibition of STAT3 signaling. Emerin interacted directly and co-localized with STAT3 in the nuclear membrane. Emerin knockdown induced STAT3 target genes Bcl2 and Survivin to increase cell survival signals and suppress hydrogen peroxide-induced apoptosis in HeLa cells. Specifically, downregulation of BAF or lamin A/C increases STAT3 signaling, suggesting that correct-localized emerin by assembling with BAF and lamin A/C acts as an intrinsic inhibitor against STAT3 signaling. In C2C12 cells, emerin knockdown induced STAT3 target gene, Pax7, and activated abnormal myoblast proliferation associated with muscle wasting in skeletal muscle homeostasis.

Our results indicate that emerin downregulates STAT3 signaling by inducing retention of STAT3 and delaying STAT3 signaling in the nuclear membrane. This mechanism provides clues to the etiology of emerin-related muscular dystrophy and could be a new therapeutic target for treatment.

Keywords: JAK, STAT3, emerin, muscular dystrophy

1. Introduction

The nuclear lamina confirms mechanical assistance for the nucleus and provides a protein network that contributes to DNA replication, gene regulation, and genome stability [1,2]. The nuclear lamina proteins scaffold hundreds of proteins, including the LAP2- emerin-MAN1-domain (LEM-D) protein family in the inner nuclear membrane [3,4]. Multiple human diseases are caused by the loss of individual nuclear lamina proteins, highlighting the importance of this network [2,5]. Emerin was initially identified as a 35 kDa, the inner nuclear transmembrane protein which interacts with structural proteins including lamin A/C, nesprin-1α/2β, and BAF [6-9]. In addition to its known function in supporting the mechanical integrity of the nuclear membrane, emerin is involved in sensing and responding to mechanical tension at nuclear membrane [10]. Emerin also plays a role in the signaling and transcriptional regulation to interact many proteins, including β-catenin, notch intracellular domain (NICD), germ cell-less (GCL), Bcl-2-associated transcription factor (Btf) , and LIM Domain Only 7 (LMO7) [11-16]. The mutations in the emerin network cause Emery-Dreifuss muscular dystrophy (EDMD) [2,17,18]. Recently, the nucleotide sequence of the emerin mutant that induces EDMD has been revealed [19]. X-linked EDMD (X-EDMD) is caused by mutations in EMD located on chromosome Xq28, and autosomal dominant EDMD (AD-EDMD) is caused by mutations in LMNA (encoding Lamin A) located on chromosome 1q11-q23 [17,18,20,21]. The loss of the emerin protein resulted in skeletal muscle wasting and cardiac defects, the character of EDMD [22,23]; however, the role of emerin loss in this disease has not been precisely elucidated.

In the present study, we assessed the emerin effect on gene expression using a transcription factor profiling PCR array covering 84 genes. Based on the positive value of transcription factor profiling, we tried to confirm the possibility of STAT3 signaling as a target pathway regulated by emerin. STAT3, a member of the STAT protein family has first discovered in interferon (IFN) signaling studies [24-26]. STAT3 is expressed at a low, basal level in virtually all the cells. A variety of stimuli can activate STAT3 signaling via phosphorylation of tyrosine 705. In general, cytokines such as interleukin-6 (IL-6) [27], leukemia inhibitory factor (LIF) [28], IL-5 [29], IFN-γ [30] and TNF-α [31] are known to activate STAT3. In addition to cytokines, growth factors such as EGF [32], TGF α [31], and PDGF [33] activate STAT3. In response to cytokines and growth factors, STAT3 is phosphorylated by receptor-associated JAK, form homo- or heterodimers, and translocate to the cell nucleus where they act as transcription activators [34]. Additionally, activation of STAT3 may occur via phosphorylation of serine 727 by Mitogen-activated protein kinases (MAPK) [35] and through c-Src non-receptor tyrosine kinase [36,37]. Among the various roles of STAT3, many downstream genes are expressed in cell survival [38-41], cell proliferation [42-44], inflammation [27,45,46], and tumorigenesis [47-52]. Especially, STAT3 signaling has been known to play a critical role in muscle wasting induced by the IL6/JAK/STAT3 signaling pathway [53,54]. STAT3 signaling is activated in skeletal muscle and promotes skeletal muscle atrophy in muscle diseases, such as Duchenne muscular dystrophy (DMD), and Merosin-deficient congenital muscular dystrophy (MDC1A) [55-57]; thus, prolonged activation of STAT3 in muscles has been shown to be responsible for muscle wasting by activating protein degradation pathways. It is important to balance the extent of STAT3 activation and the duration and location (cell types) of the STAT3 signaling when developing therapeutic interventions [58].
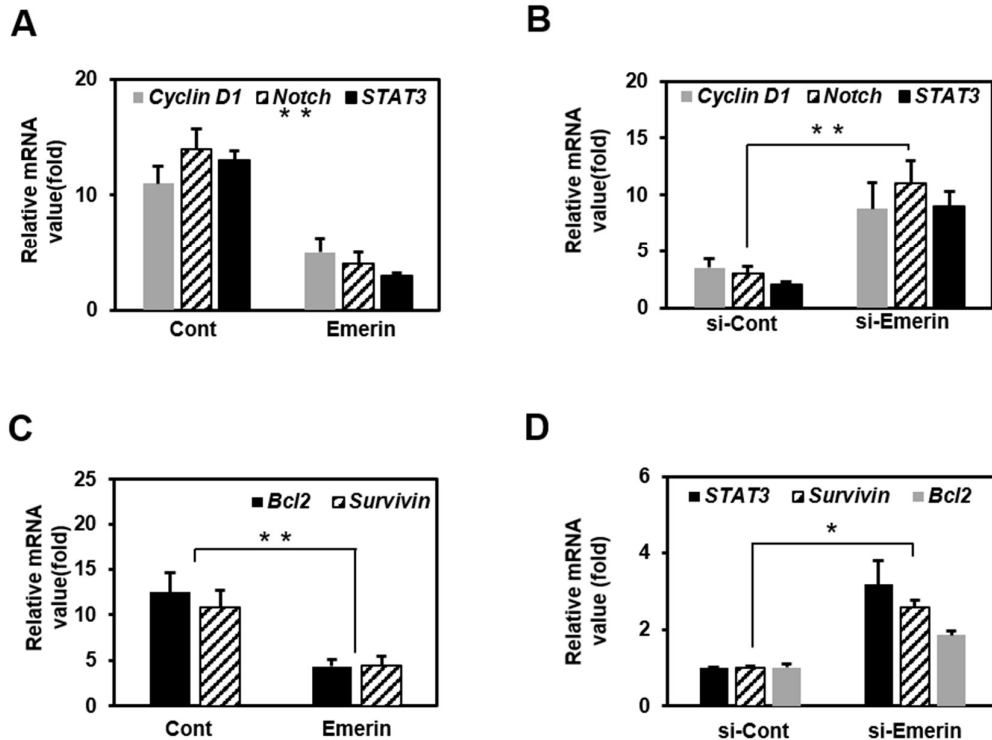
In the study, we examined the role of emerin as a transcriptional inhibitor of STAT3 target genes, like cell survival-related genes including *Bcl2* and *Survivin* in HeLa cells. We show that emerin can modulate STAT3 signaling by inducing the retention of the STAT3 at the inner nuclear membrane. Cytotoxicity studies using emerin knockdown HeLa cells confirmed the STAT3 signaling induced STAT3 target genes *Bcl2* and *Survivin* to increase cell survival signals and suppress hydrogen peroxide-induced cell death. Using mouse myoblast C2C12 cells, we show that emerin can modulate myoblast proliferation by inhibiting STAT3 signaling. These findings imply that normal STAT3 activity under emerin regulation is required for proper muscle maintenance. However, intermittent STAT3 inhibition may have promising implications for increasing muscle regeneration in emerin-related muscular dystrophy. Furthermore, all of our results suggest misregulation of the STAT3 signaling pathway, which is essential for skeletal muscle development, may lead to LEM-D protein-associated human diseases such as EMDM.
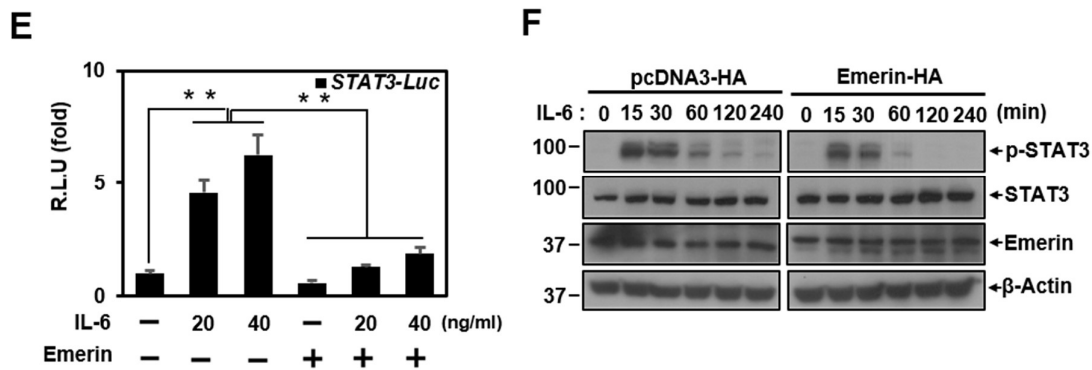
2. Results

2.1. Emerin represses STAT3 transcriptional activity

When the STAT3 signal, known to be essential for cancer cell proliferation, is activated, the cytoplasmic STAT3 protein moves to the nucleus through the nuclear inner membrane where emerin is present. In studies related to the current work, Notch and Wnt signals are known to be regulated by emerin [11,15]. Here we investigated the involvement of emerin in STAT3 signaling in the same manner as a previously reported Notch signal-related transcription factor profiling PCR array that measures the expression levels of human transcription factor genes using qRT-PCR [15]. Reduction of STAT3 expression by overexpressed Emerin was confirmed in the same pattern as decreasing Notch expression (Fig. 1A). In addition, our results showed that emerin suppresses the expression of cyclinD1, β-catenin target gene in Wnt signaling (Fig. 1A). Conversely, the knockdown of emerin by si-RNA led to upregulation of *STAT3*, *Notch*, and *cyclinD1* (Fig. 1B). These results are consistent with previous studies [11, 15] and indicate that our experimental method for testing the effect of emerin on STAT3 is appropriate. This experiment also showed that emerin is similar in regulating STAT3 signaling as a mechanism for controlling Notch signaling through spatial control of the nuclear membrane [15]. Furthermore, we examined whether emerin affects expression of *Bcl2* and *Survivin*, survival-related genes in STAT3 downstream. Emerin inhibits mRNA expression of *Bcl2* and *Survivin* (Fig. 1C). As Figure 1B, decreased emerin is increased *Bcl2* and *Survivin* of STAT3 target gene (Fig. 1D).

To determine if STAT3 signaling could be a direct target of emerin, we built a STAT3-luciferase assay system on Hela cell double-infected STAT3-Luciferase particles and Renilla particles. Treatment with IL-6 as a STAT3 activation signal in HeLa-STAT3-Luc/Ren cells increases STAT3 transcriptional activity as indicated by an increased amount of IL-6 (Fig. 1E). In a STAT3 luciferase reporter experiment, it was confirmed that IL-6 activates STAT3 transcriptional activity and that activated STAT3 is downregulated by emerin (Fig. 1E). Interestingly, the decrease in the transcriptional activity of STAT3 in the nucleus, which is dependent on emerin, resulted in a significant decrease in phosphorylated STAT3, an activated form completed in the cytoplasm. (Fig. 1F). This means that emerin may negatively affect the expression or activation process of Jak or MAPK, which are involved in STAT3 activation. A study related to our results reported the general activation of the MAPK pathway in EMD null mice, an EMDM model [59].

E

F

Figure 1. Emerin regulates the expression of STAT3 genes. (A, C) HeLa cells were transfected with vectors encoding *Emerin-HA*(1μg) in 6-well plates for 24 hours in 6 well plates. Total RNA was isolated and subjected to qRT-PCR analysis. Data were normalized to GAPDH expression. The results represent the mean ±S.D. of three independent experiments performed in triplicate. *, $P < 0.05$, **, $P < 0.01$. (B, D) HeLa cells were treated with siRNA (100 nM) against *Emerin* or *Control* (si-Cont) at 6well plates for 48 hours in 6 well plates. Total RNA was isolated and subjected to qRT-PCR analysis. Data were normalized to GAPDH expression. The results represent the mean ±S.D. of three independent experiments performed in triplicate. *, $P < 0.05$, **, $P < 0.01$. (E) HeLa cells were dual-infected with STAT3 lentiviral luciferase and Renilla lentiviral luciferase. Cells were selected by puromycin. Increasing amounts of IL-6 (0-40ng/ml) were treated HeLa cells for 6 hours before were transfected with vectors encoding *Emerin-HA* (1 μg) for 24 hours in 6 well plates. Cells were lysed subject to a luciferase assay. The luciferase reporter activity in each sample was normalized to a Renilla protein activity. *R.L.U.,* relative luciferase units. The results represent the mean ±S.D. of three independent experiments performed in triplicate. *, $P < 0.05$, **, $P < 0.01$. (F) Confluent Hela cells were treated with IL-6 (40ng/ml) for indicated times in 6 well plates. Treated cells were lysed subjected to western blotting with antibody against STAT3, p-STAT3, Emerin and β-actin.
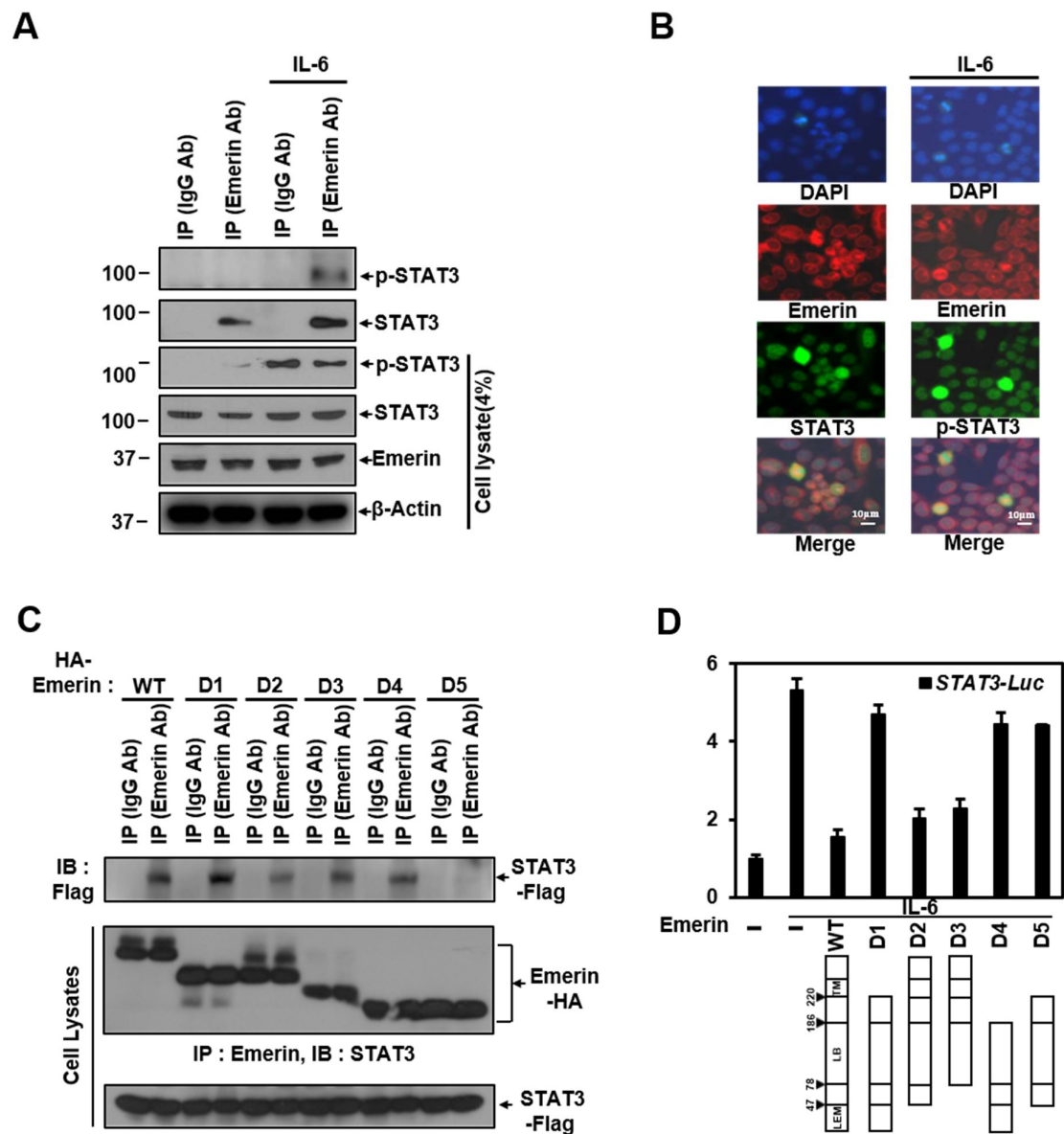
2.2. Emerin interacts with STAT3 proteins

Emerin is known to form a network in the nuclear membrane with several proteins including lamin A/C and BAF. Emerin regulates gene expression by interacting with several proteins while maintaining the integrity of the nuclear membrane [9,60]. The regulation of emerin on STAT3 transcriptional activity may have two possibilities: through direct interaction and through other proteins in the network. We performed a Co-immunoprecipitation (Co-IP) experiment to determine whether there is a direct interaction by emerin. Co-IP experiments showed that emerin and STAT3 can bind directly at the endogenous level. It can be seen that the interaction between the two proteins is through direct binding regardless of whether STAT3 is phosphorylated or not (Fig. 2A). Emerin, which is present in the nuclear membrane, is expected to bind to activated STAT3 migrating to the nucleus and regulate the pSTAT3 transcriptional activity. Immunocytochemistry (ICC) experiments showed that activated STAT3 migrates to the nucleus and some of it is located in the nuclear membrane with emerin. The orange color in the merged figure showed that emerin (red) and STAT3 (green) can interact in the same location (Fig 2B).

We also investigated which part of emerin is essential in binding to STAT3 using overexpressed the deletion mutation emerin in Co-IP experiments. Results showed that most of the mutations could bind differently in the degree to STAT3 and mutant D5 had the weakest binding. As a result of these, Trans-Membrane (TM), the parts required for Emerin's anchoring, and the LEM domain are not essential for binding. Instead, it has been shown that the lamin binding (LB) domain is involved in binding, since all deletion mutants have LB domain in common.

As shown the STAT3-luciferase assay in Fig. 1E, IL-6-induced STAT3 transcriptional activity was inhibited by emerin. Therefore, we tried to determine which emerin deletion mutations affect STAT3 transcriptional inhibition through STAT3 luciferase assay. As a result, it was shown that the Emerin

deletion mutants D2 and D3, containing TM domain and LB domain, had a significant inhibitory effect on STAT3 transcriptional activity comparing wild-type emerin (Fig. 2D). Emerin mutants D1, D4 capable of binding to STAT3 do not show inhibition of transcriptional activity means that STAT3 transcriptional activity is inhibited through emerin-STAT3 binding only when emerin is anchored to the nuclear membrane (Fig. 2C and D). These data suggest that the localization of emerin proteins to the inner nuclear membrane is important for the suppression of STAT3 signaling.
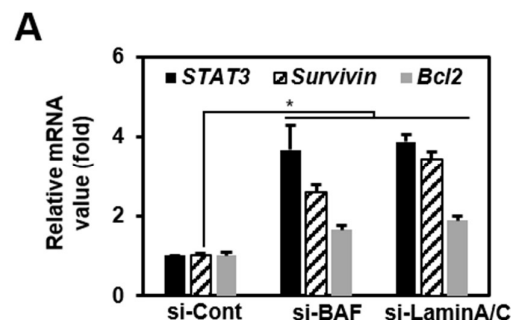

Figure 2. Emerin interact with the STAT3. (A) Confluent HeLa cells in 100mm plates with or without IL-6(40ng/ml) were lysed and immunoprecipitated with antibodies against Emerin. The precipitates were subjected to western blotting with antibody against STAT3 or p-STAT3. (B) Immunocytochemistry image stained with anti-STAT3, p-STAT3 and Emerin antibody from confluent HeLa cells in coverslip on 30mm plates with or without IL-6(40ng/ml). DAPI (blue) was used to visualize nucleus. Scale bar =10μm. (C) HeLa cells were transiently co-transfected with vectors encoding *HA-Emerin deletion mutants* (6μg) and *Flag-STAT3* (6μg) in 100mm plates for 24 hours. Cells were lysed and immunoprecipitated with antibody against HA for overnight at 4°C, and the precipitates were subjected
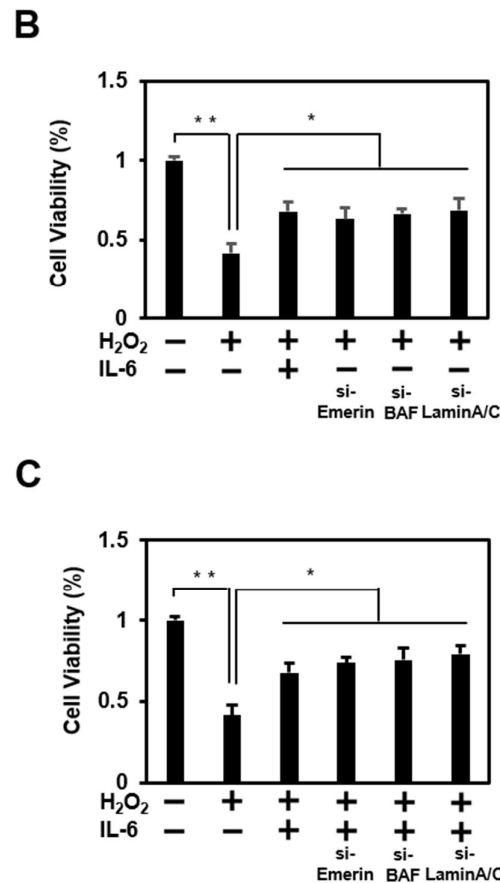
to western blotting with antibody against Flag. (D) Dual infected Hela cells were transiently transfected with vectors encoding *Emerin deletion mutnats* (1µg) for 24 hours and treated for 6 hours with IL-6 (40ng/ml) in 6 well plates. Cells were lysed subjected to a luciferase assay. The luciferase reporter activity in each sample was normalized to Renilla protein activity. *R.L.U.,* relative luciferase units. The results represent the mean ±S.D. of three independent experiments performed in triplicate. *, $P < 0.05$, **, $P < 0.01$.

2.3. Emerin- network affects cell survival through modulating STAT3 signal

Emerin localizes predominantly to the inner nuclear membrane by binding to A-type lamins (nuclear intermediate filament proteins) and a chromatin protein, BAF [61]. BAF is essential for the localization of emerin to the nuclear envelope because in the absence of BAF, emerin is sequestered in the cytosol [60, 62]. Thus, loss of BAF and lamin A/C may increase STAT3 signaling by inducing mislocalization of emerin to the cytosol. Therefore, we studied changes in the transcriptional activity of STAT3 when the emerin-network was disrupted by si-RNA, including si-Lamin A/C, and si-BAF. An increase in STAT3 expression confirmed the effect of the disrupted emerin-network through the same qRT-PCR experiment as in Figure 1D, in which si-emerin was introduced (Figure 3A). In addition, like the emerin knockdown experiment, *Bcl2* and *Survivin* expression increased in Hela cells using si-BAF and si-Lamin A/C. These results showed that when the expression of the proteins constituting the emerin-network is impaired, the entire emerin-network is also defective, thereby reducing the emerin inhibitory effect on STAT3 transcriptional activity and increasing the expression of STAT3, which leads to an increase in the expression of *Bcl2* and *Survivin*. To investigate the relationship between the expression levels of *Bcl2* and *Survivin,* the survival-related genes downstream of STAT3, and the cell survival, downregulation of emerin on the emerin-network, we treated HeLa cells with H_2O_2 to induce apoptosis and reduced emerin-localization in the nuclear inner membrane using si-emerin, si-BAF, and si-Lamin A/C. As shown in Figures 3B and C, H_2O_2 induced apoptosis in HeLa cells, and IL-6 treatment significantly suppressed H_2O_2-induced cell death. This effect seems to be from STAT3 signal activation. Furthermore, the emerin-network downregulation using si-RNA increased cell viability even without IL-6 treatment. These results suggest that emerin plays a role in survival through, at least in part, the modulation of STAT3 signaling in HeLa cells and represent using a simple schematic diagram (Fig. 5A). It shows that emerin regulates JAK-STAT signaling. STAT3, present in the cytoplasm, is activated by IL-6 to form the JAK-STAT3 complex. Activated pSTAT3 enters the nucleus and acts as a transcription activator on the target gene. Thus, the formed inner nuclear emerin-network with lamin A/C, BAF, and emerin regulates transcription factors like STAT3 that enter the inner nuclear membrane.
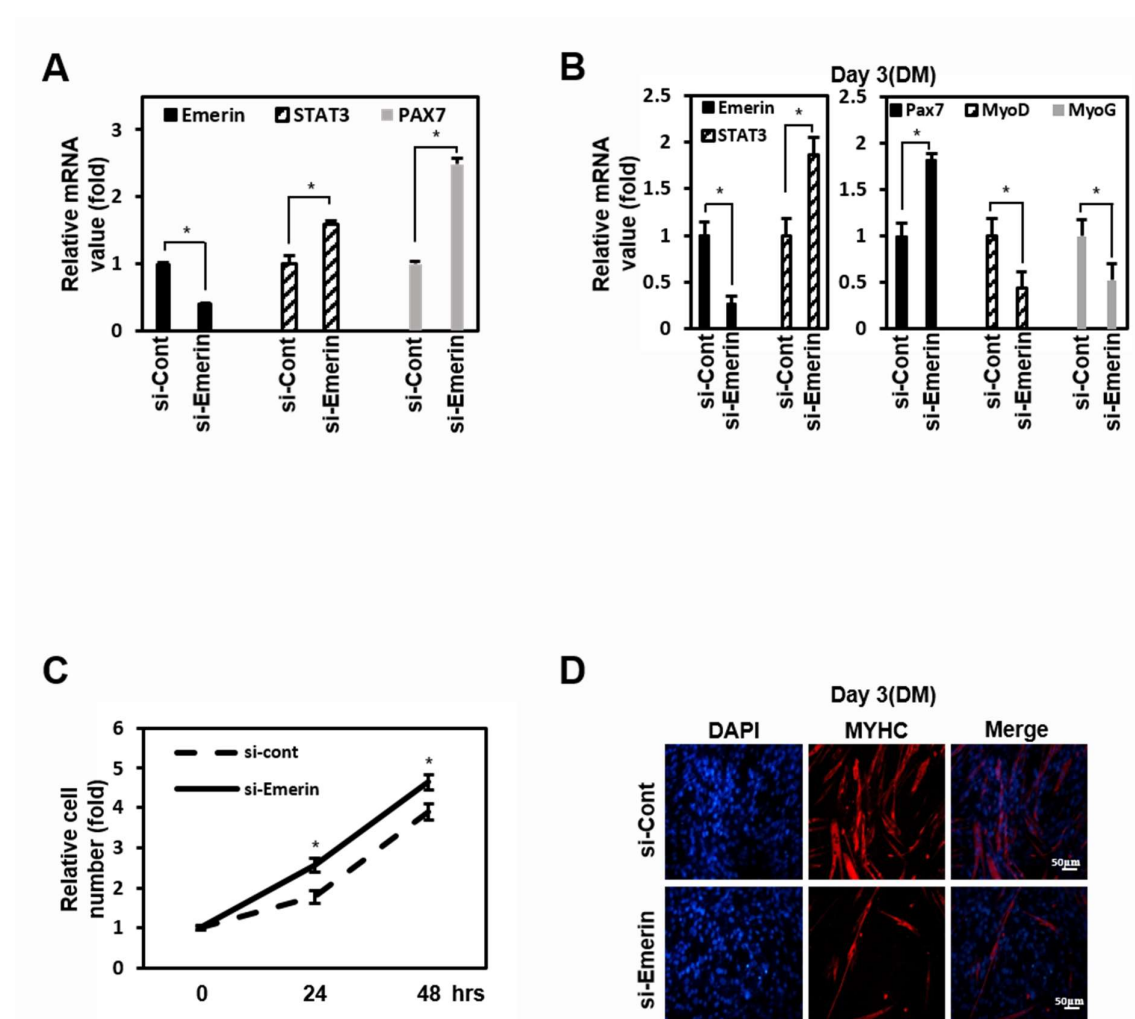
Figure 3. Endogenously expressing emerin modulates STAT3 signaling and survival in HeLa cells. (A) HeLa cells were treated with siRNA (100 nM) against *BAF, Lamin A/C* or *Control* at 6well plates for 48 hours in 6 well plates. Total RNA was isolated and subjected to qRT-PCR analysis. Data were normalized to GAPDH expression. The results represent the mean ±S.D. of three independent experiments performed in triplicate. *, $P < 0.05$, **, $P < 0.01$. (B, C) HeLa cells were treated with siRNA (100 nM) against *Emerin, BAF* or *lamin A/C* at 6well plates for 36 hours. HeLa cells were treated with or without IL-6 (40ng/ml) for 4 hours in 6 well plates. Then, HeLa cells were treated H_2O_2 (500μM) for 18 hours. Cell viability was measured by CCK-8 assay at 24 well plates. The results represent the mean ±S.D. of three independent experiments performed in triplicate. *, $P < 0.05$, **, $P < 0.01$.

2.4. Emerin regulates muscle cell proliferation through STAT3 signaling

Emerin and STAT3 are well known to play a critical role in the cause of muscle disease [17,58]. Therefore, we experimented with C2C12 myoblast cells to investigate the effect of the regulation of STAT3 by emerin on muscle cells. In C2C12 cells, knockdown emerin increased STAT3 mRNA, similar to results of HeLa cells, and PAX7 of myoblast proliferation gene was also increased (Fig 4A). In addition, the growth of C2C12 cells was promoted by STAT3, which was increased by treatment with si-RNA targeting emerin (Fig. 4C). Therefore, we experimented with C2C12 myoblast cells to investigate the effect of the regulation of STAT3 by emerin on muscle cells. In C2C12 cells, knockdown emerin increased STAT3 mRNA, similar to results of HeLa cells, and PAX7 of myoblast proliferation gene was also increased (Fig. 4A). In addition, the growth of C2C12 cells was promoted by STAT3, which was increased by treatment with si-RNA targeting emerin (Fig. 4C). As with previous reports [56, 65], increasing STAT3 inhibited muscle cell differentiation and promoted the proliferation of muscle cells by emerin knockdown. Therefore, we investigated the physiological changes in C2C12 cells due to the regulation of STAT3 by emerin in the process of C2C12 cell differentiation. After reduction of
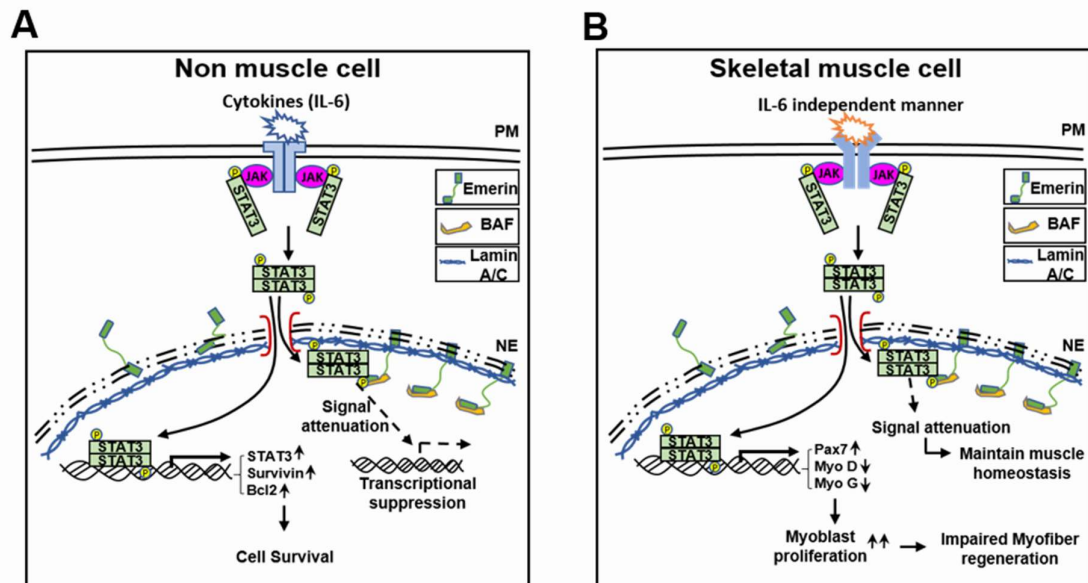
emerin using si-RNA in C2C12 cells, PAX7, MyoD, and Myogenin (MyoG) expression levels were checked for three days in differentiation medium (DM). Emerin knockdown in DM-treated C2C12 cells increased STAT3 mRNA and increased PAX7 of myoblast proliferation gene, similar to the results of the standard medium in Figure 4A. Conversely, MyoD and MyoG, the myocyte differentiation genes, decreased (Fig. 4B). Furthermore, as can be seen from the results in Figure 4B, Myosin Heavy Chain (MYHC) decreased with increasing STAT3 by si-emerin at day 3 of DM in the ICC of MYHC (Fig. 4D). However, on the 5th day of DM, as the knockdown effect by si-emerin was reduced, there was no significant increase in PAX7 or decrease in MyoD and MyoG, and there was not much difference in the level of MYHC expression in ICC results (Suppl. Fig. 1). These results suggest that emerin plays a role in muscle cell development by engaging in muscle cell proliferation through modulation of STAT3 signaling. Regarding these proposals, we expressed these with a simple schematic (Fig. 5B).

Figure 4. Emerin regulates muscle cell proliferation through STAT3 signaling. (A) C2C12 cells were treated with si-RNA (100 nM) against *Emerin* or *control* for 48 hours in 6 well plates. The total RNA was isolated and subjected to a qRT-PCR analysis. Data were normalized to *β-Actin*. The results represent the mean ±S.D. of three independent experiments performed in triplicate. *, P < 0.05, ** P < 0.001. (B) C2C12 cells were treated with si-RNA (100 nM) against *Emerin,* or *control* for 48 hours in 6 well plates. After 48 hours, cells were treated with differentiation media (DM) for 3 days. The total RNA was isolated and subjected to a qRT-PCR analysis. Data were normalized to *β-Actin*. The results represent the mean ±S.D. of three independent experiments performed in triplicate. *, P < 0.05, ** P < 0.001. (C) C2C12 cells were treated with si-RNA (100 nM) against *Emerin* or *control* for 24 or 48 hours

in 6 well plates. Cells were fixed each time and relative cell number counted ecch time points. The results represent the mean ±S.D. of three independent experiments performed in triplicate. *, P < 0.05, ** P < 0.001. (D) C2C12 cells were treated with si-RNA (100 nM) against *Emerin,* or *control* for 48 hours in 6 well plates. After 48 hours, cells were treated with differentiation media (DM) for 3 days. Immunocytochemistry image stained with anti- MYHC antibody from emerin-depleted C2C12 cells at day 3 of differentiation. DAPI (blue) was used to visualize nucleus. Scale bar=50 μm.

Figure 5. A schematic diagram describing the role of Emerin in the STAT3 signaling pathway. (A, B) Emerin regulates STAT3 transcriptional activity on non-muscle cells or skeletal muscle cells.

3. Discussion

Skeletal muscle development is a complex regulatory process that fine-tunes the balance of catabolic and anabolic processes regulated by muscle genes and transcription factors. Depending on the stringent temporal sequence expression, these transcription factors precisely control the proliferation, differentiation, and fusion of muscle cells [63-65]. However, muscular dystrophy impairs the microscopic homeostasis, which slows down muscle generation and repair leading to an increased catabolic process resulting in loss of skeletal muscle mass by increasing proteolysis through the ubiquitin-proteasome autophagy-lysosome system [58]. Thus, representative muscular dystrophy, X-EMDM, is presumed to cause problems in several biological phenomena due to the lack of emerin.

In the present study, we executed transcription profiling analysis to explore the role of emerin in gene regulation. We found that emerin plays a role in the transcriptional suppression of many genes, including those of the Notch, Wnt, and STAT signaling pathways [Fig. 1A, B and Suppl. table. 1]. These results are consistent with previously reported results because emerin can negatively regulate gene expression through the recruitment of transcription inhibitors. For example, the direct interaction of emerin and β-catenin triggers the downregulation of Wnt signaling [11]. Conversely, in the absence of emerin, the level of nuclear β-catenin increases, leading to upregulation of the target gene [11]. In addition, Emerin induces nuclear envelope localization of LMO7, a transcriptional activator for muscle differentiation, and inhibits transcriptional function [13]. Our transcription profiling analysis showed significant relevance to previous studies related to myogenesis, such as the temporal balance between Notch and Wnt signaling orchestrates the precise progression of muscle precursor cells along the myogenic lineage pathway [66].

Transcription factor Profiling analysis also shows that the level of expression of STAT families by emerin is generally low (Suppl. table. 1). We were able to confirm once again that it inhibits STAT3 transcriptional activity in HeLa cells (Fig. 1C and E). STAT3 signaling has been reported to play a

critical role in satellite cell myogenic capacity and self-renewal [67, 68]. In addition, activated STAT3 promotes skeletal muscle atrophy in muscle diseases [55] while acting as an oncogene in non-muscular cells [69, 70]. An exciting aspect of the results of transcription profiling analysis is that in addition to significantly reducing the expression of stat3, overexpression of emerin also decreased the expression of MyoD and MYF5, which are involved in skeletal muscle development. This result was from HeLa cells in a typical medium that induces cell proliferation (Suppl. table. 1). However, even when STAT3 expression was increased in emerin knockdown C2C12 myoblast using DM medium that promotes differentiation, the expression of MyoD and MyoG decreased (Fig. 4B). This phenomenon suggests that even muscle differentiation is related to STAT3 released from emerin, causing upregulates Pax7 promoting cell proliferation (Fig. 5B). All of this reinforces the suggestion that LEM-D protein-associated human diseases like EMDM are associated with the misregulation of signaling pathways essential for skeletal muscle cell proliferation and differentiation.

Mutations in the genes encoding emerin (*EMD*) and lamin A/C (*LMNA*) can cause different forms of EDMD. The functional link between the two proteins and the fact that they are both implicated in very similar diseases suggested that they work through a convergent pathway. Previous reports have shown that the EDMD model, mouse embryonic fibroblasts (MEF) from *Lmna*-/- mice, has a fast-growing phenotype similar to emerin null fibroblasts [71]. We have shown that loss of lamin A/C and BAF leads to erroneous localization and loss of function of emerin to the cytoplasm, so upregulated STAT3 signaling may also contribute to cell survival in HeLa cells. Based on these results, we suggest that the cause of each EDMD in different gene mutations is due to the same expansion that forms a proliferating myoblasts pool by upregulated STAT3 signaling. In this regard, we have shown that emerin can down-regulate myoblast proliferation by inhibiting STAT3 signaling in C2C12 cells (Fig. 4). As shown in the emerin-STAT3 model in Figure 5, attenuated STAT3 activity by interacting emerin in nuclear inner membrane is sufficient to fulfill its essential functions in gene expression regulation during myoblast differentiation, thus supporting the importance of emerin in myogenesis.

In conclusion, our results strongly suggest that defects in muscle cell proliferation regulation due to incorrect regulation of STAT3 signaling may be one cause of EDMD pathology.

4. Materials and methods
4.1. Antibodies and Chemicals

For immunoblotting and immunocytochemistry, primary antibodies for emerin (#sc-15378, 1:4500), Lamin A/C (#sc-20681, 1:4500), BAF (#sc-166324, 1:5000) and GAPDH (#sc-47724, 1:5500) were purchased from Santa Cruz (Santa Cruz Biotechnology, CA). Primary antibodies specific for pSTAT (#ab76315, 1:3000) was purchased from Abcam (Cambridge, UK). Primary antibodies specific for HA (1:5000), Flag (1:5000), and β-actin (1:5000) were purchased from MBL (USA, Woburn, MA. Primary antibodies specific for STAT3 (1:3000) were purchased from Cell signaling (USA, Danvers, MA). Primary antibodies for Myosin Heavy Chain (MYHC, #MAB4470, 1:50) was purchased from R&D systems (Bio-techne. MN). For viability assay, cells were treated H_2O_2 for cell death, and Thiazoyl blue tetrazolium bromide (MTT) was purchased from Bio Basic Canada.

4.2. Cell culture and transfection

HeLa Cells (American type culture collection; ATCC) were cultured in DMEM (Biowest, L0103-500) supplemented with 10% FBS (Welgene, South Korea) and 1% Antibiotic Antimycotic Solution, 100X (Corning, USA, Manassas, VA). Transfection was executed using the Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY) according to the manufacturer`s instruction. The transfected cells were cultured for 24-48 hours, washed with DPBS, and harvested with lysis buffer (Invitrogen, Grand Island, NY). C2C12 cells were a gift from Yong-Gyu Ko at Korea University. C2C12 cells were cultured in DMEM supplemented with 10% FBS and 1% Antibiotic Antimycotic Solution, 100X. For induction of differentiation, fully confluent C2C12 cells were cultured in DMEM supplemented with 2% horse serum (Sigma-Aldrich, MO) for 3 days.

4.3. Plasmid constructs

The human emerin cDNA was (provided by 21C Frontier Human gene bank) amplified by RT-PCR and inserted into the restriction enzyme sites of pcDNA3-HA for biochemical studies. For construction of emerin deletion mutants, the corresponding regions were amplified by RT-PCR and inserted into the restriction enzyme sites of pcDNA3-HA and pcDNA3-FLAG.

4.4. Transcription factor profiling assay

Total RNA was isolated using Trizol reagent (Life Technologies), and 1 µg of total RNA was used for cDNA synthesis. The human transcription factor profiling PCR array was performed according to the manufacturer's protocol (#PAHS-075ZC-2; Qiagen, Valencia, CA). Data were obtained using the manufacturer's software.

4.5. Luciferase reporter assay

Hela cells were dual-infected with STAT3 lentiviral luciferase and Renilla lentiviral luciferase (#CCS-9028L, Qiagen, Hilden, Germany), selected by puromycin (stable cell line-Hela STAT3 Luc/Ren). Cells were grown to 70% - 80% cell confluence in 6well plates and transiently transfected with encoding emerin and incubated 24 hours at 37°C CO_2 incubator. Then, cells were treated IL-6 6 hours, lysed in 5 × Passive Lysis Buffer (Promega), and analyzed for dual luciferase activity with a GloMax® 96well microplate luminometer system (Promega). The luciferase reporter activity in each sample was normalized to Renilla protein activity (Promega).

4.6. Immunoblotting

For western blot analysis, all proteins were separated by SDS-PAGE (Polyacrylamide gel electrophoresis) and transferred on PVDF (Polyvinylidene difluoride) membranes (Millipore, USA, Billerica, MA). Membranes were blocked for 1 hour at R.T with a solution of 4% non-fat milk powder or 4% BSA in TBS contained 0.05% Tween-20 (TBST). The membranes were incubated with 1st antibody in blocking solution O.N at 4 °C. The membranes were washed 3 times with TBST and incubated with 2nd antibody for 2 hours at R.T. After washing 3 times with TBST, the membranes were developed using the ECL (Electrochemiluminescence) detection system (Bio-Rad, USA, Hercules, CA).

4.7. Immunocytochemistry (ICC)

HeLa cells were plated on glass cover slips and then transfected with 1 µg of vector. After incubation for 24-48 hours, then cells were fixed with 4% PFA (paraformaldehyde), permeabilized with 0.1% Triton X-100 in PBS and then incubated with blocking Sol. After incubation O.N with 1st HA antibody (1:200) in blocking solution, cells were washed and incubated with TRITC or FITC - conjugated 2nd antibodies (1:200) for 1 hours at R.T. After staining with DAPI (Life technology, Thermo Fisher Scientific Inc), cells were observed under a microscope.

4.8. Quantitative RT-PCR (qRT-PCR)

Total RNA was separated using the RiboEx (Gene all, South Korea), and 1 µg of total RNA was used for cDNA synthesis. cDNA was amplified using primer pairs for human *STAT3* (forward 5′-TTGACAAAGACTCTGGGGAC-3′ and reverse 5′-CAGGGAAGCATCACAATTGG-3′), human *Notch1* (forward 5′-TACGTGTGCACCTGCCGGG-3′, reverse 5′-CGTTTCTGCAGGGGCTGGGG - 3′), human *Survivin* (forward 5′-CCTTCACATCTGTCACGTTC-3′ and reverse 5′-GAAGCTGTA ACAATCCACCC-3′), human *Bcl2* (forward 5′-GTGGCCTCTAAGATGAAGGA-3′ and reverse 5′-TGCGGATGATCTGTTTGTTC-3′), human *Cyclin D1* (forward 5′-GGATTGTGGCCTTCTTTGAG GA-3′ and reverse 5′-AGGTACTCAGTCATCCACAG-3′), human *GAPDH* (forward 5′- AAAAGCA GCCCTGGTGACC-3′ and reverse 5′-GTCGGAGTCAACGGATTTGG-3′), mouse *emerin* (forward 5'-CCACCAAGACATACGGGGAG-3' and reverse 5'-TCTTGGCCATAGATGAGGCG-3'), mouse *PAX7* (forward 5'-CGACTCTGGATTCGTCTCC-3' and reverse 5'-GGCCTTGGCCAAGAGGG-3'), mouse *MyoD* (forward 5'-GGCTACGACACCGCCTACTAC-3' and reverse 5'-GGTCTGGGTTCCC TGTTCTG-3'), mouse *Myogenin* (*MyoG*) (forward 5'-CCTGGAAGAAAAGGGACTGG-3' and reverse 5'-CGCTCAATGTACTGGATGGC-3'), mouse *STAT3* (forward 5'-AGGAGTCTAACAAC GGCAGCCT-4' and reverse 5'-GTGGTACACCTCAGTCTCGAAG-3') and mouse *β-Actin* (forward 5'-GGCTGTATTCCCCTCCATCG-3' and reverse 5'-CCAGTTGGTAACAATGCCATGT-4'). qRT-

PCR was performed using the StepOne Real-Time PCR System. Reactions were amplified using the primers described above and a HiPi Real-Time PCR 2x Master MIX-SYBR green (ELPis, South Korea) according to the manufacturer's instruction.

4.9. Small interfering RNA

Negative siRNA was non-targeting siRNA for human, rat and mouse. The human *emerin* (#1047199), mouse *emerin* (#13726-1), human *BAF* (#1011344), human *Lamin A/C* (#1033333), and negative control (#SN-1002) siRNA oligo were purchased from Bioneer (South Korea). Transfection was performed with Lipofectamine 2000 in HeLa cells and C2C12 cells according to the manufacturer's protocol. The sequences for si-RNA targeting were as follows; human *emerin* (sense sequence 5′-AGUCGAAUUCAAGUCAGAG-3′, and antisense sequence, 5′-CUCUGACUUGAAUUCACU-3′), mouse *emerin* (sense sequence 5'-GAGCAAGGACUAUAAUGA-3', and antisense sequence 5'-AUCAUUAUAGUCCUUGCUC-3') human *BAF* (sense sequence 5′ACUUCUGCCAGUUAGAGC-3′, and antisense sequence, 5′-GCUCUAACUGGCUAGAAGU-3′) and human *Lamin A/C* (sense sequence, 5′-CUCUGACUUGAAUUC CU-3′ and antisense sequence, 5′-AGUCGAAUUCAAGUCA GAG-3′).

4.10. Statistical analysis

All results data were presented as the mean ± S.D. Statistical significance was determined with the Student T-test with a significance level of P 0.05. The data for transcription qRT-PCR array, Dual-Luciferase assay and viability assay were presented as the mean of three independent experiments.

Institutional Review Board Statement: The study was conducted according to the guidelines of the

Declaration of Helsinki, and approved by the Local Ethics Committee, Warsaw, Poland (protocol

code 37/2012, date of approval 26.06.2012 and protocol code 1/2016, date of approval 26.01.2016).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest

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