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*Supplementary material*

# Extracellular Vesicles Derived from Acidified Metastatic Melanoma Cells Stimulate Growth, Migration, and Stemness of Normal Keratinocytes

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**Citation:** Bychkov, M.L.; Kirichenko, A.V.; Mikhaylova, I.N.; Paramonov, A.S.; Yastremsky, E.V.; Kirpichnikov, M.P.; Shulepko, M.A.; Lyukmanova, E.N. Extracellular Vesicles Derived from Acidified Metastatic Melanoma Cells Stimulate Growth, Migration, and Stemness of Normal Keratinocytes. *Biomedicines* **2022**, *10*, 660. https://doi.org/10.3390/biomedicines10030660

Academic Editor: Paul Rösch

Received: 2 December 2021
Accepted: 9 March 2022
Published: 12 March 2022

**Table S1.** Primers, used for qPCR experiments.

| Gene | Primer | | Amplicon size, bp |
|---|---|---|---|
| | Forward | Reverse | |
| *S18 SSU RNA* | CTCAACACGGGAAAC-CTCAC | CGCTCCACCAACTAA-GAACG | 110 |
| *RPL13a* | *TCAAAGCCTTCGCTAG-TCTCC* | GGCTCTTTTTGCCCG-TATGC | 104 |
| *EGFR* | GAAATCATACGCGGCAG-GAC | TGAGGGAGCG-TAATCCCAAG | 91 |
| *PDGFRA* | GACATCATGCGGGACTCG AA | CATGGGCAGCTCTGGG-TAAG | 180 |
| *TNFA* | AGGCACTCCCCCAAAA-GATG | GCTCCTCCACTT-GGTGGTTT | 219 |
| *BDNF* | GGCGAGCAGAG-TCCATTCAG | TCGTCAGAC-CTCTCGAACCT | 614 |
| *VEGFA-* | AGGAGGAGGG-CAGAATCATCA | GGCACACAGGATGGCTTG AA | 144 |
| *ITGA2* | GGTCATCAGGGCAC-TATCCG | GGCTCCTAAAGGCTCCAT CG | 70 |
| *ITGA3* | GGGC-TACCCTATTCCTCCGA | CAGCTCCGAG-TCAATGTCCA | 285 |
| *ITGAV* | TCCCATCAGTGGTTT-GGAGC | AGCTGAC-GTGATCTACATGG | 154 |
| *HSP60* | GGACGCTGACGCGAAGA | TAAGCCCGAGTGAGATGA GGA | 153 |
| *KLF4* | TGCGGCAAAAC-CTACACAAAG | GTTCATCTGAGCGGGCGA AT | 121 |

**TableS2.** Primers used for analysis of miRNA expression

| miRNA | Stem-loop primer | Forward primer | Reverse primer |
|---|---|---|---|
| *U6 snRNA* | CGCTTCACGAATTT-GCGTGTCA | GCTTCGG-CAGCACATA-TACTAAAAT | CGCTTCAC-GAATTT-GCGTGTCAT |
| *miR-7* | CAC-CGTTCCCCGCCGTCGG TGACAACA | CGCCCTG-GAAGACTAG-TGAT | CCGTCGGTGA CAACAAAAT |
| *miR-21* | GTCGTATCCAGTG-CAGGGTCCGAGGTAT TCGCACTGGATAC-GACTCAACA | GCCCGCTAGC TTATCAGACT GATG | CAGTG-CAGGGTCCGA GGT |
| *miR-31* | GTCGTATCCAGTG-CAGGGTCCGAGGTAT TCGCACTGGATAC-GACAGCTAT | GCCG-CAGGCAA-GATGCTGGC | |
| *miR-96* | GTCGTATCCAGTG-CAGGGTCCGAGGTAT TCGCACTGGATAC-GACAGCAAA | GCCCGCTTT-GGCACTAG-CACATT | |

| | | | |
|---|---|---|---|
| *miR-135b* | GTCGTATCCAGTG-CAGGGTCCGAGGTAT TCGCACTGGATAC-GACTCACAT | GCCCGC-TATGGCTTTC ATTCCT | |
| *miR-203* | GTCGTATCCAGTG-CAGGGTCCGAGGTAT TCGCACTGGATAC-GACCTAGTGGTC | GTATCCAG-TG-CAGGGTCCG A | CGAC-GGTGAAATGT TTAG |
| *miR-221* | CAC-CGTTCCCCGCCGTCGG TGGAAACC | CGGGCAGC-TACATT-GTCTG | CGTCGGTG-GAAAC-CAGCA |
| *miR-451* | CAC-GGAACCCCGCCGACC GTGAACTCA | CGCCGAAAC-CGTTACCAT | GCCGAC-CGTGAACTCA GTAAT |

**Table S3**. The siRNA sequences

| **Gene** | **siRNA sequence** | |
|---|---|---|
| | **sense** | **antisense** |
| *EGFR* | UGAUCUGUCACCACAUAAU UACGG | CCCGUAAUUAUGUGGU GACAGAUCA |
| | UUAGAUAAGACUGCUAAGG CAUAGG | CCUAUGCCUUAGCAGUC UUAUCUAA |
| | UUUAAAUUCACCAAUACCU AUUCCG | CGGAAUAGGUAUUGGU GAAUUUAAA |
| Scramble | UUCUCCGAACGUGUCACGU | ACGUGACACGUUCGGAG AA |

**Table S4.** Analysis of mRNA contained in EVs derived from the mel H cells cultivated at pH 6.5 and pH 7.4. Expression is shown as normalized mRNA level ± SEM ($n$ = 8).

| **Gene** | **EVs (pH 7.4)** | **EVs (pH 6.5)** |
|---|---|---|
| ***EGFR*** | **-** | **-** |
| ***PDGFRA*** | **-** | **-** |
| ***TNFA*** | **-** | **-** |
| ***BDNF*** | **-** | - |
| ***VEGFA*** | **-** | $1.14 \times 10^{-6} \pm 5.2 \times 10^{-7}$ |
| ***ITGA2*** | **-** | - |
| ***ITGA3*** | **-** | $2.92 \times 10^{-5} \pm 4.3 \times 10^{-6}$ |
| ***ITGV*** | **-** | $2.42 \times 10^{-6} \pm 1.3 \times 10^{-7}$ |
| ***HSP60*** | **-** | $1.99 \times 10^{-8} \pm 1.8 \times 10^{-9}$ |

**Table S5.** Analysis of mRNA contained in EVs derived from the mel Kor cells cultivated at pH 6.5 and pH 7.4. Expression is shown as normalized mRNA level ± SEM ($n$ = 8).

| **Gene** | **EVs (pH 7.4)** | **EVs (pH 6.5)** |
|---|---|---|
| ***EGFR*** | - | - |
| ***PDGFRA*** | - | - |

| | | |
|---|---|---|
| ***TNFA*** | - | - |
| ***BDNF*** | $2.56 \times 10^{-10} \pm 1.5 \times 10^{-10}$ | - |
| ***VEGFA*** | $3 \times 10^{-7} \pm 4.9 \times 10^{-8}$ | $8.21 \times 10^{-12} \pm 7 \times 10^{-12}$ |
| ***ITGA2*** | - | $5.06 \times 10^{-6} \pm 4.5 \times 10^{-7}$ |
| ***ITGA3*** | - | $1.52 \times 10^{-5} \pm 4.5 \times 10^{-6}$ |
| ***ITGV*** | - | $3.6 \times 10^{-6} \pm 4.6 \times 10^{-7}$ |
| ***HSP60*** | - | $4.34 \times 10^{-7} \pm 1.3 \times 10^{-7}$ |

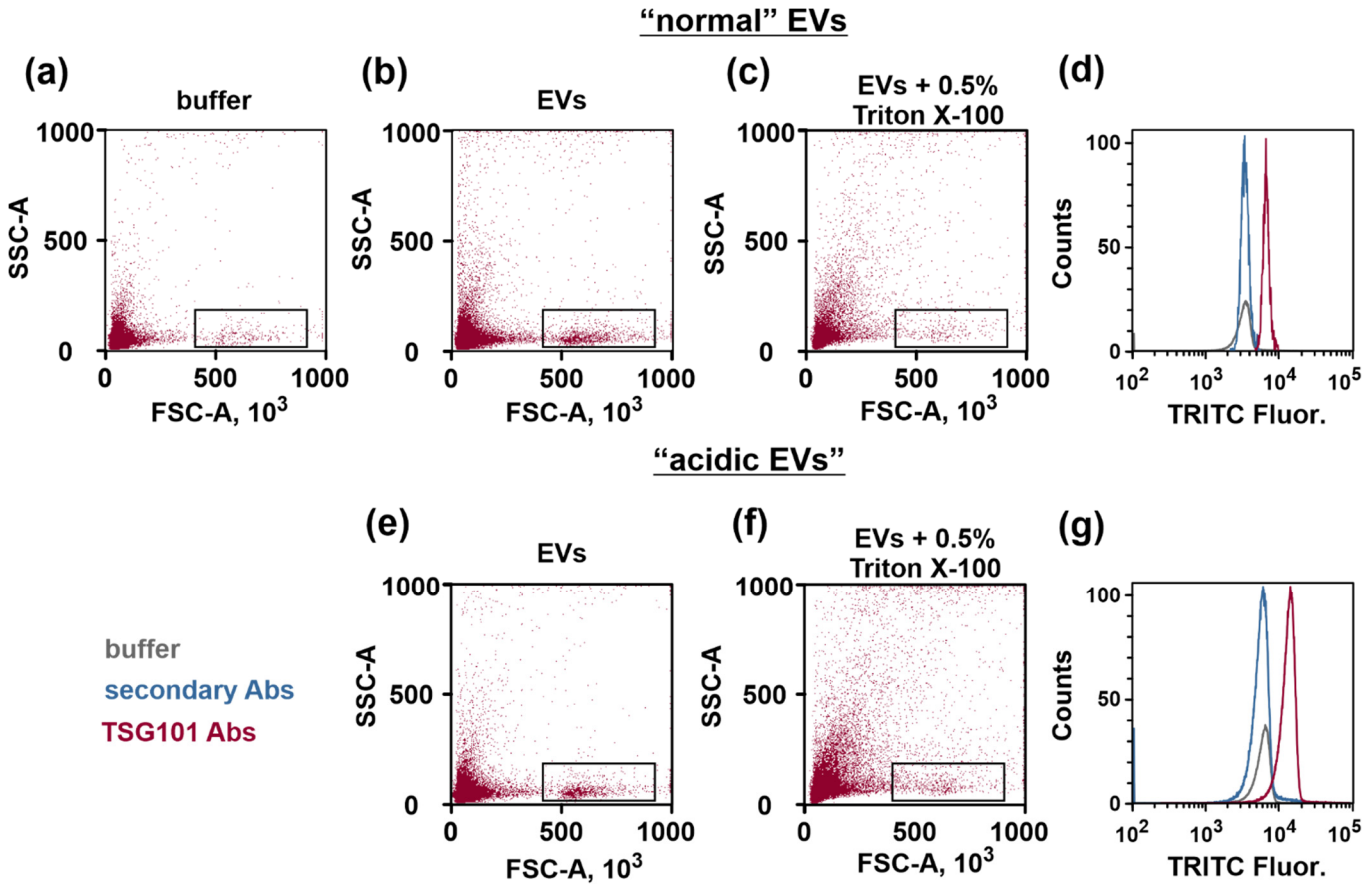


**Figure S1.** Exosome characterization by flow cytometry: the double-filtered FC buffer (**a**); "normal" (**b**) and "acidified" (**e**) EVs; "normal" (**c**) and "acidified" (**f**) EVs after the 30 min incubation with Triton X-100; "normal" (**d**) and "acidified" (**g**) EVs stained with the antibody to TGS101 are shown.

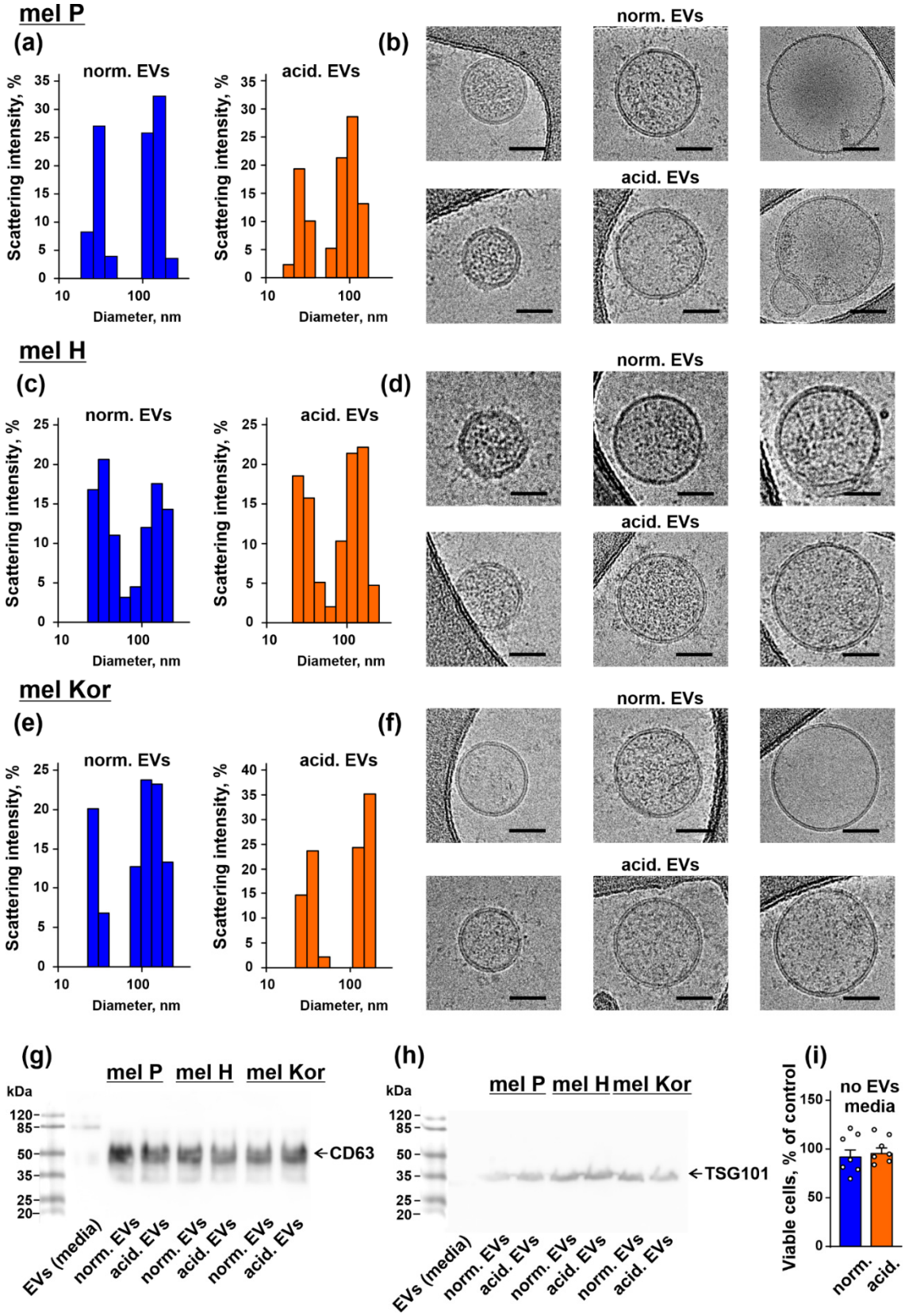


**Figure S2.** Characterization of EVs derived from the mel P (**a, b**), mel H (**c, d**), and mel Kor (**e, f**) cells: EVs size analysis according to dynamic light scattering (**a,c,e** for mel P, mel H and mel Kor cells, respectively), EVs visualization by cryo-EM (**b, d, e** for mel P, mel H and mel Kor cells, respectively, scale bar 25 nm); **g, h** – analysis of the expression of the exosome markers CD63 (**g**) and TSG101 (**h**) in EVs by Western blotting. EVs from the media (without cells) was used as the negative control; (**i**) influence of "EVs" from the FCS-supplemented media on growth of the Het-1A cells. Data presented as % of the control (untreated cells) ± SEM ($n = 7$).

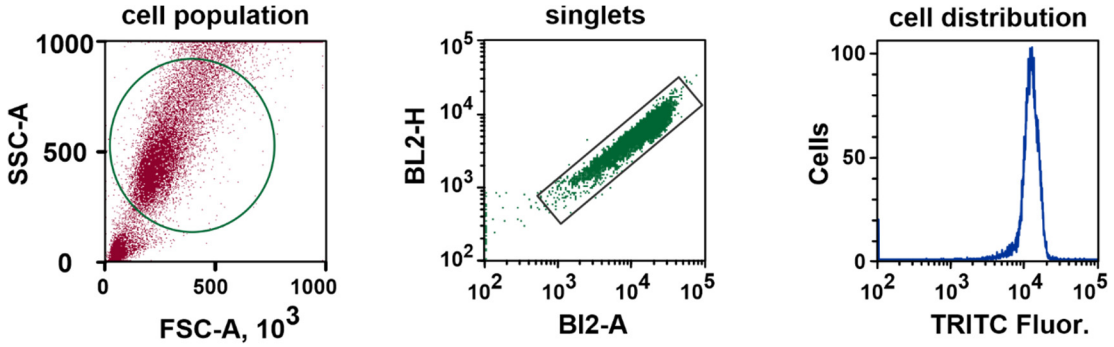


**Figure S3.** Gating strategy for the analysis of the EVs influence on the expression of the prooncogenic markers in the keratinocytes.

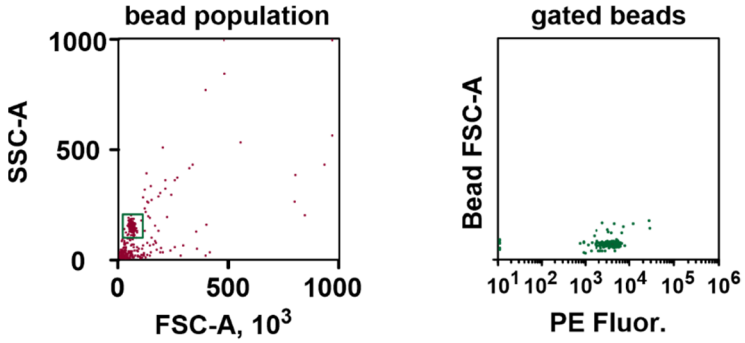


**Figure S4.** Gating strategy for the analysis by the Bio-Plex magnetic bead assay of the intracellular pathways activated in the keratinocytes.

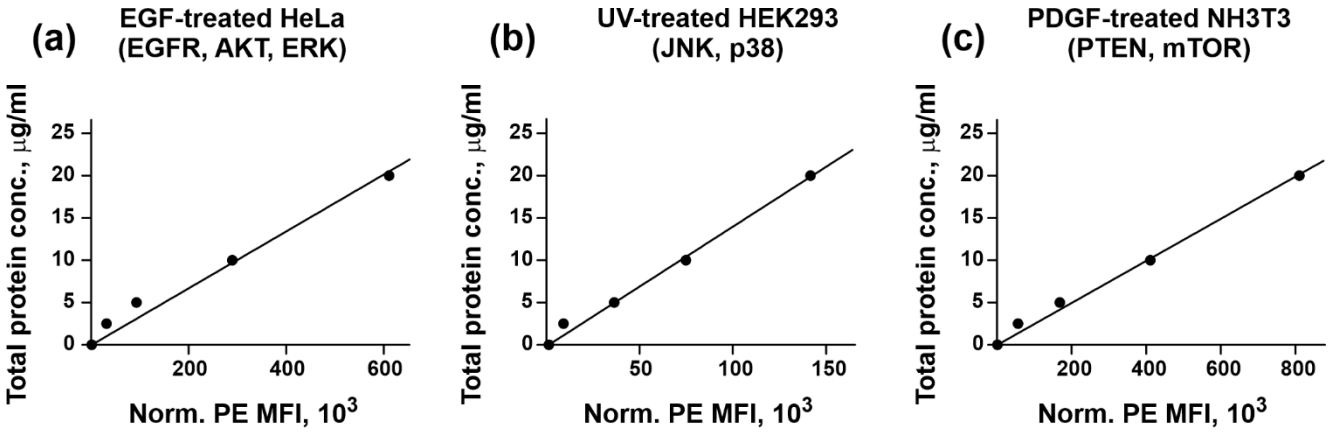


**Figure S5.** The Bio-Plex assay linearity analysis. Cell lysates were serially diluted and the protein phosphorylation was detected according to the manufacturer protocol. After that the linear regression was performed in the GraphPad Prism 9.2 software.

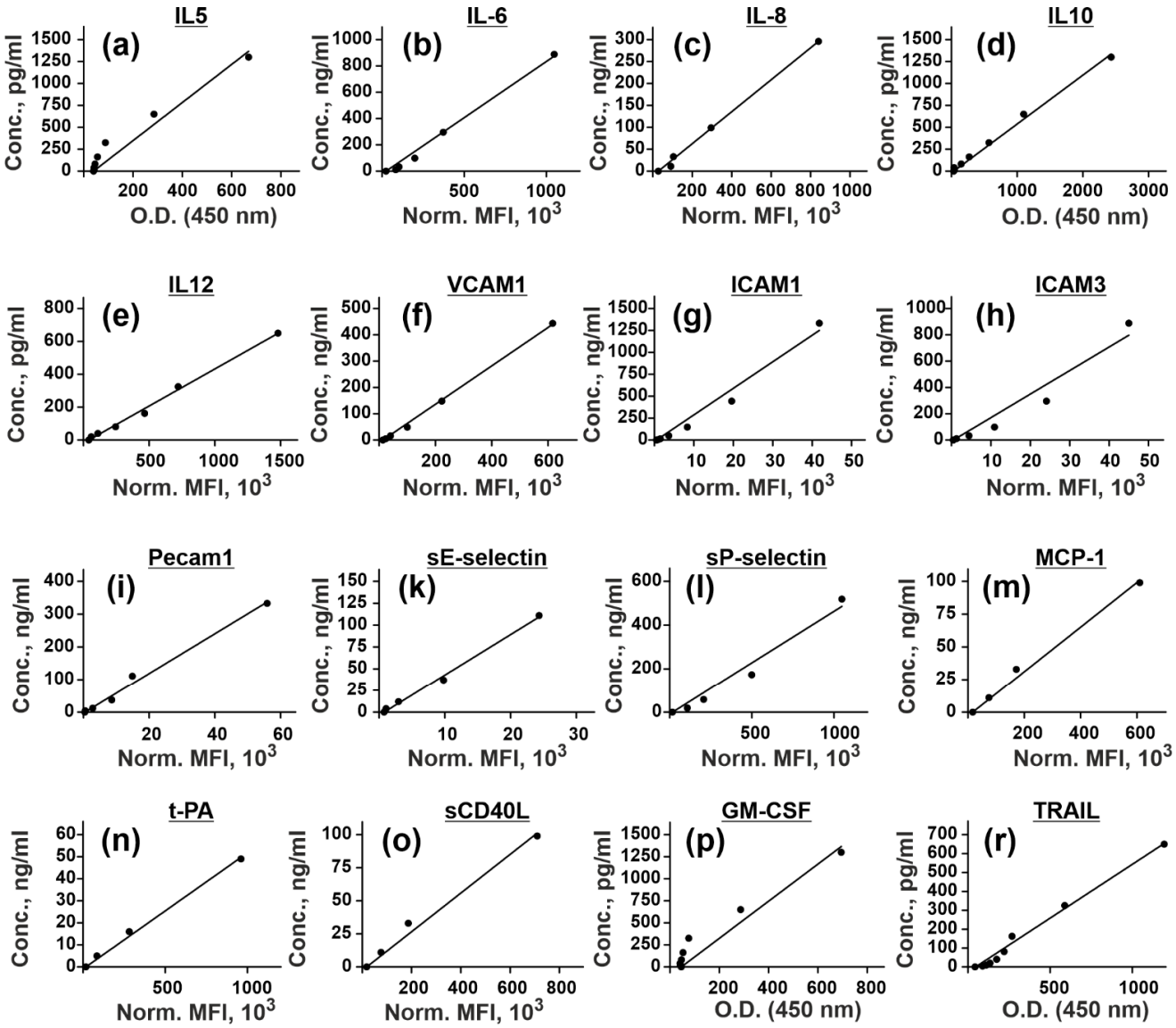


**Figure S6.** The ELISA's and Flow Cytomix kits assays linearity analysis. Serial dilution of the standard proteins was subjected to the ELISA or Flow cytomix assays according to the manufacturer instructions. Concentration of IL5 (**a**), IL10 (**d**), IL12 (**e**), GM-CSF (**p**), and TRAIL (**r**) was assayed by ELISA. Concentration of IL6 (**b**), IL8 (**c**), sVCAM-1 (**f**), sICAM-1 (**g**), sICAM-3 (**h**), sPECAM-1 (**i**), sE-selectin (**k**), sP-selectin (**l**), MCP-1 (**m**), t-PA (**n**), and sCD40L (**o**) was analyzed by the Flow Cyto-mix kits. After that the linear regression was performed in the GraphPad Prism 9.2 software.

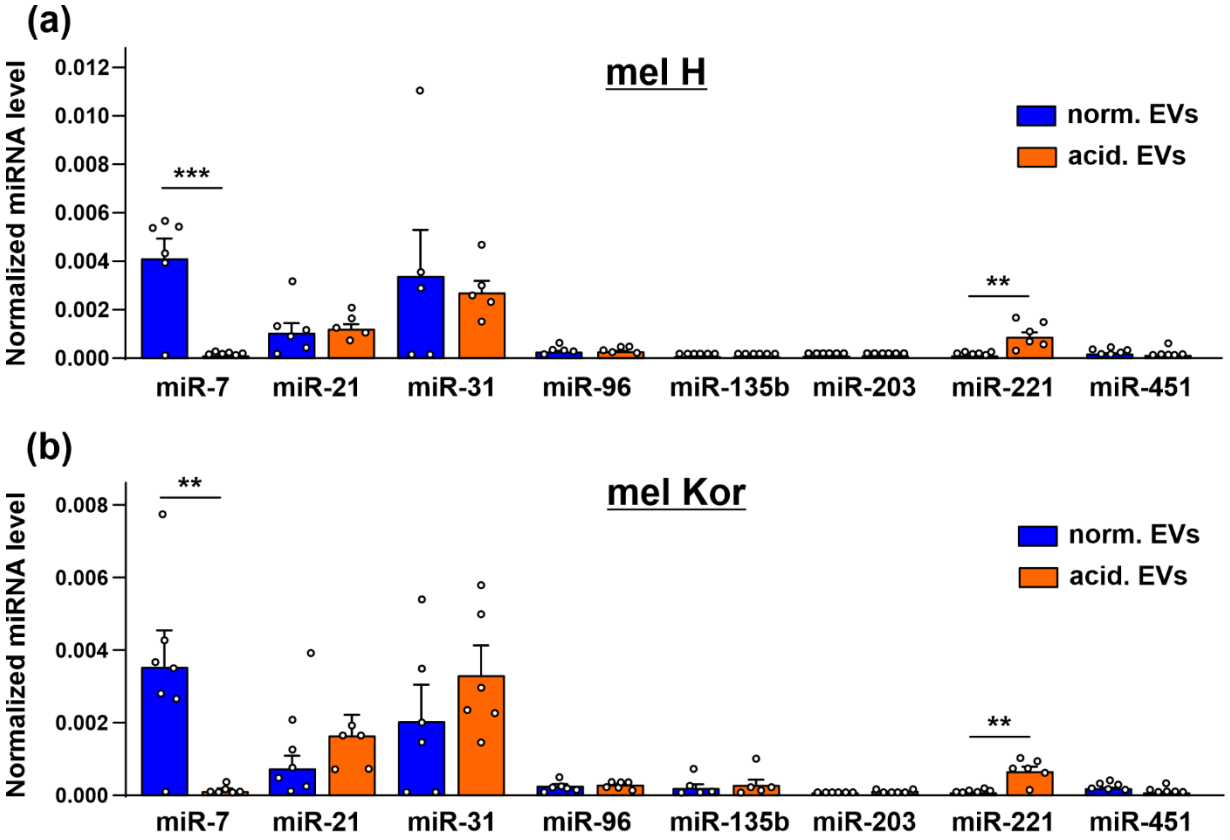


**Figure S7.** Analysis of the miRNA expression in EVs derived from the metastatic melanoma cells mel H (**a**) and mel Kor (**b**) cultivated at pH 6.5 ("acidified") and pH 7.4 ("normal"): Expression of different miRNA in "normal" and "acidified" EVs was assayed by qPCR with stem-loop primers and normalized to the U6 non-coding RNA. Data presented as the relative miRNA level ± SEM ($n$ = 5-6). ** ($p < 0.01$) and *** ($p < 0.001$) indicate significant difference between the data groups according to the two-tailed t-test.

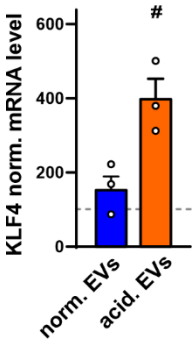


**Figure S8.** PCR analysis of the *KLF* expression in the keratinocytes treated by "normal" and "acidified" EVs derived from the mel P cells. *KLF4* expression was normalized to the expression of the *RPL13a* and *S18* genes and presented as the relative expression ± SEM (n = 3). # ($p < 0.05$) indicates significant difference between the treated and untreated cells (control, shown by dashed line) according to the one sample t-test.

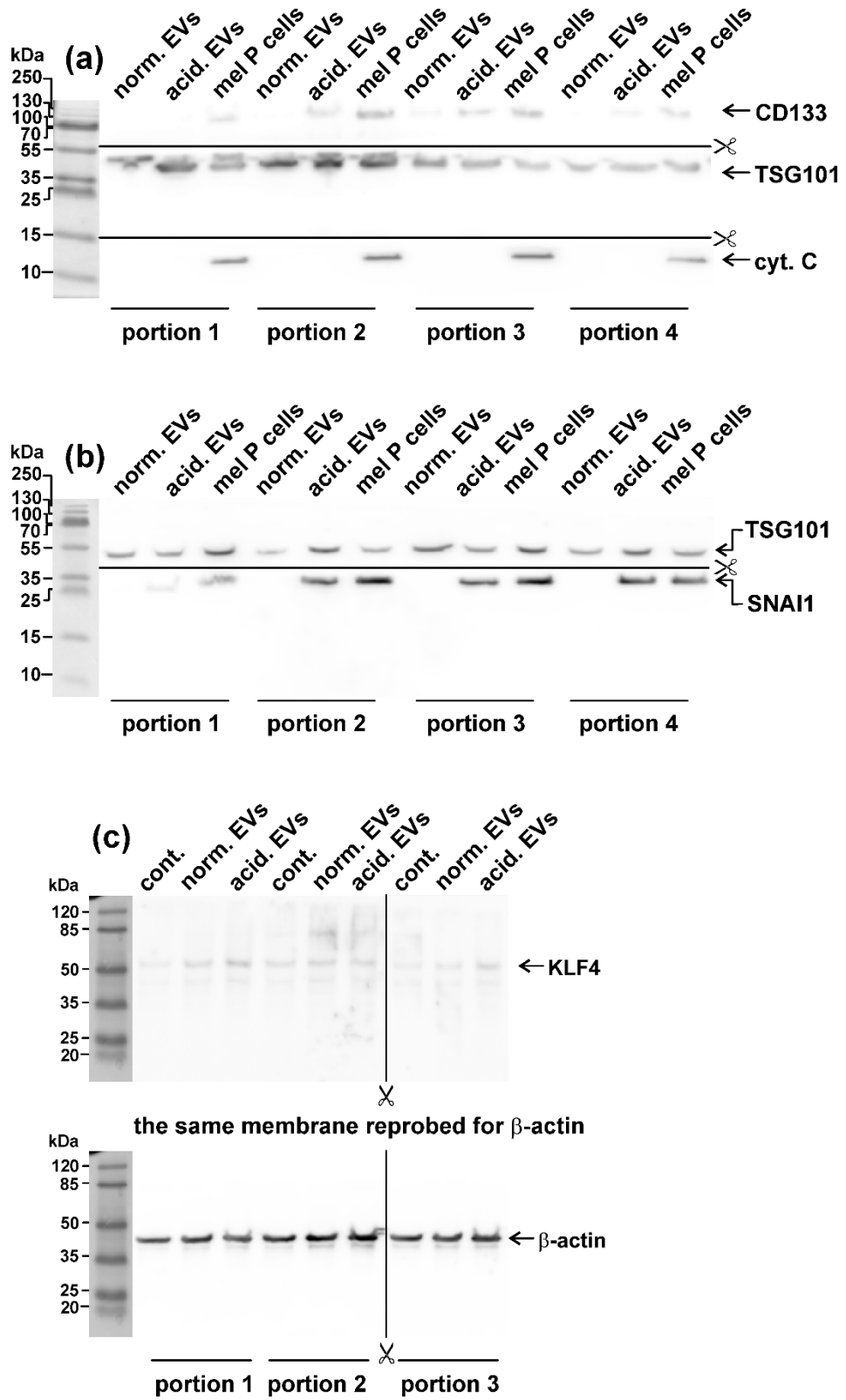


**Figure S9.** Whole membranes used for the analysis of the CD133 (**a**), SNAI1 (**b**), and KLF (**c**) expression by Western blotting. TSG101 was used as the exosomal marker, cytochrome C (**a**) served as the negative and positive control for the protein detection in EVs and the mel P cells, respectively.

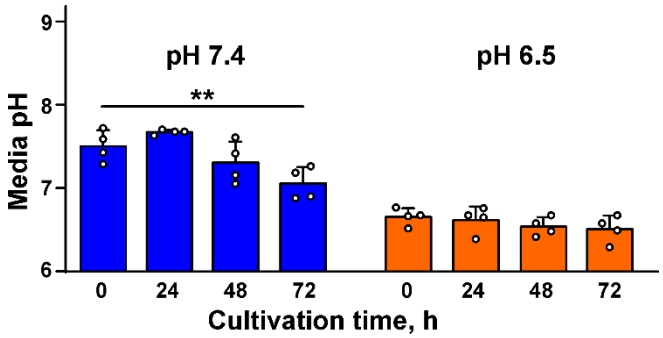


**Figure S10.** Media pH changes upon the cultivation of the mel P cells in the "normal" and "acidic" media ($n$ = 4). ** ($p$ < 0.01) indicates significant difference between the data groups by the one-way ANOVA followed by Tukey's post hoc test.

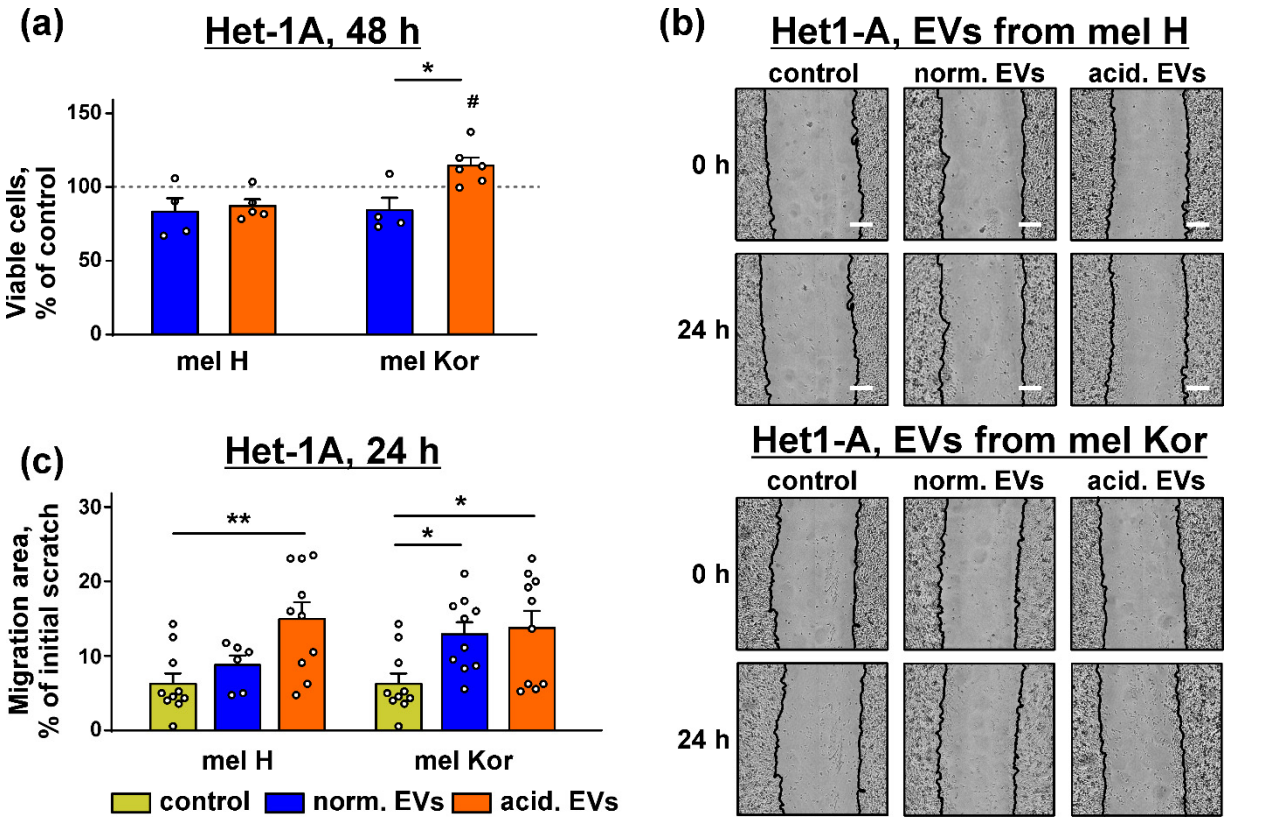


**Figure S11.** Effect of "normal" and "acidified" EVs on growth and migration of the keratinocytes. (**a**) Influence of "normal" and "acidified" EVs on viability of the Het-1A cells upon the 48 h incubation according to the WST-1 assay. Data were normalized to viability of the untreated cells (control, shown by dashed line). Data are % of the untreated cells ± SEM ($n$ = 4-6), # ($p$ < 0.05) indicates significant difference from the untreated cells by the one-sample t-test, * ($p$ < 0.05) indicates significant difference between the data groups by the two-tailed t-test; (**b**)representative pictures of the scratch test for the Het-1A cells upon the 24 h incubation with "normal" and "acidified" EVs derived from the mel H and mel Kor cells; (**c**) scratch square occupied by the migrating Het-1A cells. Data are presented as % of the scratch surface, occupied by the migrating cells ± SEM ($n$ = 6-10), * ($p$ < 0.05) and ** ($p$ < 0.01) indicate significant difference between the data groups by the one-way ANOVA followed by Tukey's post hoc test.

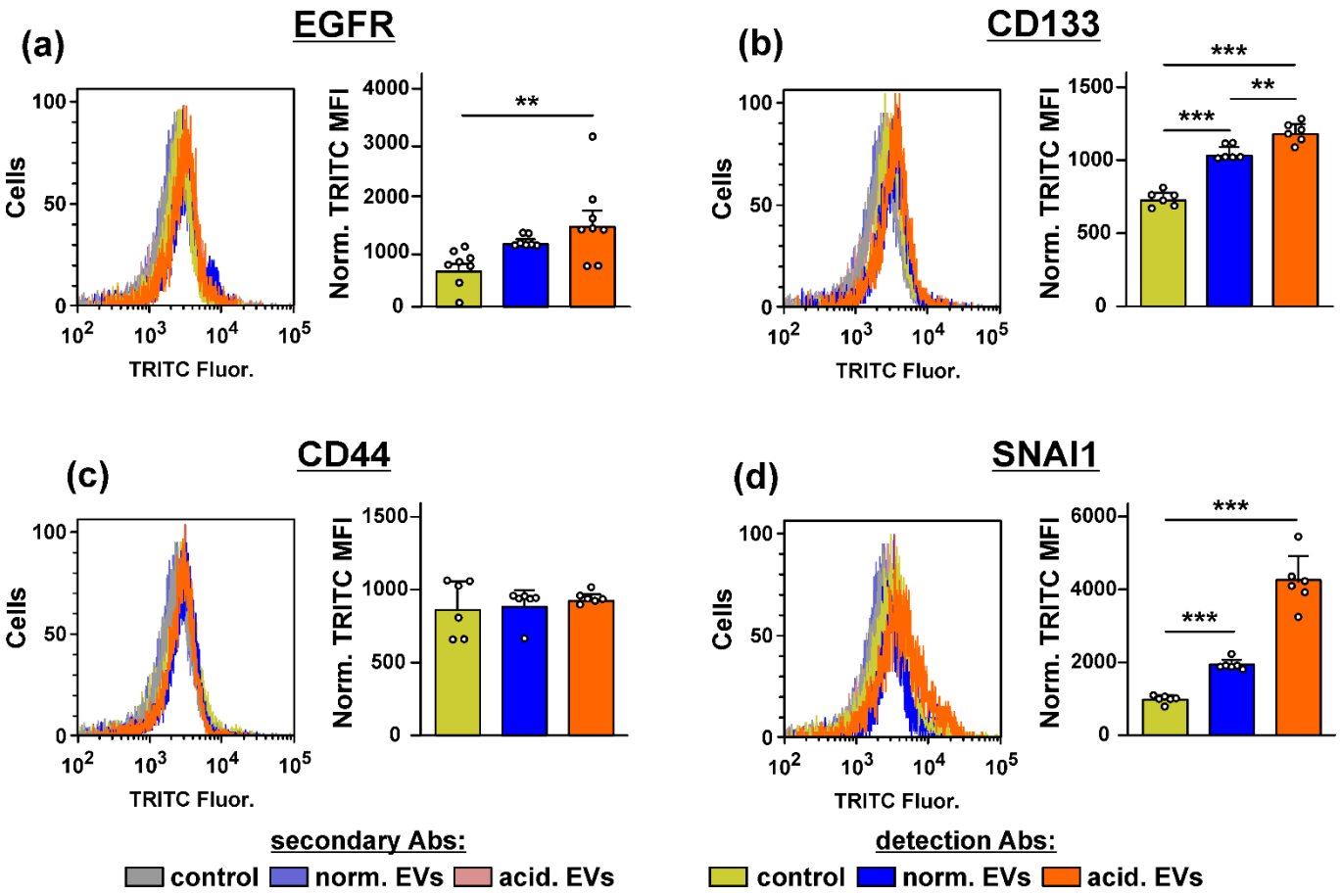


**Figure S12.** Influence of "normal" and "acidified" EVs on the expression of the EGFR (**a**), CD133 (**b**), CD44 (**c**), and SNAI1 (**d**) proteins in the keratinocytes. Representative cell distribution histograms of the EGFR, CD133, CD44, and SNAI1 proteins stained in the keratinocytes and the expression level of the corresponding proteins are shown on the left and right panels, respectively. Data presented as normalized MFI ± SEM ($n$ = 6). ** ($p < 0.01$) and *** ($p < 0.001$) indicate significant difference between the data groups by the one-way ANOVA followed by Tukey's post hoc test.

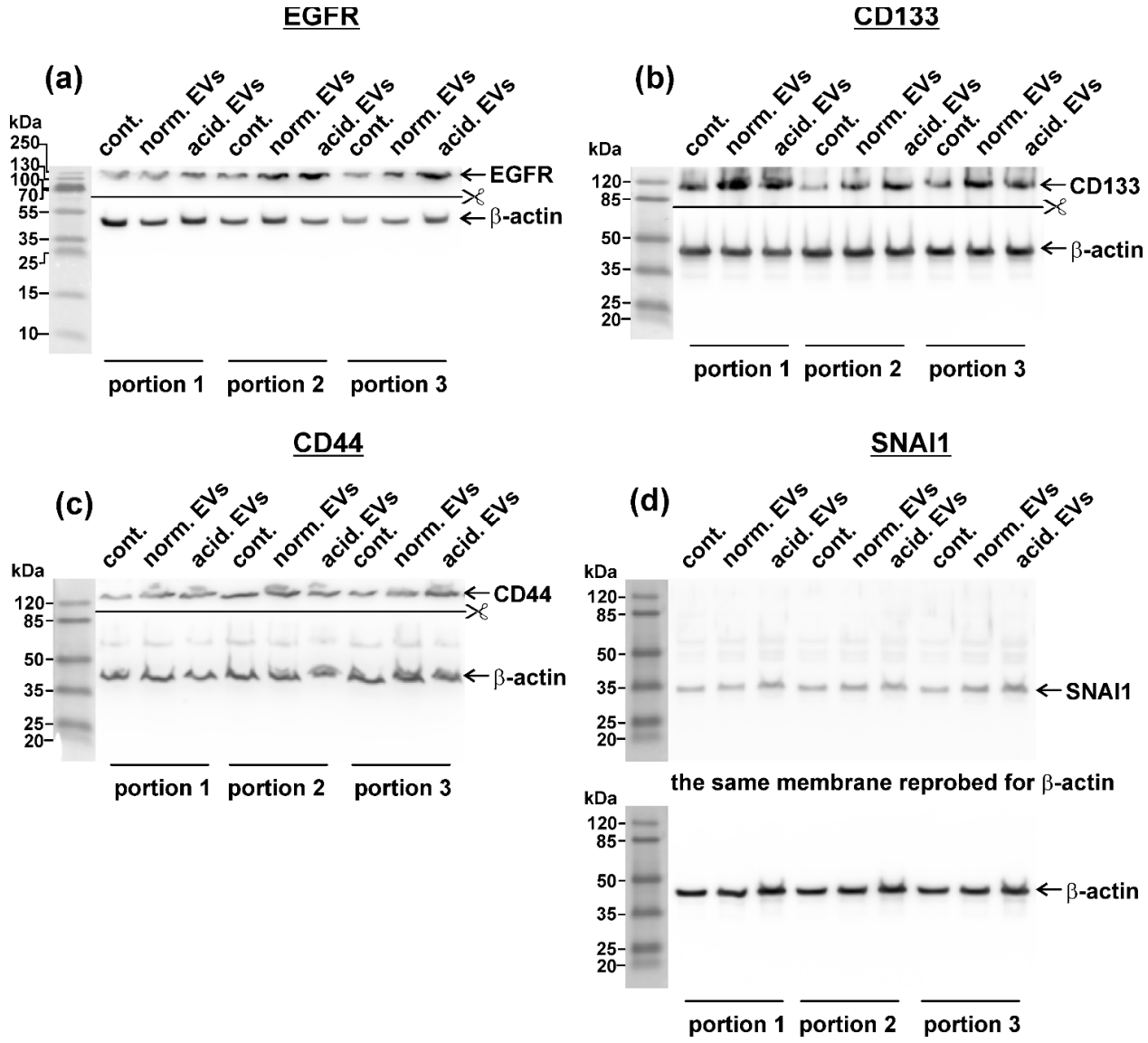


**Figure S13.** Whole membranes used for the analysis of the influence of "normal" and "acidified" EVs on the expression of the EGFR (**a**), CD133 (**b**), CD44 (**c**), and SNAI1 (**d**) proteins in the keratinocytes.