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SUPPLEMENTARY INFORMATION

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Self-assembled peptide–poloxamine nanoparticles enable in vitro and in vivo genome restoration for cystic fibrosis

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SUPPLEMENTARY TABLES

Supplementary Table 1. The structure of all synthetic peptides used in current study.

Name	Sequence
Peptide 0	Lipoic acid-W**KKRRRRKK***VKRKKKP*
Peptide 1	**KKRRRRKK***VKRKKKP*
Peptide 2	Lipoic acid
Peptide 3	Lipoic acid-W**KKRRRRKK***VKRKTKP*
Peptide 4	Lipoic acid-W**KKKKRRRRRKKKK**GAC*SERSMN*FCG
Peptide 5	Lipoic acid-W**KKKKRRRRRKKKK**GAC*YGLPHK*FCG
Peptide 6	Lipoic acid-W**KKRRRRRRRRKK**GAC*SERSMN*FCG
Peptide 7	Lipoic acid-W**KKRRRRRRRRKK**GAC*YGLPHK*FCG
Peptide 8	Lipoic acid-W**LHHHKKLLHHHKKL**GAC*SERSMN*FCG
Peptide 9	KETWWETWWTEWWTEW**KKKKRRRRRKKKK**GAC*SERSMN*FCG
Peptide 10	VVVVVV**KRRRRK**GAC*SERSMN*FCG
Peptide 11	$C_{13}H_{27}CONH$-**KKKKRRRRRKKKK**GAC*SERSMN*FCG
Peptide 12	Cholesteryl-**KKKKRRRRRKKKK**GAC*SERSMN*FCG

Sequences given in italics are targeting moieties. Underlined sequences represent anchor moieties. Sequences given in bold represent cationic moieties.

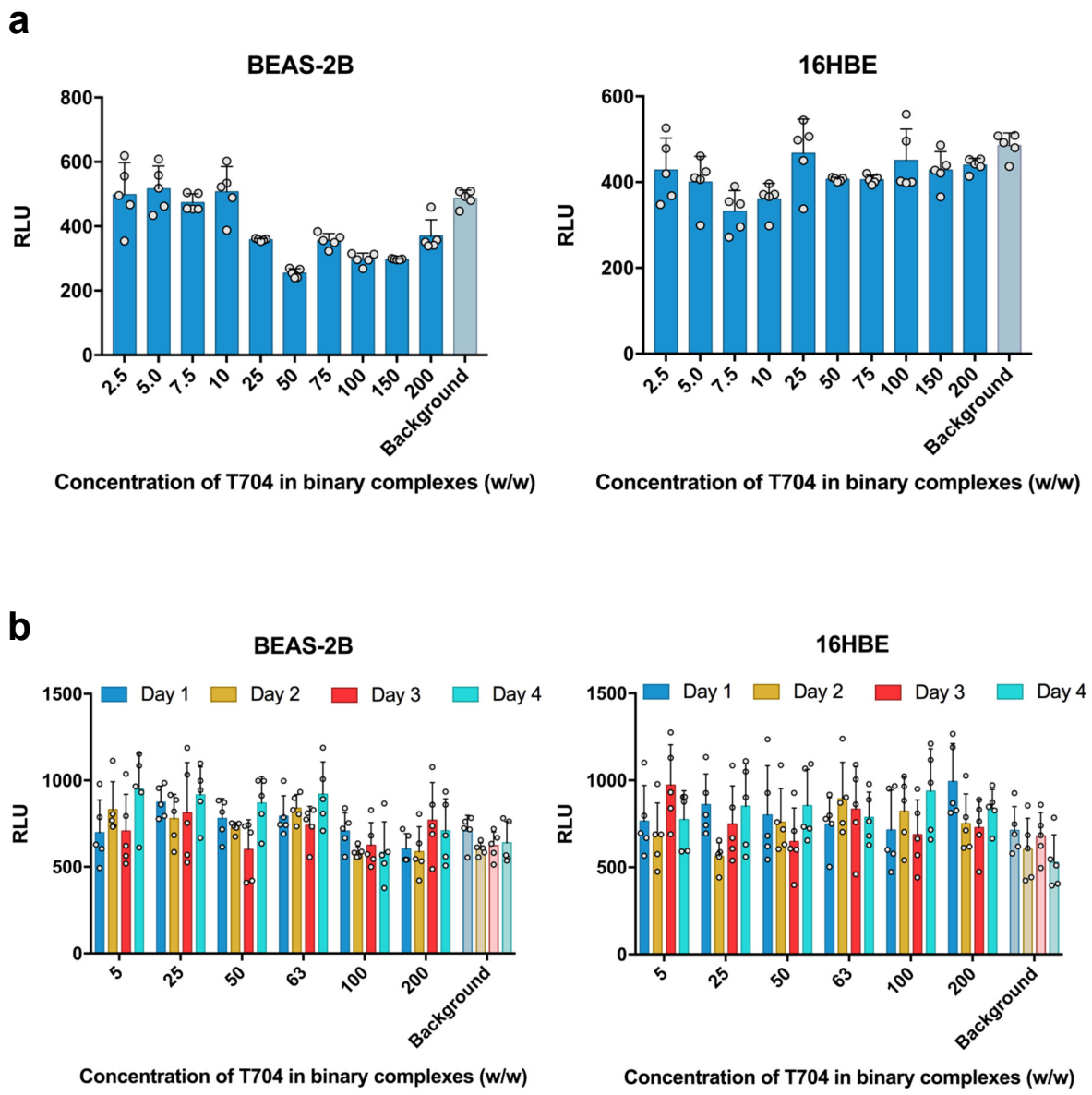
Supplementary Table 2. Physical characterization (size and zeta potential) of binary and ternary complexes containing mRNA or pDNA.

Complex	Particle size (nm)	Zeta-potential (mV)	PDI
mRNA + T704	65.2 ± 6.5	-23.0 ± 1.6	0.449
mRNA + T704 + Peptide 0 (N/P=10)	56.3 ± 5.1	19.7 ± 1.2	0.235
mRNA + T704 + Peptide 1 (N/P=20)	20.4 ± 8.8	13.1 ± 1.4	0.877
mRNA + T704 + Peptide 2 (no N/P)*	69.8 ± 2.7	-21.3 ± 2.2	0.485
mRNA + T704 + Peptide 3 (N/P=10)	49.7 ± 6.1	15.0 ± 1.8	0.355
mRNA + T704 + Peptide 4 (N/P=5)	49.5 ± 4.9	16.4 ± 0.3	0.238
mRNA + T704 + Peptide 5 (N/P=2.5)	57.5 ± 5.5	17.3 ± 0.5	0.346
mRNA + T704 + Peptide 6 (N/P=5)	54.4 ± 9.9	18.8 ± 2.1	0.241
mRNA + T704 + Peptide 7 (N/P=2.5)	49.4 ± 4.3	17.8 ± 0.6	0.417
mRNA + T704 + Peptide 8 (N/P=20)	874.2 ± 65.3	2.01 ± 0.1	0.478
mRNA + T704 + Peptide 9 (N/P=5)	45.8 ± 2.9	11.9 ± 2.1	0.286
mRNA + T704 + Peptide 10 (N/P=5)	1014.4 ± 235.3	1.44 ± 0.1	0.799
mRNA + T704 + Peptide 11 (N/P=5)	20.7 ± 2.4	19.7 ± 0.6	0.902
mRNA + T704 + Peptide 12 (N/P=20)	36.2 ± 13.4	17.6 ± 1.2	0.467
mRNA + Peptide 9 (N/P=5)	164.6 ± 10.6	19.4 ± 1.2	0.178
pDNA + T704	206 ± 18.0	-19.0 ± 2.3	0.518
pDNA + T704 + Peptide 0 (N/P=20)	70.2 ± 3.6	23.4 ± 0.5	0.332
pDNA + T704 + Peptide 1 (N/P=20)	59.8 ± 8.1	10.4 ± 0.9	0.616
pDNA + T704 + Peptide 2 (no N/P)*	232.9 ± 9.3	-20.4 ± 0.8	0.554
pDNA + T704 + Peptide 3 (N/P=10)	66.8 ± 11.3	20.9 ± 0.5	0.470
pDNA + T704 + Peptide 4 (N/P=5)	69.3 ± 1.1	21.3 ± 0.8	0.241
pDNA + T704 + Peptide 5 (N/P=2.5)	82.3 ± 12.7	19.6 ± 0.9	0.196
pDNA + T704 + Peptide 6 (N/P=5)	72.6 ± 1.5	23.4 ± 2.0	0.199
pDNA + T704 + Peptide 7 (N/P=2.5)	85.1 ± 9.4	13.1 ± 0.5	0.203
pDNA + T704 + Peptide 8 (N/P=5)	1086.0 ± 83.7	-1.3 ± 0.2	0.380
pDNA + T704 + Peptide 9 (N/P=5)	58.3 ± 4.2	17.3 ± 1.1	0.263
pDNA + T704 + Peptide 10 (N/P=15)	1140 ± 586.1	11.3 ± 1.6	0.564
pDNA + T704 + Peptide 11 (N/P=10)	49.5 ± 4.0	13.6 ± 2.8	0.402
pDNA + T704 + Peptide 12 (N/P=15)	53.8 ± 8.6	18.3 ± 1.6	0.358
pDNA + Peptide 9 (N/P=5)	214.4 ± 7.9	23.5 ± 1.3	0.135

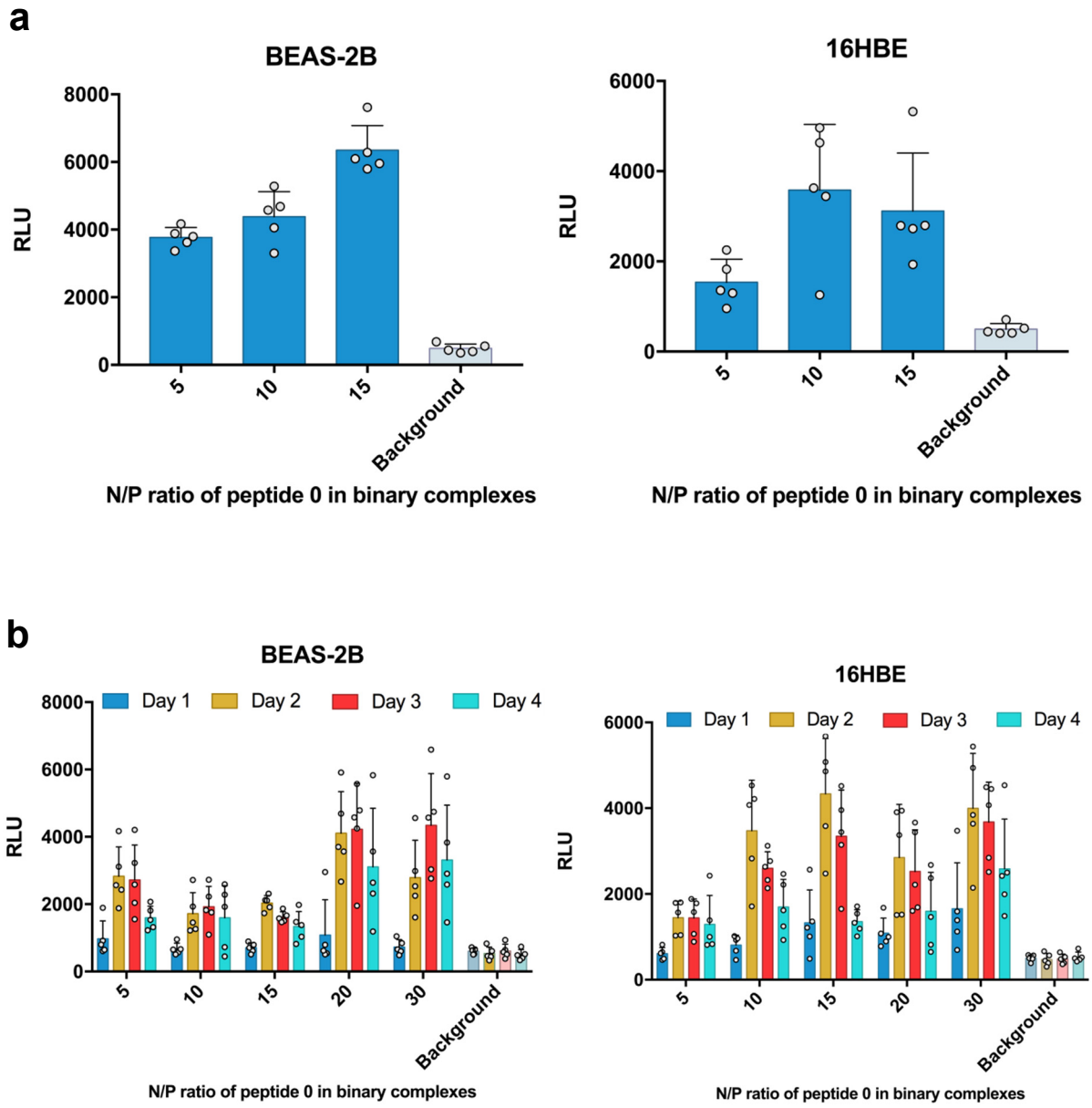
All the complexes used in the assay were prepared with the T704 at the ratio of 63 (w/w, relative to nucleic acid) and the peptide at the respective N/P ratio indicated in the bracket using methods described in "METHODS". The mRNA or pDNA concentration in all complexes was fixed at 10 μg/ml. Where applicable, the results are expressed as mean ± standard deviation (SD) of three independent experiments.

*: Peptide2 was applied with the same molar concentration of the peptide0 counterpart to form TC formulation.

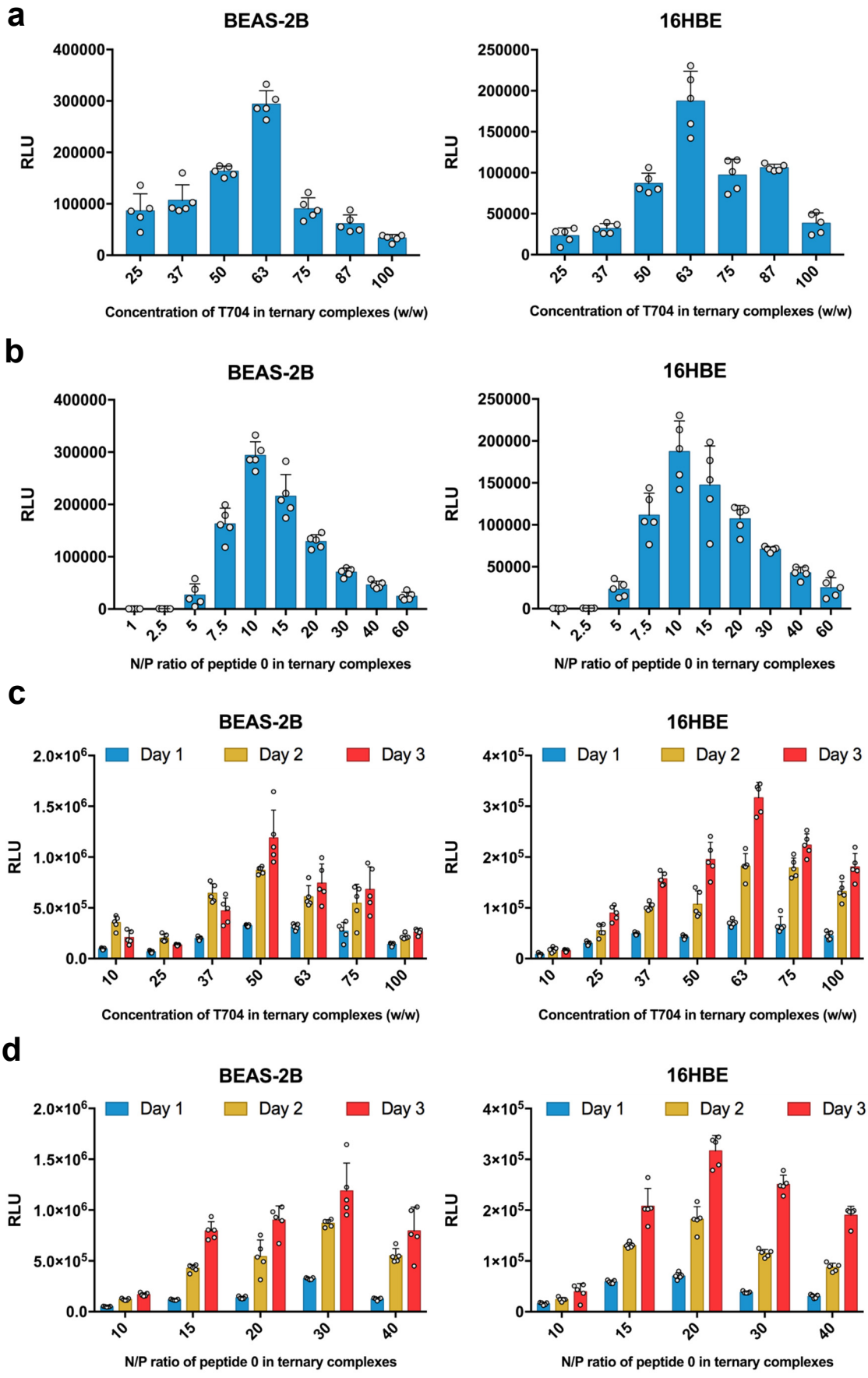
SUPPLEMENTARY FIGURES



Supplementary Fig. 1 | *In vitro* transfection profile of T704-based binary complexes in cultured human bronchial epithelial cells (BEAS-2B and 16HBE). (a) 400 ng/well MetLuc-mRNA was incubated with T704 at different concentrations from 2.5 to 200 (w/w, the concentration of T704 is always defined as the weight to weight ratio of T704 to nucleic acids in the following text) in Tyrode's solution. After 30 min incubation at room temperature (RT), samples were added into 96-well plate containing pre-seeded cells and incubated with the cells in OptiMEM (without serum and antibiotics) for 4 h at 37 ℃ in a humidified atmosphere (95% air, 5% CO_2). The luciferase activity in 50 µl supernatants from transfected cells was assayed after 24 h and its activity was expressed in relative light units (RLU). **(b)** MetLuc-pDNA/T704 binary complexes were prepared in the same way as the mRNA counterpart. The luciferase activity in 50 µl supernatants from transfected cells was measured at 24 h, 48 h, 72 h and 96 h after the transfection and its activity was expressed in RLU. Supernatants from untreated cells were used as the background samples. Data represent the mean ± SD, n=5 independent experiments.



Supplementary Fig. 2 | *In vitro* transfection profile of peptide0-based binary complexes in BEAS-2B and 16HBE cells. (a) 400 ng/well MetLuc-mRNA was incubated with peptide0 at different N/P ratios from 5 to 15 in nuclease-free water (NF-water). After 30 min incubation at RT, samples were added into 96-well plate containing pre-seeded cells and incubated with the cells in OptiMEM (without serum and antibiotics) for 4 h at 37 ℃ in a humidified atmosphere (95% air, 5% CO_2). The luciferase activity in 50 µl supernatants from transfected cells was measured after 24 h and its activity was expressed in RLU. **(b)** MetLuc-pDNA/peptide0 binary complexes were prepared in the same way as the mRNA counterpart, the luciferase activity in 50 µl supernatants from transfected cells was measured 24 h, 48 h, 72h and 96 h after transfection and its activity was expressed in RLU. Supernatants from untreated cells were used as the background samples. The data are given as the mean ± SD, n=5 independent experiments.


a
BEAS-2B
16HBE
RLU
400000
300000
200000
100000
0
250000
150000
50000
25
37
50
63
75
87
100
Concentration of T704 in ternary complexes (w/w)
b
BEAS-2B
16HBE
RLU
1
2.5
5
7.5
10
15
20
30
40
60
N/P ratio of peptide 0 in ternary complexes
c
BEAS-2B
16HBE
Day 1
Day 2
Day 3
RLU
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0.0
4×10^5
3×10^5
2×10^5
1×10^5
10
25
37
50
63
75
100
Concentration of T704 in ternary complexes (w/w)
d
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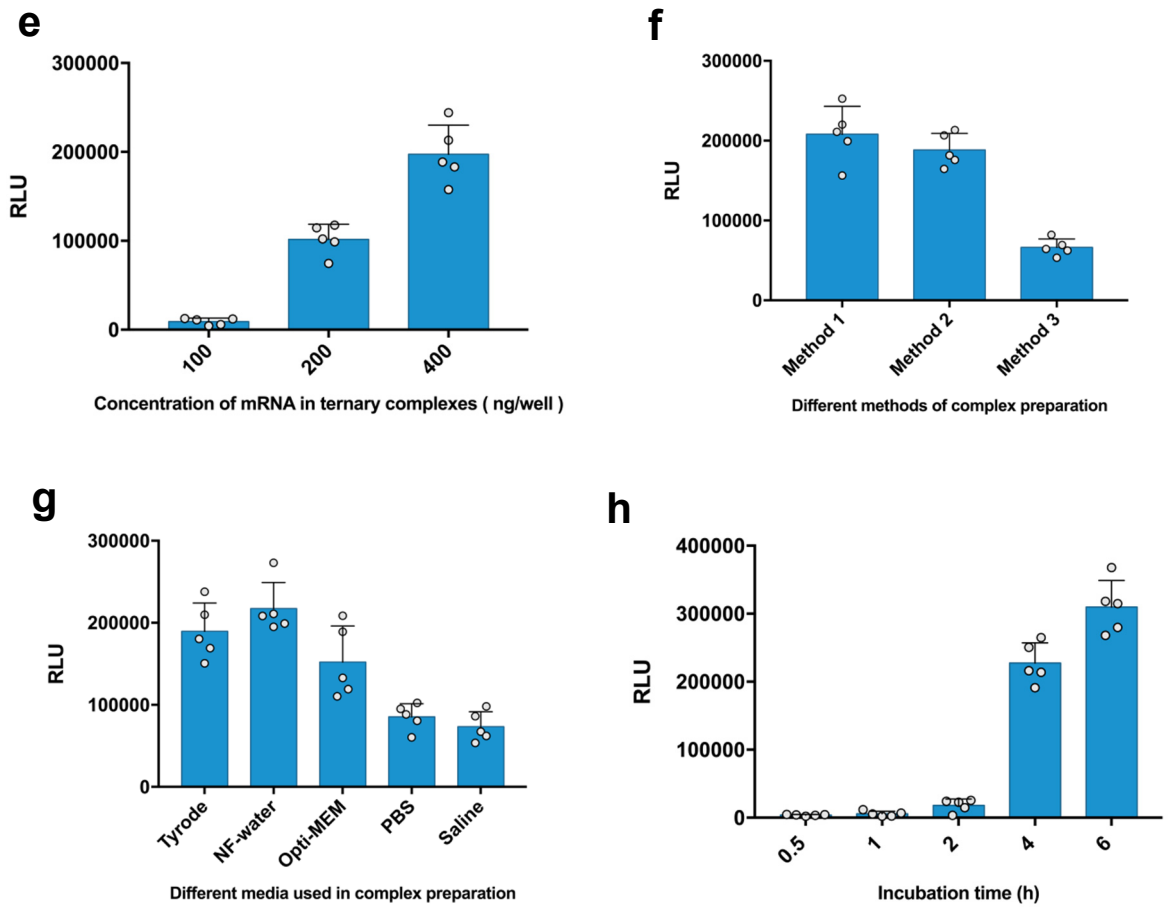
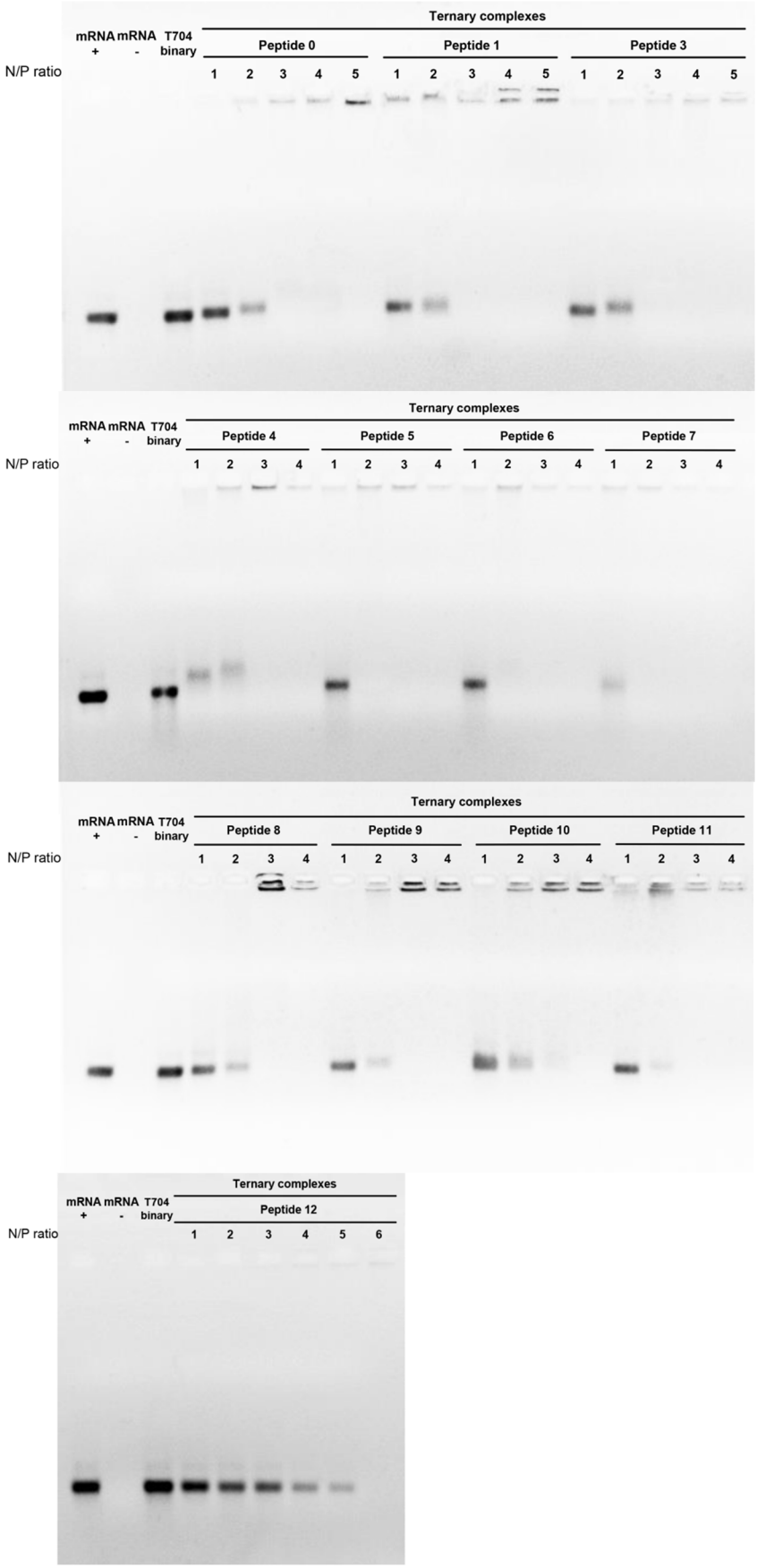
16HBE
Day 1
Day 2
Day 3
RLU
10
15
20
30
40
N/P ratio of peptide 0 in ternary complexes

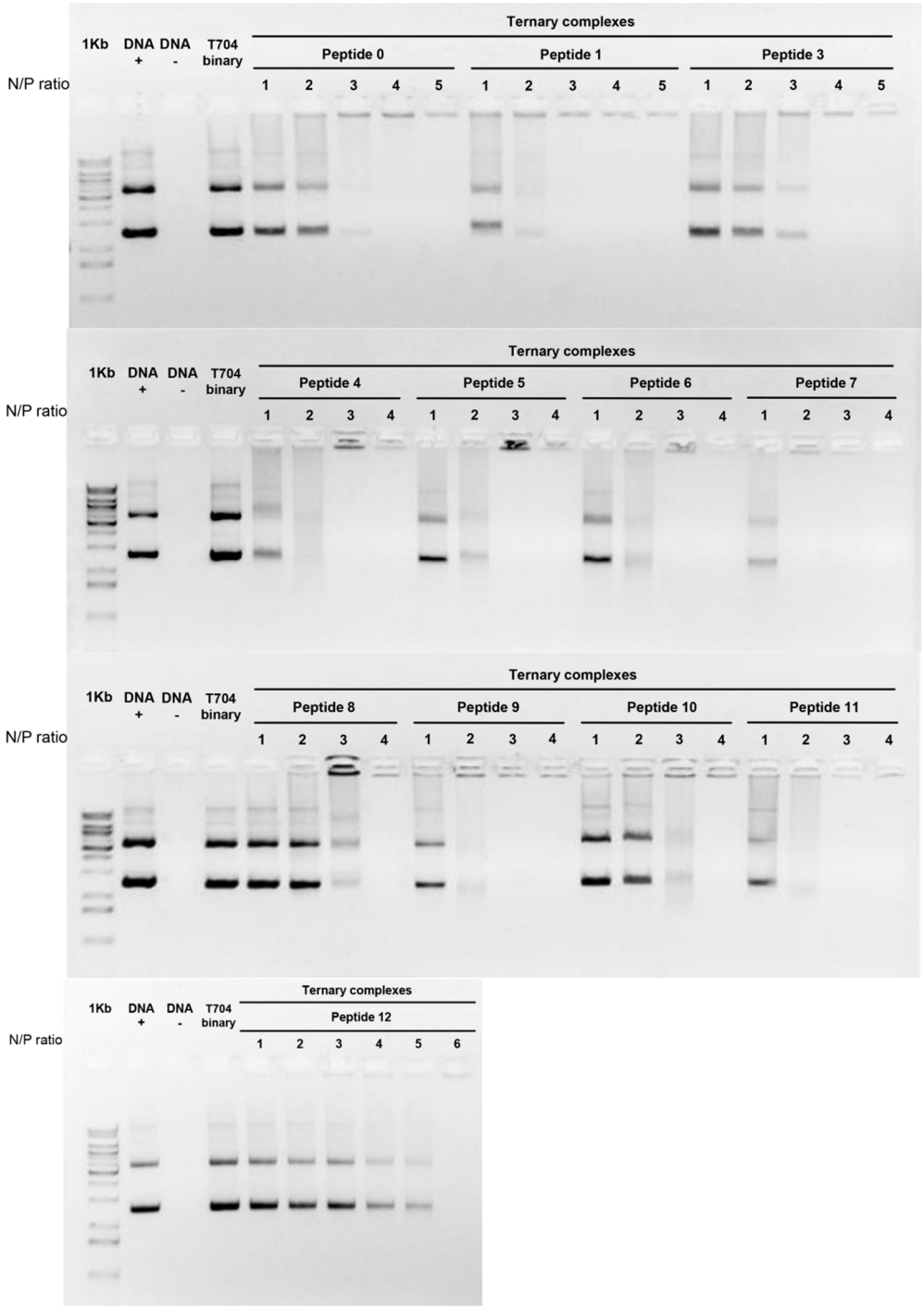


Supplementary Fig. 3 | Investigations on parameters influencing *in vitro* transfection efficiency of the ternary complex. (a) The influence of T704 concentration (w/w, relative to nucleic acids) on the transfection of mRNA-based ternary complex in BEAS-2B and 16HBE cells. The N/P ratio of peptide component was fixed at 10; **(b)** The effect of peptide0 concentration on the transfection efficiency of mRNA-based ternary complex in BEAS-2B and 16HBE cells. The concentration of T704 component was fixed at 63 (w/w, relative to nucleic acids); **(c)** Transfection efficiency of pDNA-based ternary complexes consisting of T704 at different concentrations (w/w, relative to nucleic acids) in BEAS-2B and 16HBE cells. The N/P ratio of peptide component in the case of BEAS-2B cells transfection was 30 and in the case of 16HBE cells transfection was 20; **(d)** Evaluation of pDNA expression mediated by ternary complexes comprising different concentrations of peptide0 in BEAS-2B and 16HBE cells. The concentration of T704 component in the case of BEAS-2B cells transfection was 50 (w/w) and in the case of 16HBE cells transfection was 63 (w/w); The transfection efficiency of mRNA-based ternary complex depending on **(e)** the concentration of mRNA in ternary complexes; on **(f)** different methods of complex preparation: Method 1: T704 and peptide0 at the optimum concentration were first mixed and incubated for 20 min, followed by adding equal volume of mRNA solution with another 20 min incubation at RT. Method 2: In the first step, T704 and mRNA solution were mixed and incubated for 20 min at RT, an equal volume of peptide0 were added and incubated for another 20 min at RT in the second step. Method 3: Peptide0 was incubated with mRNA for 20 min at RT, then an equal volume of T704 solution was introduced and incubated for another 20 min to form the ternary complex; on **(g)** different media used in complex preparation; and on **(h)** incubation time with 16HBE cells. For mRNA transfection, the luciferase activity was assayed 24 h after transfection. The luciferase activity mediated by pDNA transfection was measured on day 1, day 2 and day 3 after transfection. The results represent the mean ± SD of five independent experiments.

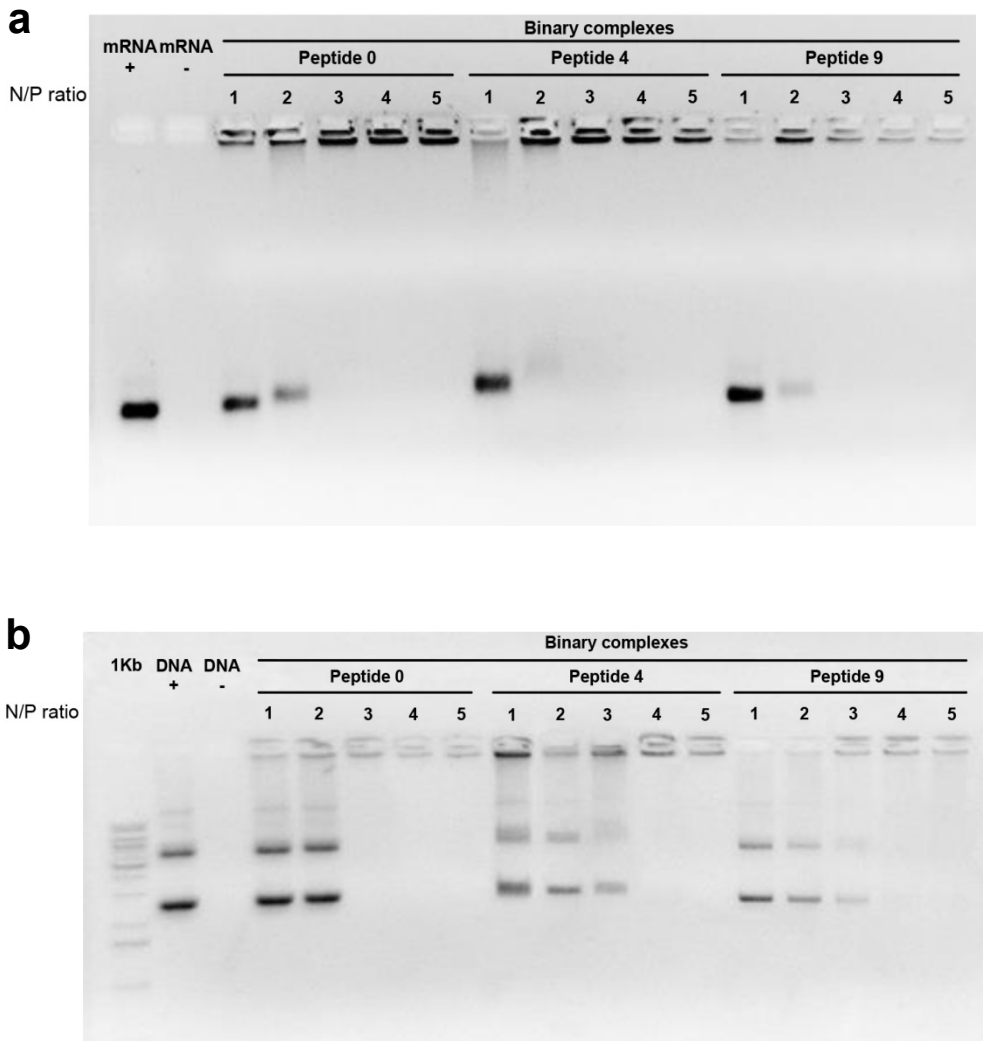
a


Ternary complexes
mRNA + mRNA - T704 binary
Peptide 0
Peptide 1
Peptide 3
N/P ratio
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
Ternary complexes
mRNA + mRNA - T704 binary
Peptide 4
Peptide 5
Peptide 6
Peptide 7
N/P ratio
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
Ternary complexes
mRNA + mRNA - T704 binary
Peptide 8
Peptide 9
Peptide 10
Peptide 11
N/P ratio
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
Ternary complexes
mRNA + mRNA - T704 binary
Peptide 12
N/P ratio
1 2 3 4 5 6

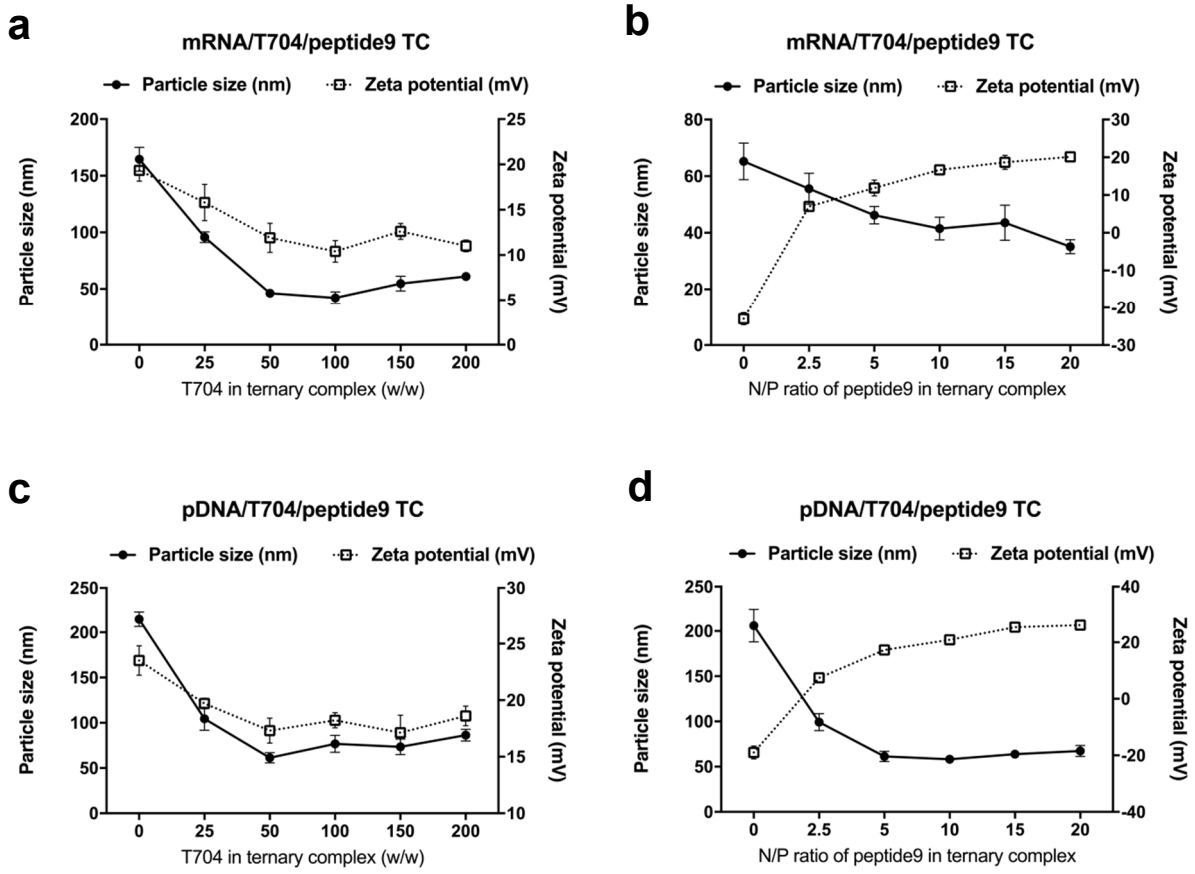
b



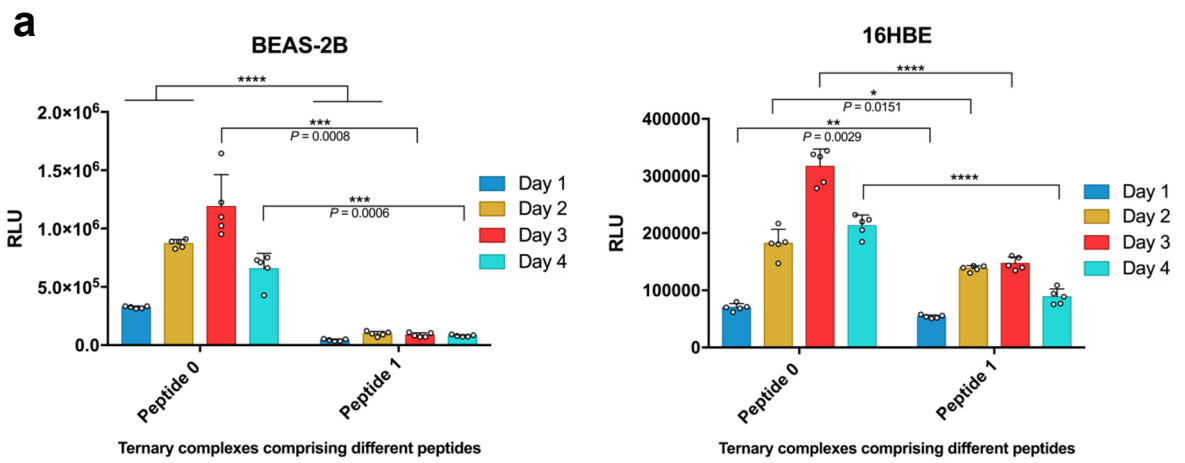
Supplementary Fig. 4 | Supplementary agarose gel retardation assay of ternary complexes. (a) mRNA condensation studies using ternary complexes comprising different peptides at various N/P ratios. Binary complex consisting of mRNA and T704 at the same concentration served as a binary control. Naked mRNA was used as a positive control (+) and NF-water was used as a negative control (-). **(b)** pDNA condensation studies using ternary complexes comprising different peptides at various N/P ratios. Binary complex prepared by pDNA and T704 at the same concentration served as a binary control. 1 Kb Plus DNA standard was used as a reference (1Kb), naked pDNA was used as a positive control (+) and NF-water was used as a negative control (-). The data in **a**,**b** were repeated six times independently with similar results.



Supplementary Fig. 5 | Agarose gel retardation assay of peptide-based binary complexes. (a) mRNA condensation study using binary complexes prepared by peptide0, peptide4 and peptide9 at various N/P ratios. Naked mRNA was used as a positive control (+), with NF-water as a negative control (-). **(b)** pDNA condensation studies using binary complexes prepared by peptide0, peptide4 and peptide9 at various N/P ratios. 1 Kb Plus DNA standard was used as a reference (1Kb), naked pDNA was used as a positive control (+) and NF-water was used as a negative control (-). The data in **a**,**b** were repeated four times independently with similar results.



Supplementary Fig. 6 | The influence of T704 or peptide concentration on the particle size (filled circles) and zeta potential (open squares) of ternary complexes. **(a)** The particle size and zeta potential of mRNA/T704/peptide9 ternary complexes with an increased amount of T704 (w/w, relative to mRNA). The N/P ratio of peptide9 was fixed at 5. **(b)** The particle size and zeta potential of mRNA/T704/peptide9 ternary complexes with an enhanced N/P ratio of peptide9. The amount of T704 was fixed at 63 (w/w, relative to mRNA). **(c)** The particle size and zeta potential of pDNA/T704/peptide9 ternary complexes with an increased amount of T704 (w/w, relative to pDNA). The N/P ratio of peptide9 was fixed at 5. **(d)** The particle size and zeta potential of pDNA/T704/peptide9 ternary complexes with an enhanced N/P ratio of peptide9. The amount of T704 was fixed at 63 (w/w, relative to pDNA). The amount of mRNA or pDNA used to prepare ternary complexes was fixed at 10 μg/ml. The data are shown as the mean ± SD (n=3 independent experiments).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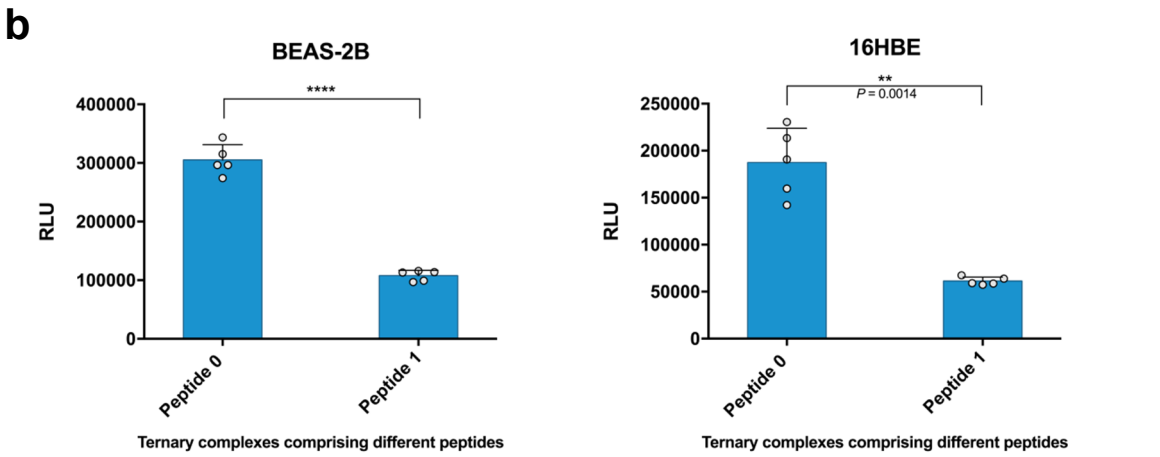
BEAS-2B
16HBE
RLU
Day 1
Day 2
Day 3
Day 4
P = 0.0008
P = 0.0006
P = 0.0151
P = 0.0029
Peptide 0
Peptide 1
Ternary complexes comprising different peptides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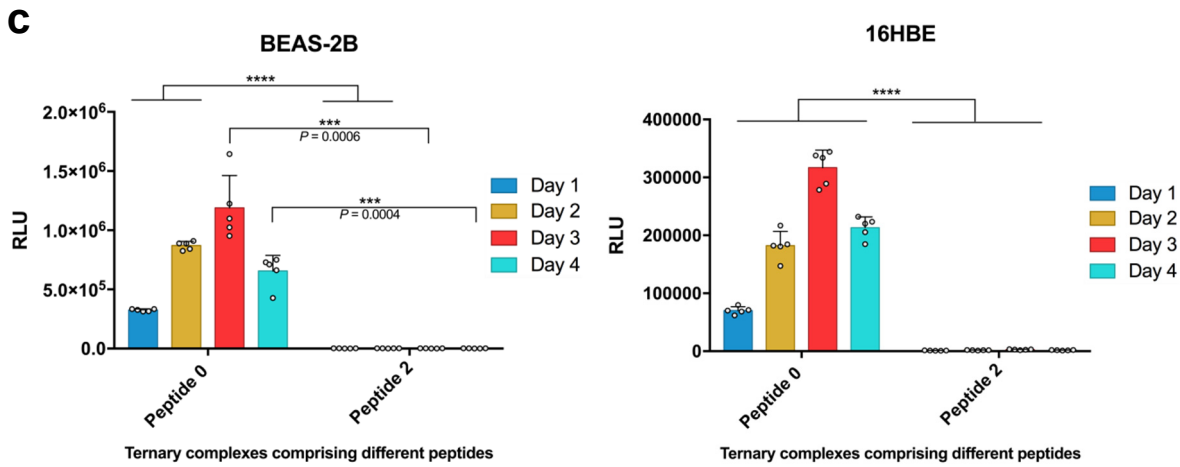
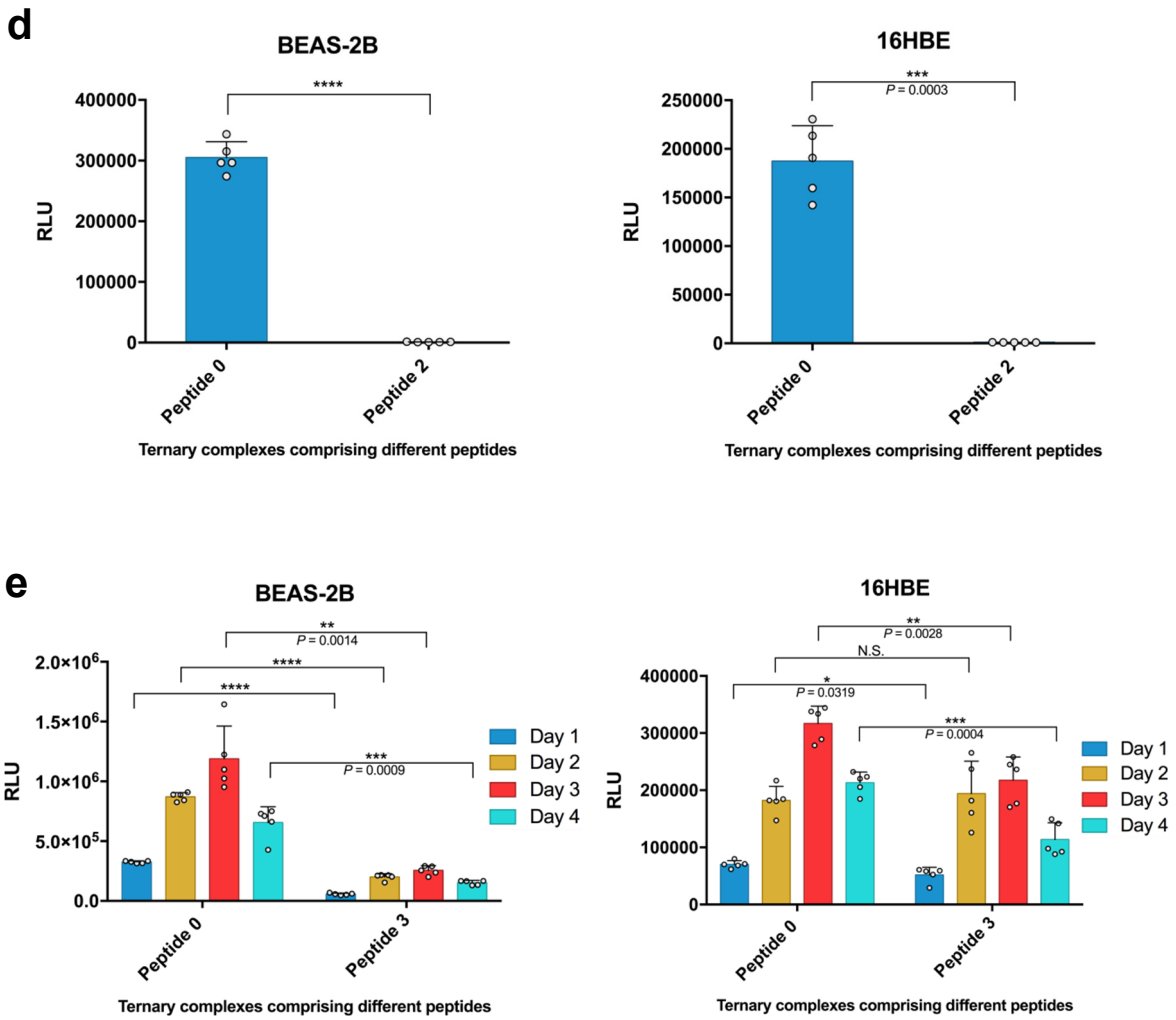
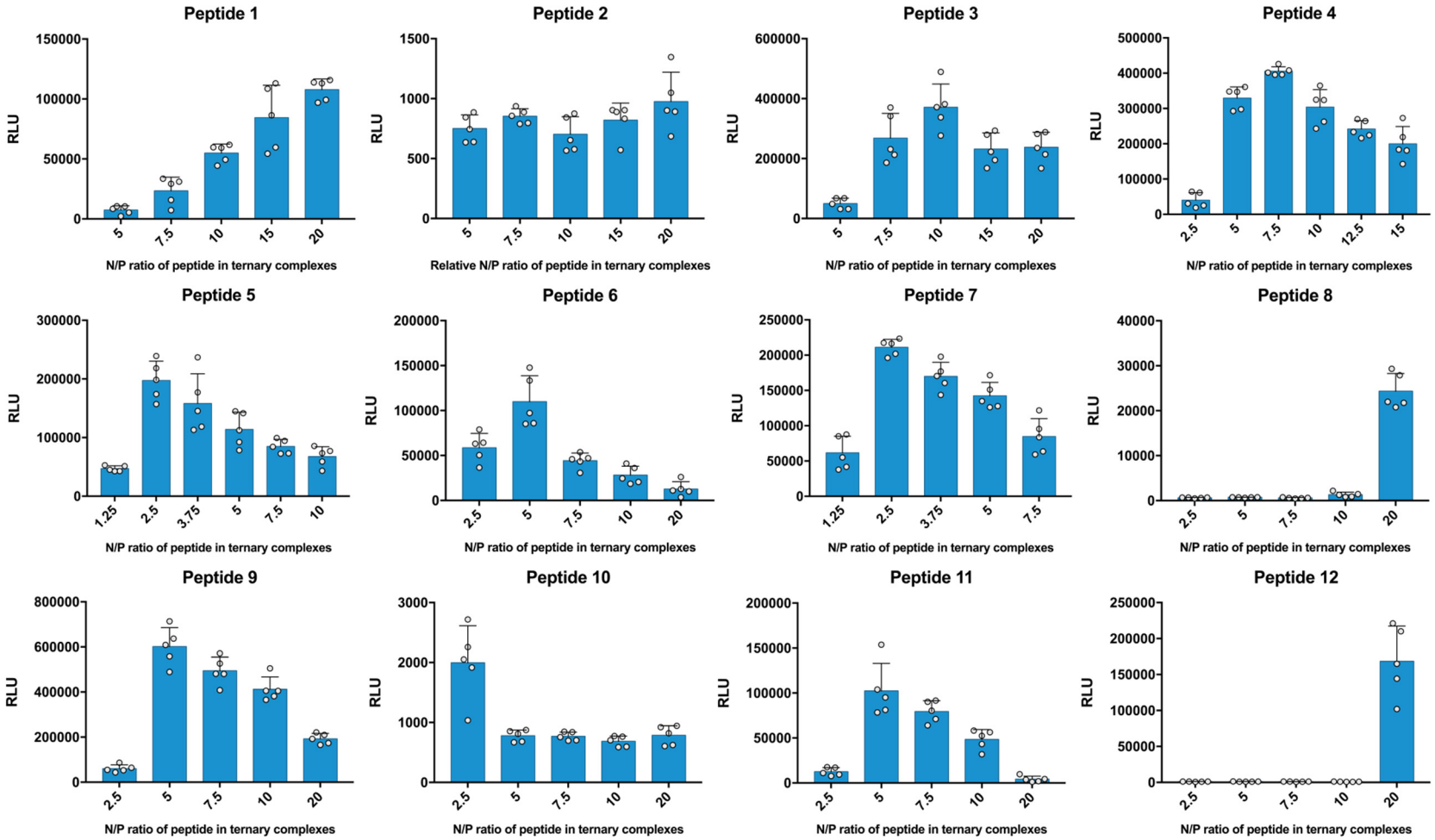
16HBE
RLU
P = 0.0014
Peptide 0
Peptide 1
Ternary complexes comprising different peptides

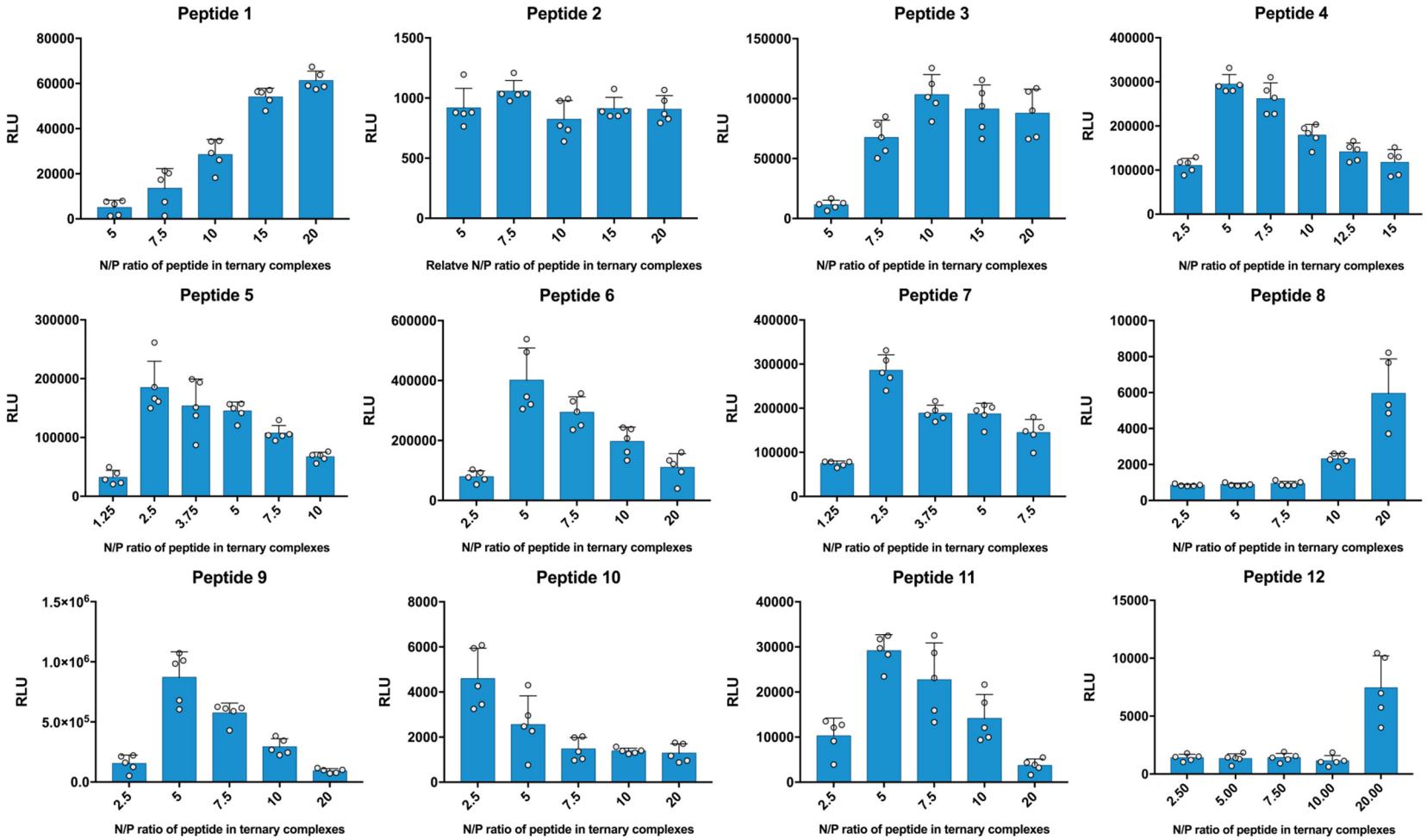

c
BEAS-2B
16HBE
RLU
Day 1
Day 2
Day 3
Day 4
P = 0.0006
P = 0.0004
Peptide 0
Peptide 2
Ternary complexes comprising different peptides



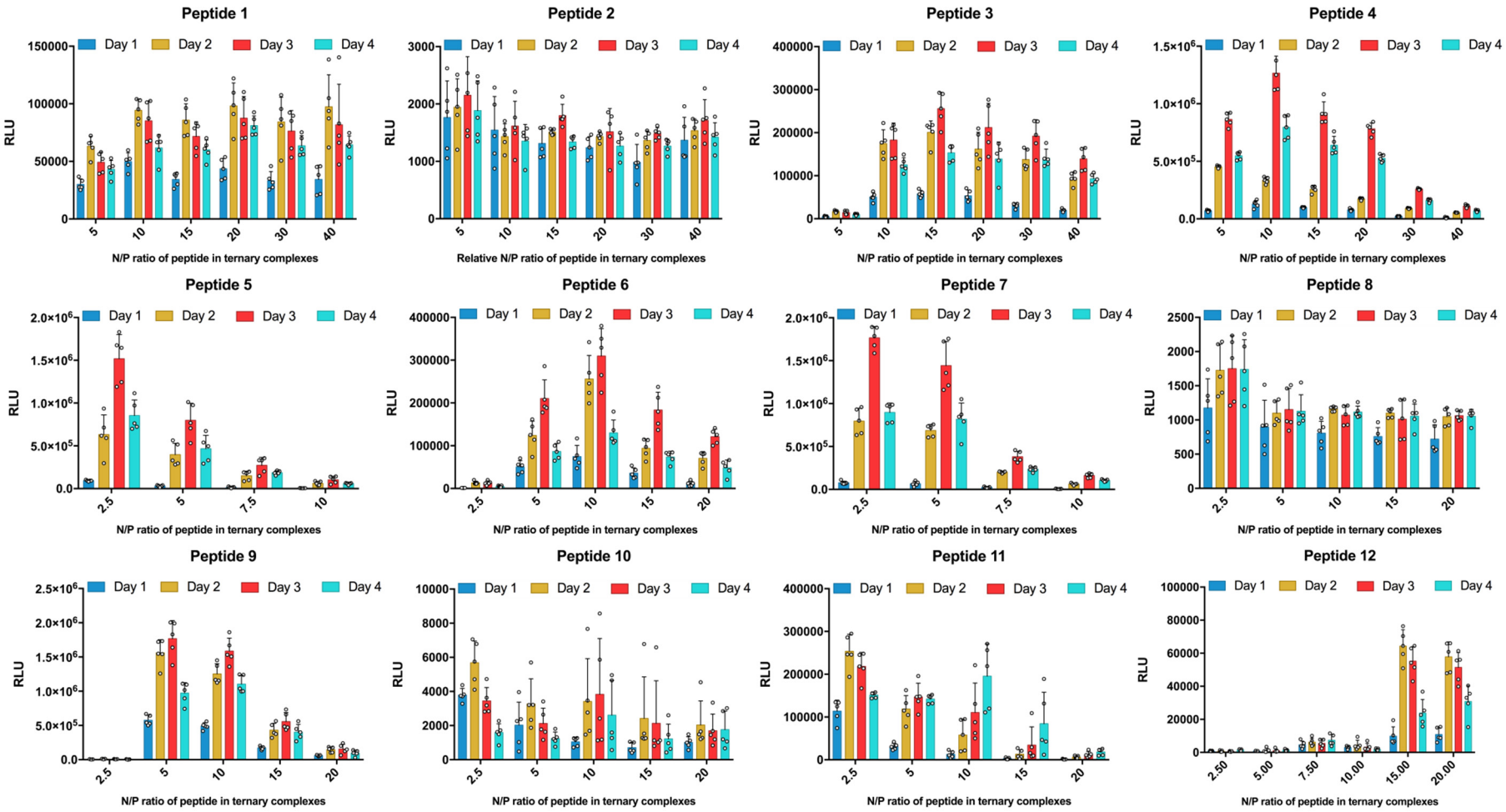
Supplementary Fig. 7 | Evaluation on the important role of each moiety that constitutes peptide0. The function of "anchor moiety" in peptide0 was proved by comparing the optimum **(a)** pDNA and **(b)** mRNA transfection efficiency of peptide0-based ternary complexes with that of peptide1-based ternary complexes in BEAS-2B and 16HBE cells. Peptide1 which did not have the hydrophobic "anchor moiety" was a negative control of peptide0. The function of "cationic moiety" in peptide0 was studied by comparing the optimum **(c)** pDNA and **(d)** mRNA transfection efficiency of peptide0-based ternary complexes with peptide2-based counterparts in BEAS-2B and 16HBE cells. Peptide2 which did not have positively charged amino acids at neutral pH served as a negative control of peptide0. **(e)** The function of "targeting moiety" (nuclear localization signal, NLS) in peptide0 was evaluated by comparing the optimum pDNA transfection efficiency of peptide0-based ternary complex with that of peptide3-based ternary complex in BEAS-2B and 16HBE cells. Peptide3 containing a transport deficient NLS was a negative control of peptide0. The luciferase activity mediated by mRNA transfection was detected 24 h after transfection. The luciferase activity mediated by pDNA transfection was measured on day 1, day 2, day 3 and day 4 after transfection. The data are given as the mean ± SD, n=5 independent experiments. N. S. meant not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ by two-tailed unpaired *t*-test for the indicated comparisons (where applicable, the exact *P* values are shown in the figures).



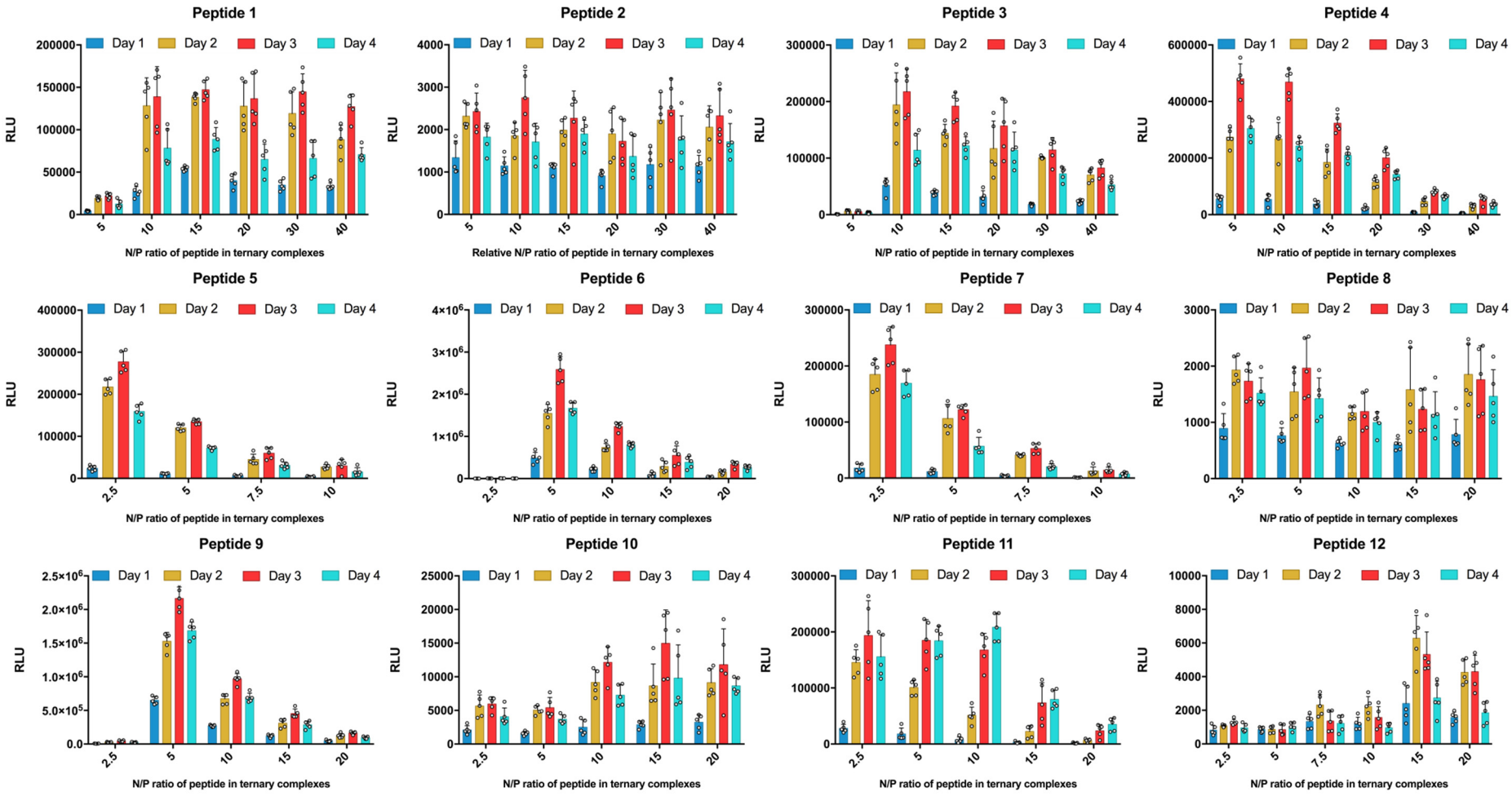
Supplementary Fig. 8 | Transfection efficiency of mRNA-based ternary complexes (comprising different peptides) in BEAS-2B cells. Peptide1 to peptide12 at different N/P ratios (as peptide2 do not contain positively charged amino acids at neutral pH, peptide2 was applied with the same molar concentration of peptide0-based counterpart accordingly) were incubated with T704 at the concentration of 63 (w/w) respectively, then followed by adding MetLuc-mRNA (400ng/well). The luciferase activity was measured 24 h after transfection and was expressed in RLU. Data represent the mean ± SD of five independent experiments.



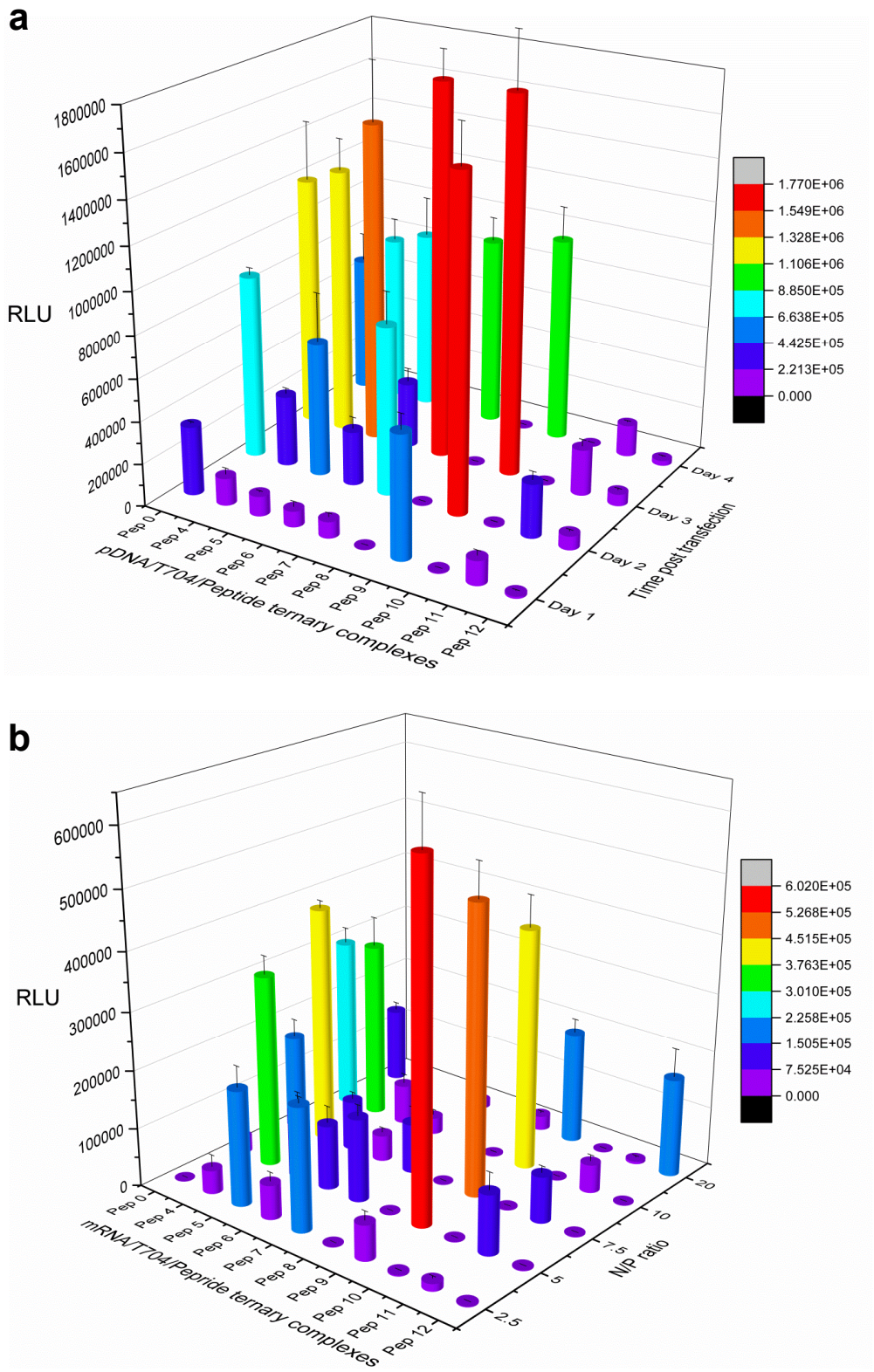
Supplementary Fig. 9 | Transfection efficiency of mRNA-based ternary complexes (comprising different peptides) in 16HBE cells. Peptide1 to peptide12 at different N/P ratios (peptide2 was applied with the same molar concentration of peptide0-based counterpart) were incubated with T704 at the concentration of 63 (w/w) respectively, then followed by adding MetLuc-mRNA (400ng/well). The luciferase activity was measured 24 h after transfection and was expressed in RLU. Data represent the mean ± SD of five independent experiments.



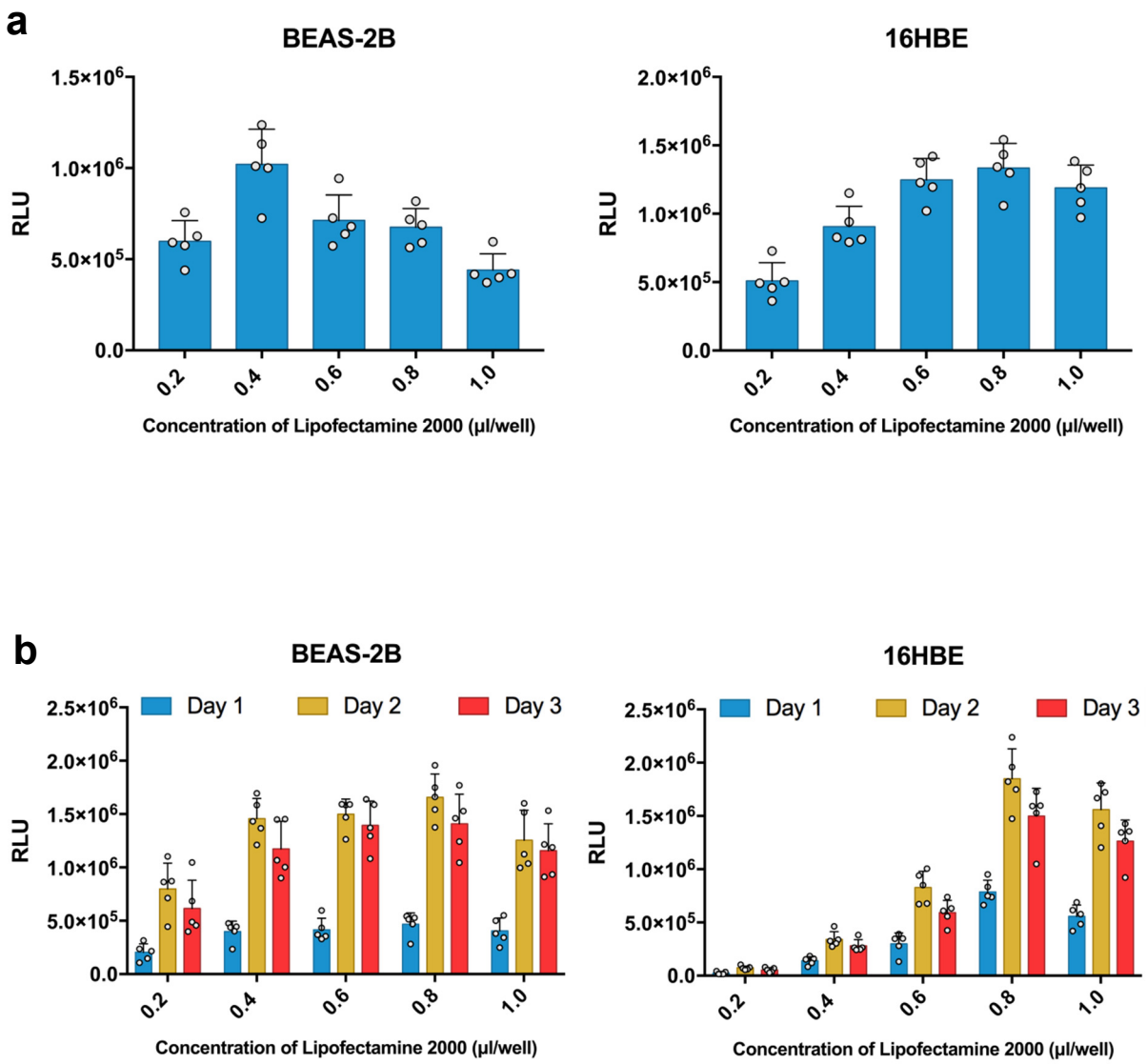
Supplementary Fig. 10 | Transfection efficiency of pDNA-based ternary complexes (comprising different peptides) in BEAS-2B cells. Peptide1 to peptide12 at different N/P ratios (peptide2 was applied with the same molar concentration of peptide0-based counterpart) were incubated with T704 at the concentration of 50 (w/w) respectively, then followed by adding MetLuc-pDNA (400ng/well). The same transfection procedure as described above was applied. The luciferase activity was measured 24 h, 48 h, 72 h and 96 h after transfection and was expressed in RLU. Data represent the mean ± SD of five independent experiments.



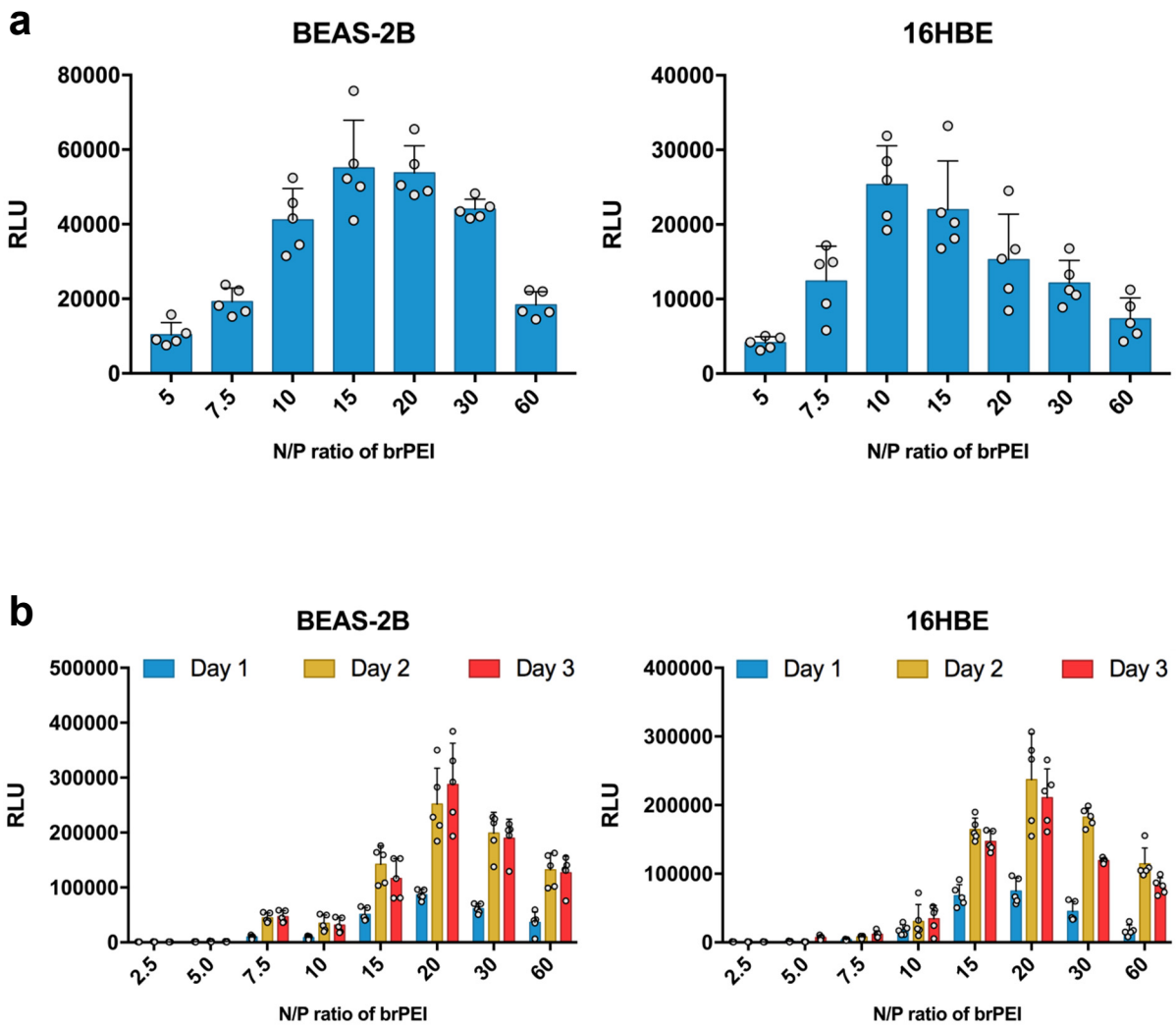
Supplementary Fig. 11 | Transfection efficiency of pDNA-based ternary complexes (comprising different peptides) in 16HBE cells. Peptide1 to peptide12 at different N/P ratios (peptide2 was applied with the same molar concentration of peptide0-based counterpart) were incubated with T704 at the concentration of 63 (w/w) respectively, then followed by adding Metluc-pDNA (400ng/well). The same transfection procedure as described above was applied. The luciferase activity was measured 24 h, 48 h, 72 h and 96 h after transfection and was expressed in RLU. Data represent the mean ± SD of five independent experiments.



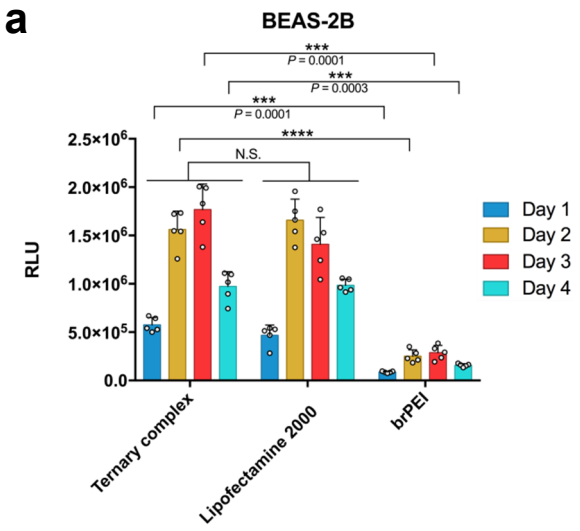
Supplementary Fig. 12 | Supplementary transfection efficiency of ternary complexes comprising different peptides in BEAS-2B cells. (a) MetLuc-pDNA expression mediated by ternary complexes containing different peptides at the optimum N/P ratio. The luciferase activity was measured on day 1, day 2, day 3 and day 4 after transfection. The concentration of T704 in ternary complexes was 50 (w/w). **(b)** MetLuc-mRNA expression mediated by ternary complexes consisting of different peptides at different N/P ratios. The luciferase activity was assayed 24 h after transfection. The concentration of T704 in ternary complexes was fixed at 63 (w/w). Data represent the mean ± SD of five independent experiments.

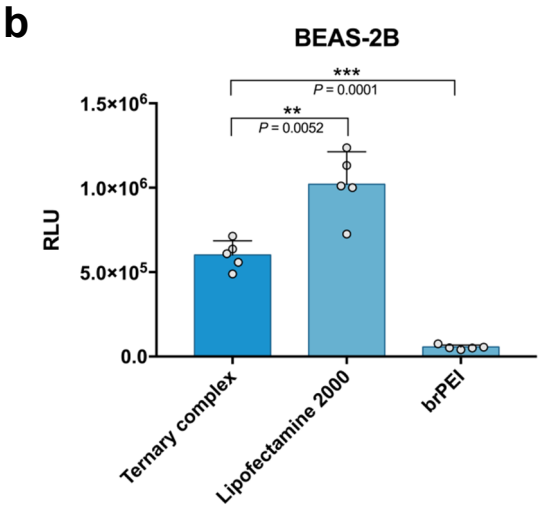


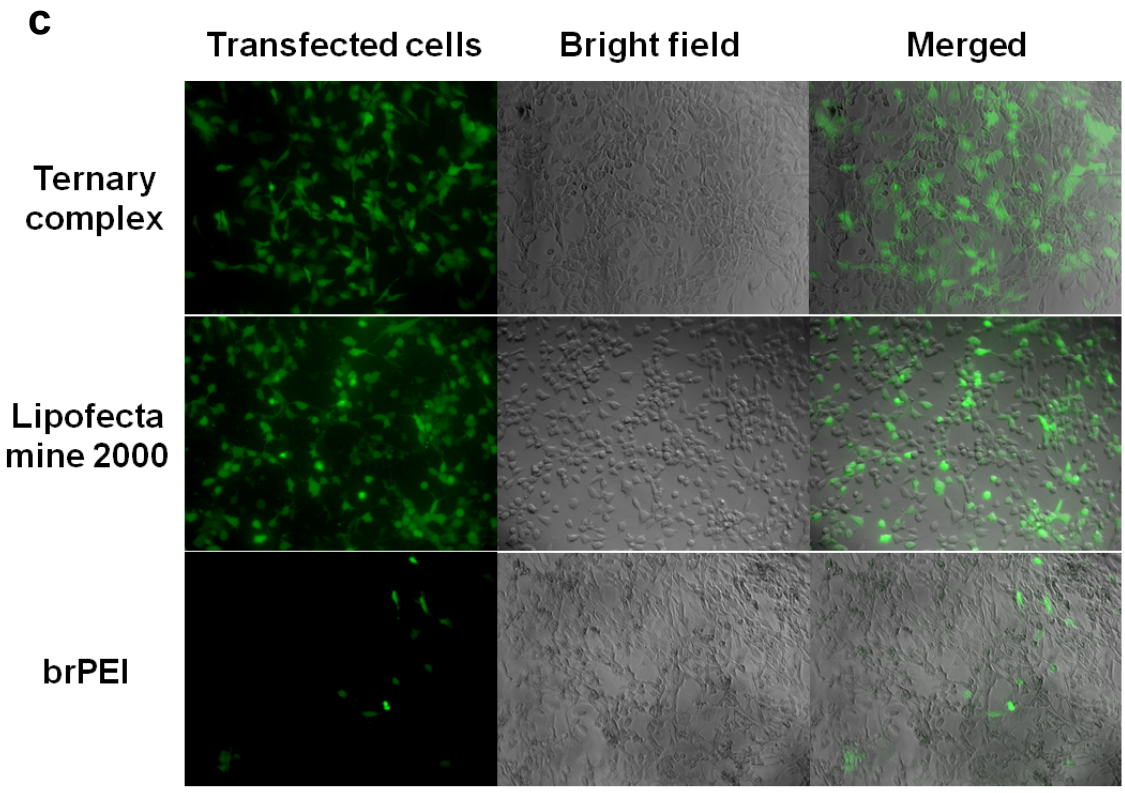
Supplementary Fig. 13 | *In vitro* transfection profile of Lipofectamine2000 (a state-of-the-art gene delivery vehicle)-based lipoplexes in BEAS-2B and 16HBE cells. (a) 400 ng/well MetLuc-mRNA was incubated with Lipofectamine2000 at different concentrations from 0.2 to 1.0 µg/well (relative to 96-well plate) in OptiMEM. After 30 min incubation at RT, samples were added into 96-well plate containing pre-seeded cells and incubated with the cells in OptiMEM (without serum and antibiotics) for 4 h at 37 ℃ in a humidified atmosphere (95% air, 5% CO_2). The luciferase activity in 50 µl supernatants from transfected cells was detected after 24 h and its activity was expressed in RLU. **(b)** MetLuc-pDNA/Lipofecamine2000 lipoplex was prepared in the same way as the mRNA counterpart, the luciferase activity was measured 24 h, 48 h and 72 h after transfection and its activity was expressed in RLU. Data represent the mean ± SD of five independent experiments.

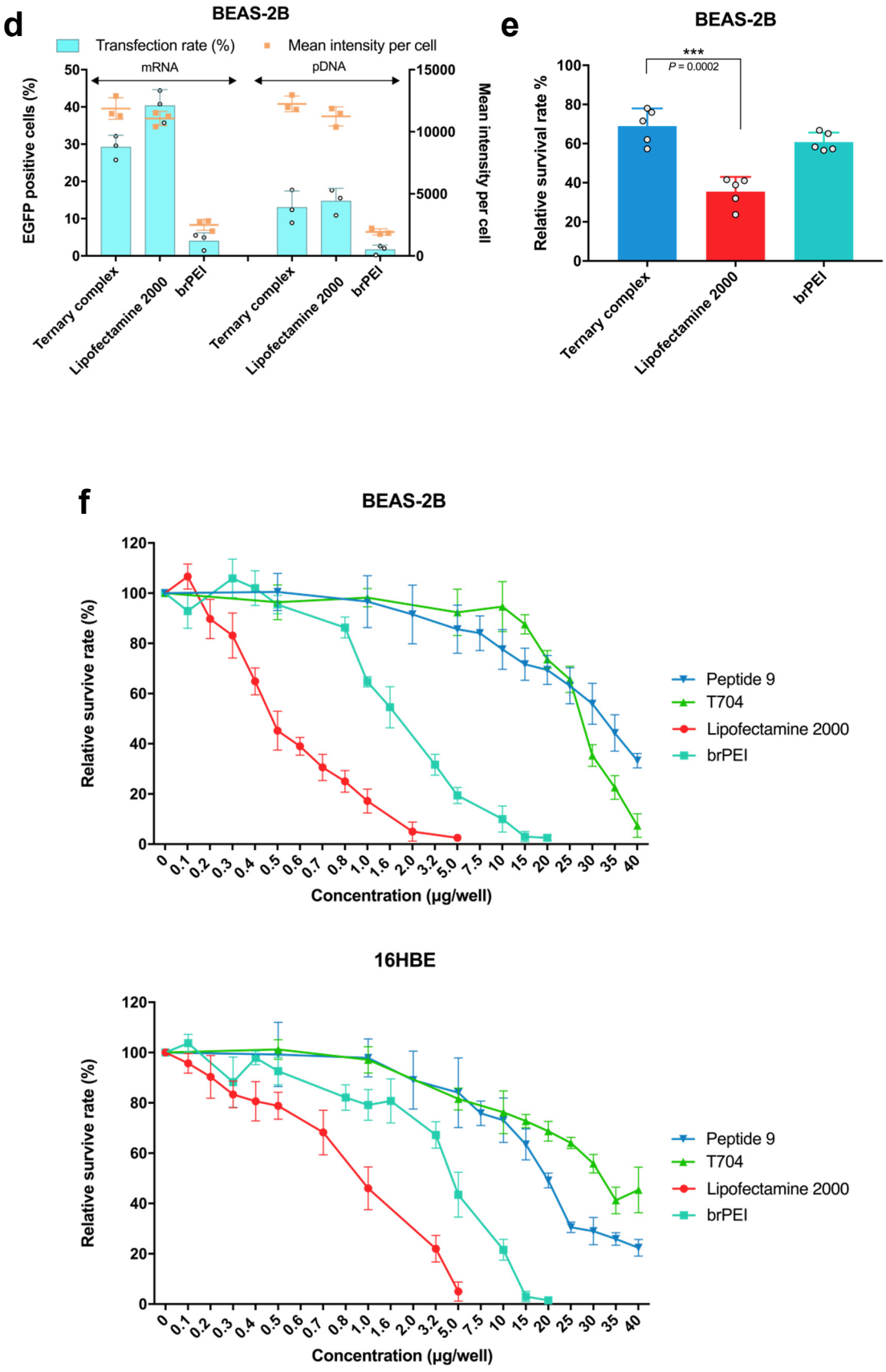


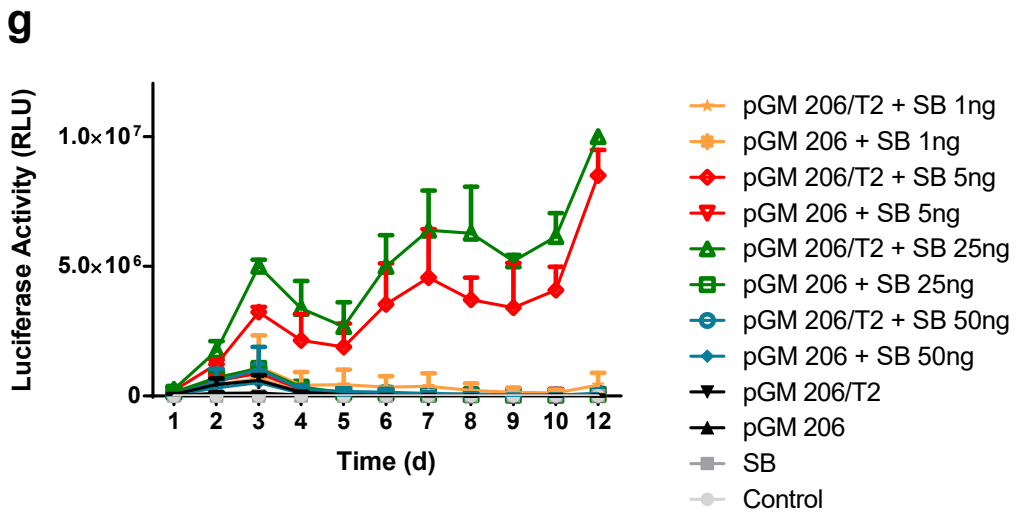
Supplementary Fig. 14 | *In vitro* transfection profile of 25 kDa brPEI (a polymer-based gold standard in gene delivery) based polyplexes in BEAS-2B and 16HBE cells. (a) 400 ng/well MetLuc-mRNA was incubated with brPEI at different N/P ratios from 5 to 60 in nuclease-free water. After 30 min incubation at RT, samples were added into 96-well plate containing pre-seeded cells and incubated with the cells in OptiMEM (without serum and antibiotics) for 4 h at 37 ℃ in a humidified atmosphere (95% air, 5% CO_2). The luciferase activity in 50 µl supernatants from transfected cells was assayed after 24 h and its activity was expressed in RLU. **(b)** MetLuc-pDNA/brPEI polyplex was prepared in the same way as the mRNA counterpart, the luciferase activity was measured 24 h, 48 h and 72 h after transfection and its activity was expressed in RLU. The data are given as the mean ± SD of five independent experiments.


a
BEAS-2B
***
P = 0.0001
***
P = 0.0003
***
P = 0.0001
****
N.S.
2.5×10^6
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0.0
RLU
Day 1
Day 2
Day 3
Day 4
Ternary complex
Lipofectamine 2000
brPEI

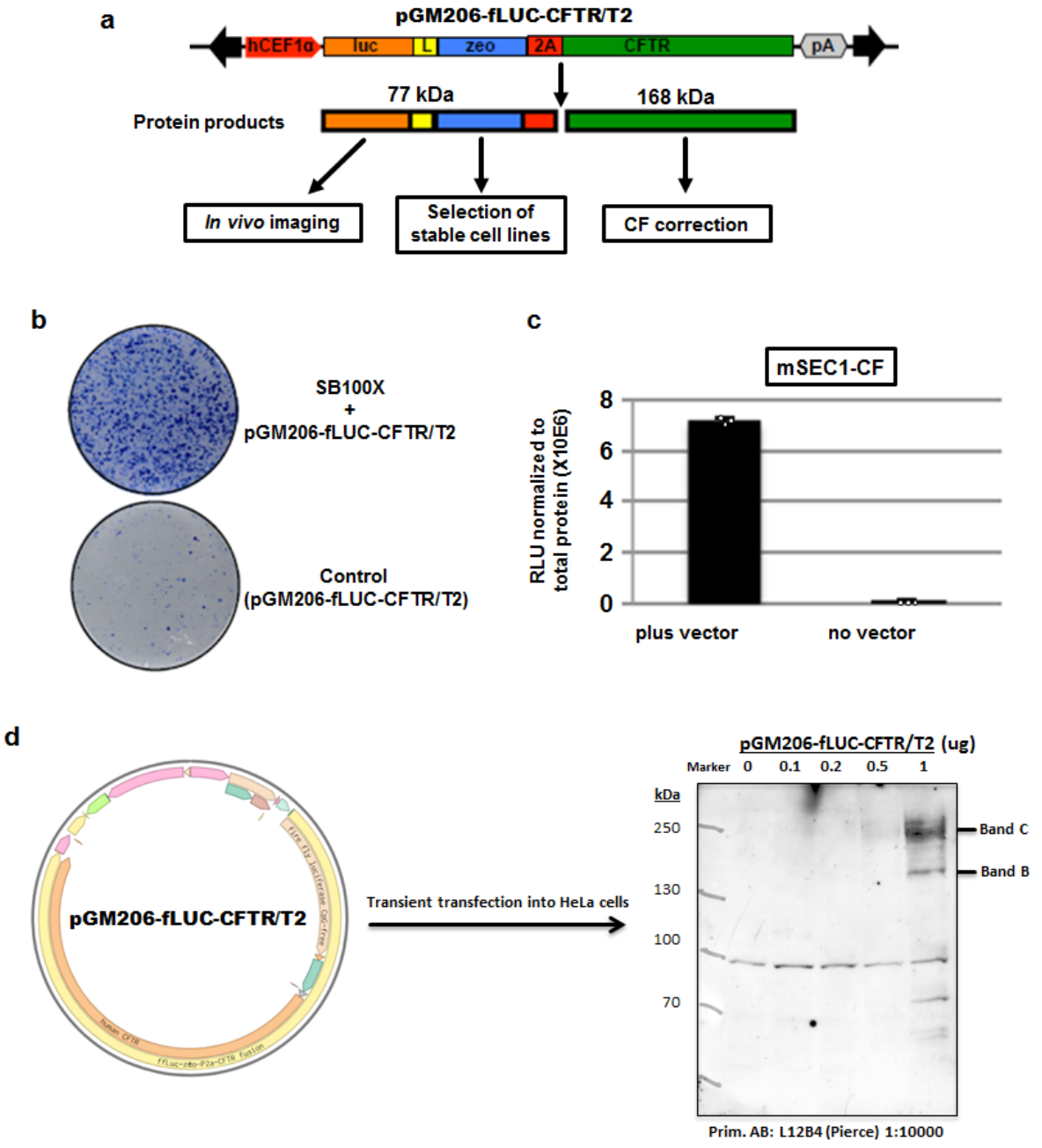

b
BEAS-2B
***
P = 0.0001
**
P = 0.0052
1.5×10^6
1.0×10^6
5.0×10^5
0.0
RLU
Ternary complex
Lipofectamine 2000
brPEI


c
Transfected cells
Bright field
Merged
Ternary complex
Lipofecta mine 2000
brPEI

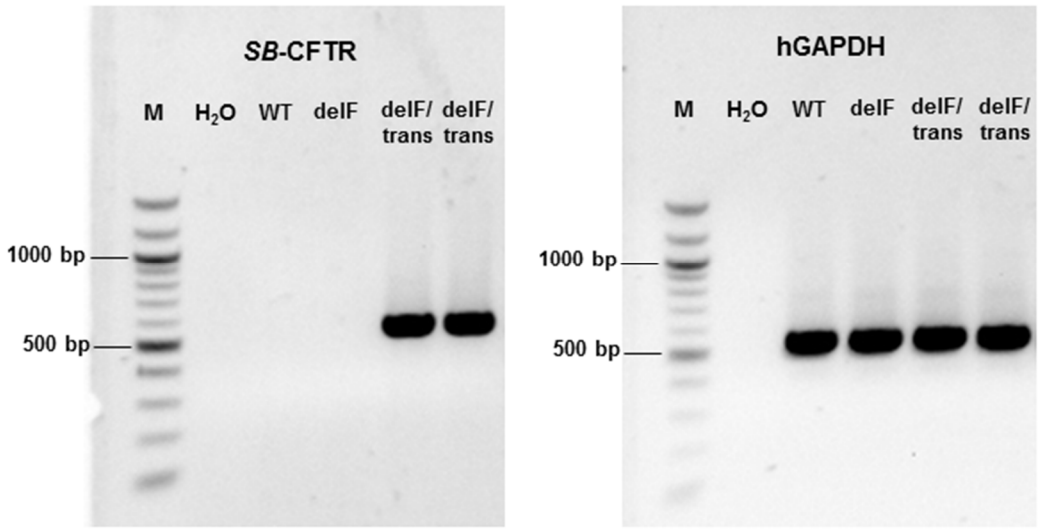

d
BEAS-2B
Transfection rate (%)
Mean intensity per cell
mRNA
pDNA
EGFP positive cells (%)
Mean intensity per cell
Ternary complex
Lipofectamine 2000
brPEI
e
BEAS-2B
***
P = 0.0002
Relative survival rate %
f
BEAS-2B
Relative survive rate (%)
Concentration (μg/well)
Peptide 9
T704
Lipofectamine 2000
brPEI
16HBE



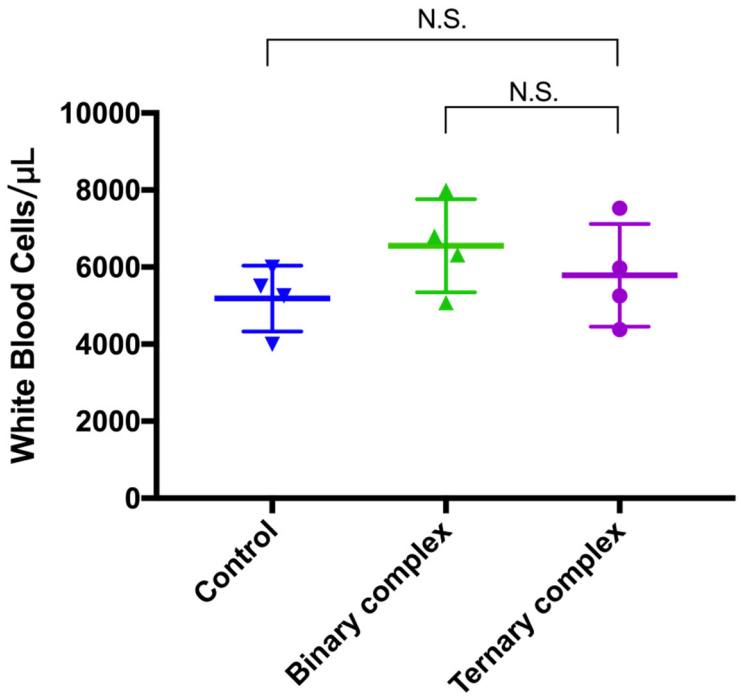
Supplementary Fig. 15 | Supplementary *in vitro* transfection efficiency and cytotoxicity of ternary complexes (T704 + peptide9 + pDNA or mRNA). Comparisons of the *in vitro* transfection efficiency induced by **(a)** pDNA/T704/peptide9 or **(b)** mRNA/T704/peptide9 ternary complexes and other nonviral counterparts in BEAS-2B cells. All formulations were prepared at the optimum ratio to ensure their maximum transfection efficiency on each cell line. N. S. meant not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by two-tailed unpaired *t*-test for the indicated comparisons (where applicable, the exact *P* values are shown in the figures). **(c)** Transgene expression of EGFP-mRNA-based ternary complex, Lipofectamine2000-based lipoplex and brPEI-based polyplex in BEAS-2B cells determined by fluorescent microscope. The data in **c** were repeated eight times independently with similar results. **(d)** Quantitative investigations on the EGFP positive cells and their mean fluorescence intensity in EGFP-mRNA-based or EGFP-pDNA-based formulations transfected BEAS-2B cells via flow cytometry analysis. Bars represent the rate of EGFP-positive cells, orange dots refer to the mean fluorescence intensity per cell. Data are given as the mean ± SD of three independent experiments. **(e)** The cytotoxicity of ternary complex, Lipofectamine2000-based lipoplex and brPEI-based polyplex (prepared under conditions showing highest transgene expression) against BEAS-2B cells. Two-tailed unpaired *t*-test, $^{***}P = 0.0002$. **(f)** The cytotoxicity of T704, peptide9, Lipofectamine2000 and brPEI at different concentrations (normalized to μg/well) against BEAS-2B and 16HBE cells. The MTT assay was performed 24 h after 4 h incubation. **(g)** Long-term expression of luciferase in BEAS-2B cells transfected by *SleepingBeauty* (*SB*)-transposon system with various combinations. The amount of pDNA was fixed at 250 ng/well. "SB" represents *SB*100X-mRNA. Untreated cells were used as "control". Where applicable, the results represent the mean ± SD of five independent experiments.



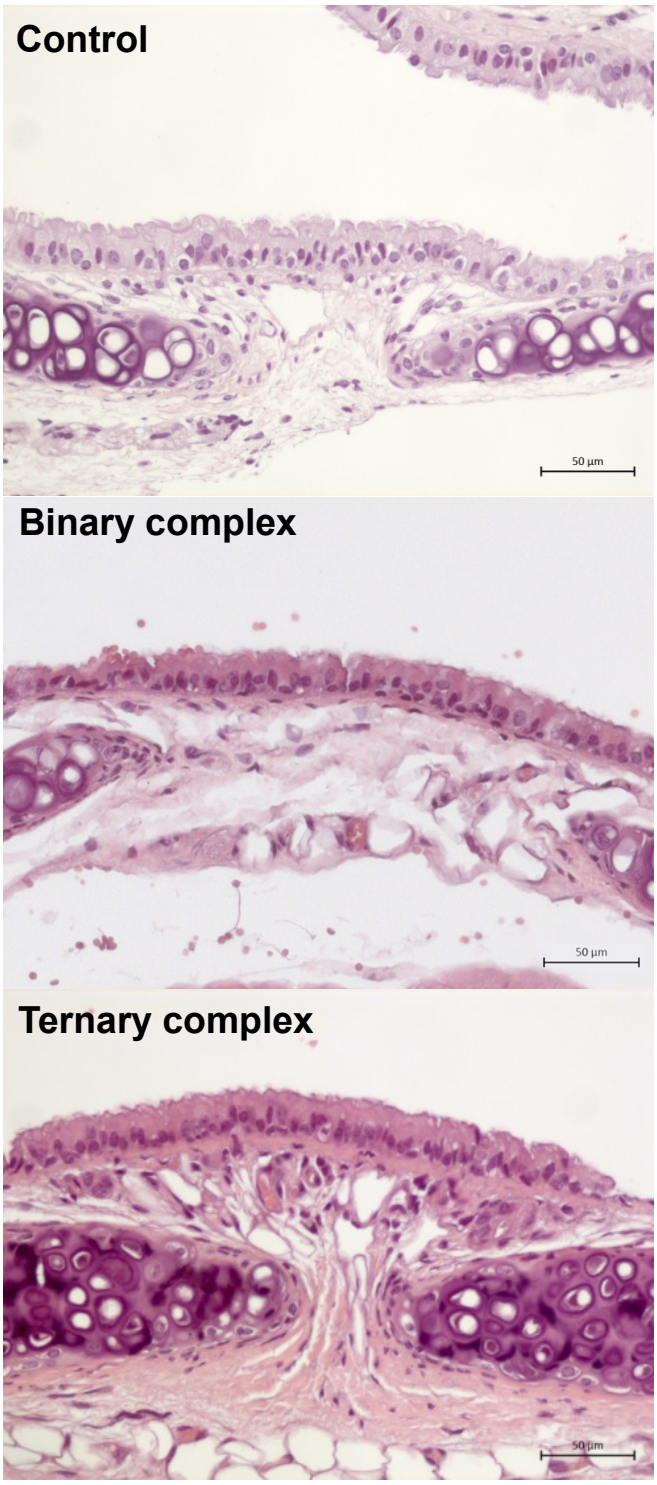
Supplementary Fig. 16 | Preliminary functional tests of pGM206-fLUC-CFTR/T2. (a) Vector design and protein products. **(b)** *SB*100X-catalyzed transposon integration in mouse mSEC1-CF cells after zeocin selection. **(c)** The firefly luciferase activity in transgenic mSEC1-CF cells compared to non-transgenic cells. The results are expressed as the mean ± SD of three biologically independent samples. **(d)** Western blot analysis of CFTR within pGM206-fLUC-CFTR/T2 transfected Hela cells. The results of **d** were repeated eight times independently with similar results.



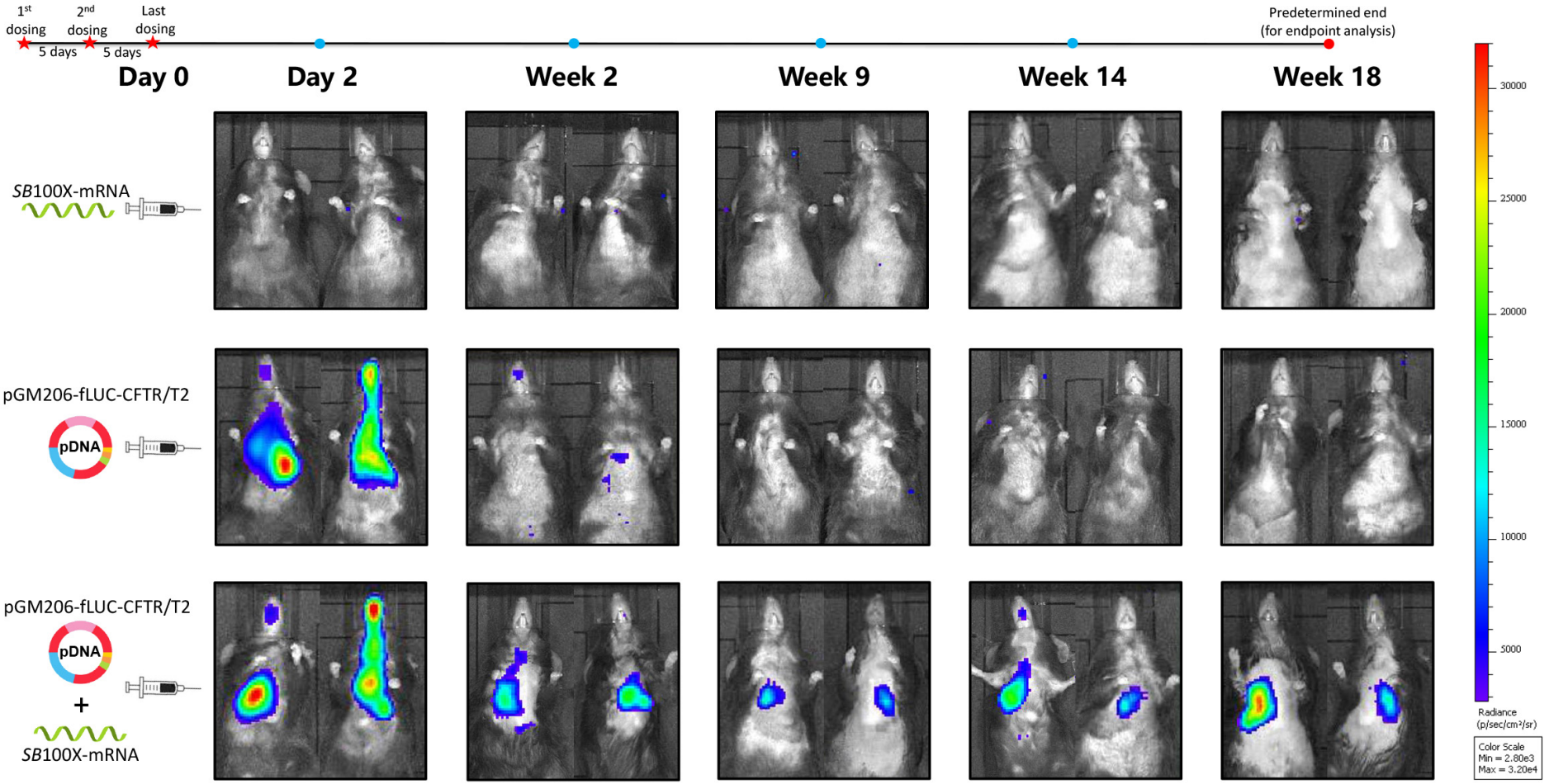
Supplementary Fig. 17 | Representative gel of 1% agarose gel electrophoresis of reverse transcription-PCR product for *Sleeping Beauty* transposon specific CFTR (*SB*-CFTR) gene in cultured cells. Lane M represents 100 bp ladder. Lane H_2O is a nuclease-free water-based negative control. Lane WT represents the sample derived from untreated CFBE-WT cells. Lane delF is the sample prepared from untreated CFBE-delF cells. Lane delF/trans is the sample obtained from *SB*-transposon system (pGM206-fLUC-CFTR/T2+*SB*100X-mRNA) transfected CFBE-delF cells (two weeks after transfection). Human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) was applied as an internal marker. The data were repeated three times independently with similar results.



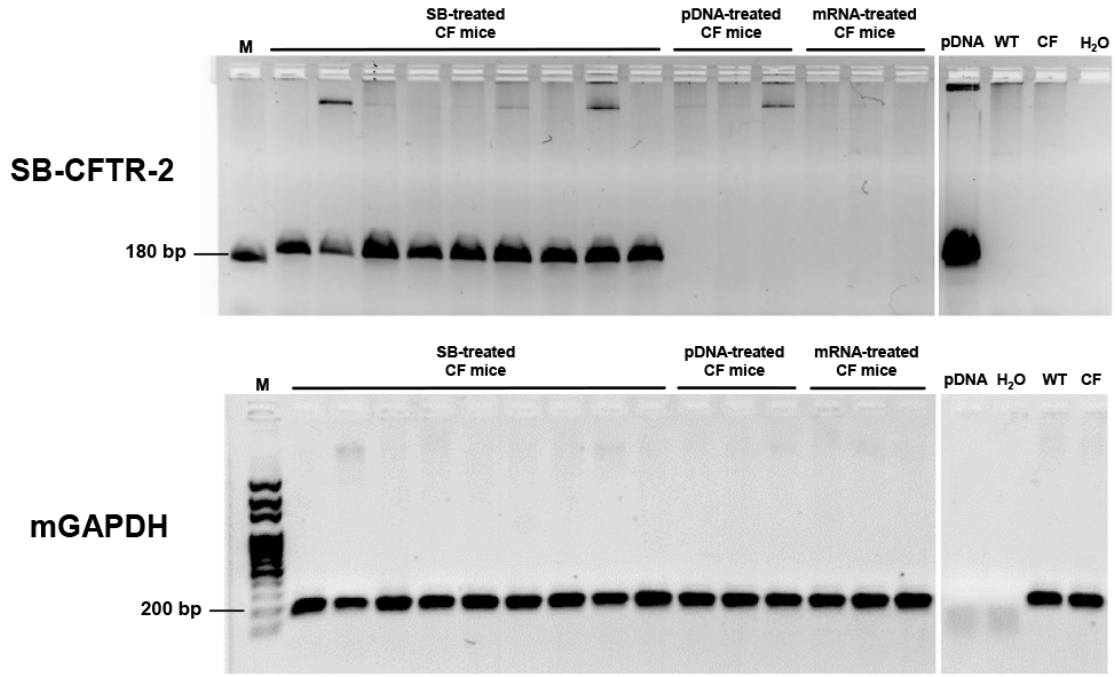
Supplementary Fig. 18 | White blood cell counts (24 h after dosing) detected in blood samples from NF-water (control), mRNA/T704-based binary and ternary complexes treated mice. The result from each sample was measured and quantitatively compared. The data represent the mean ± SD of four biologically independent animals. N. S. meant not significant for the indicated comparisons, by two-tailed unpaired *t*-test.



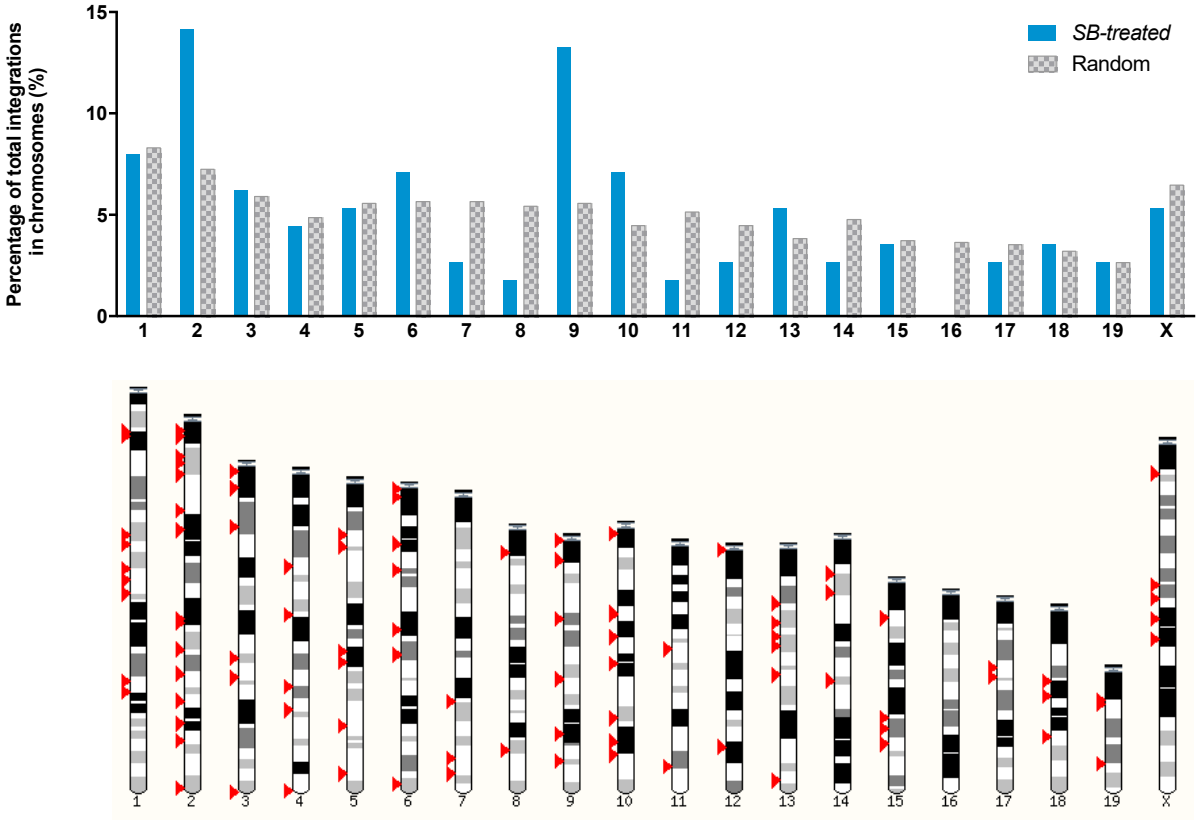
Supplementary Fig. 19 | Representative histopathologic analysis of trachea sections of CF mice. Samples were obtained from mice treated with NF-water (control), T704-based binary and ternary complex 48 h after dosing. Haematoxylin-Eosin (H&E) staining, ×40 magnification. The data were repeated six times independently with similar results.



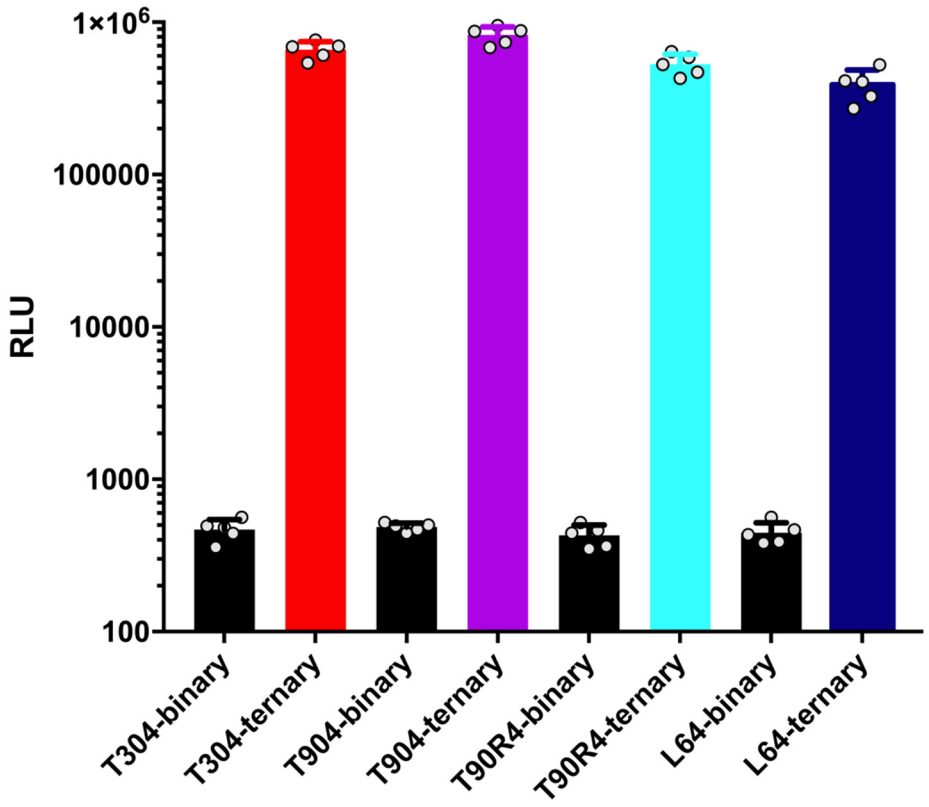
Supplementary Fig. 20 | The *SB*-transposon system mediated persistent and stable luciferase reporter gene expression in CF mouse airways. pDNA containing a T2 *SB* transposon encoding both firefly luciferase and human CFTR (pGM206-fLUC-CFTR/T2) was co-delivered to CF mouse airways with mRNA encoding *SB* transposase (*SB*100X-mRNA) via ternary complex-based formulations. Mice treated with the single formulation of pGM206-fLUC-CFTR/T2 or *SB*100X-mRNA were applied as controls. Luciferase expression at indicated time points was measured by non-invasive imaging. The monitoring process continued 18 weeks until the predetermined end. The experiment scheme was displayed in the upper line. Two representative bioluminescence images from each group were illustrated at indicated time points. The experiments were repeated three times independently with similar results.



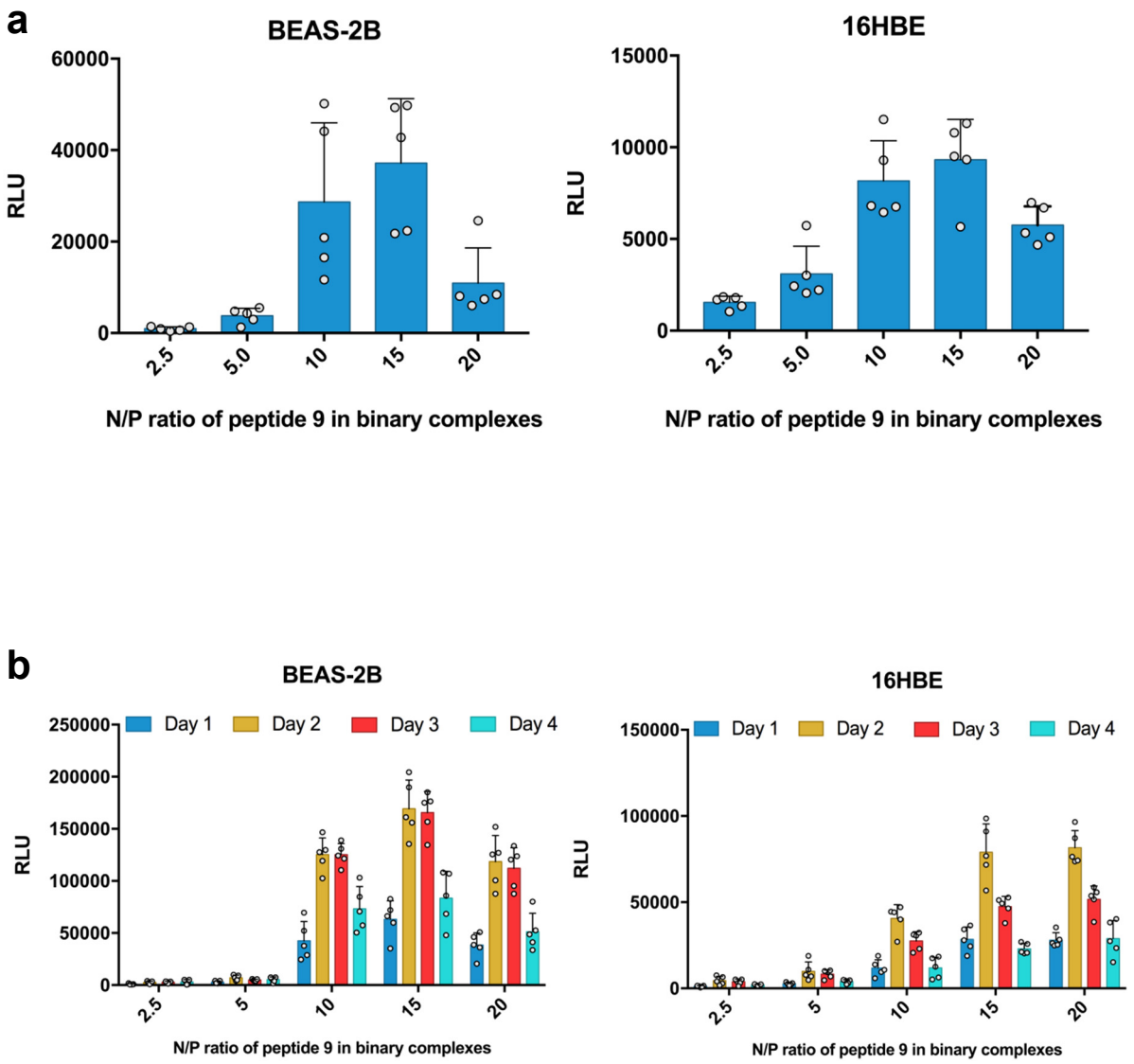
Supplementary Fig. 21 | Conventional PCR for *SB*-transposon system specific CFTR-gene in WT mouse, untreated CF mouse, (pGM206-fLUC-CFTR/T2+*SB*100X-mRNA) co-treated, pGM206-fLUC-CFTR/T2-treated, *SB*100X-mRNA-treated CF mice eighteen weeks after dosing (Upper panel). Lanes "SB-treated CF mice" represent the sample obtained from (pGM206-fLUC-CFTR/T2+*SB*100X-mRNA) co-transfected CF mice (n=9 biologically independent animals). Lanes "pDNA-treated CF mice" represent counterparts prepared from pGM206-fLUC-CFTR/T2-treated CF mice (n=3 biologically independent animals). Lanes "mRNA-treated CF mice" represent counterparts obtained from *SB*100X-mRNA-treated CF mice (n=3 biologically independent animals). Each lane within above-mentioned groups represents one animal. Lane M represents the marker. Lane pDNA is a pGM206-fLUC-CFTR/T2-based positive control. Lane H_2O is a nuclease-free water-based negative control. Lane WT represents the sample derived from untreated wild-type mouse. Lane CF is the sample obtained from untreated CF mouse. Mouse glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) was applied as an internal marker (Lower panel).



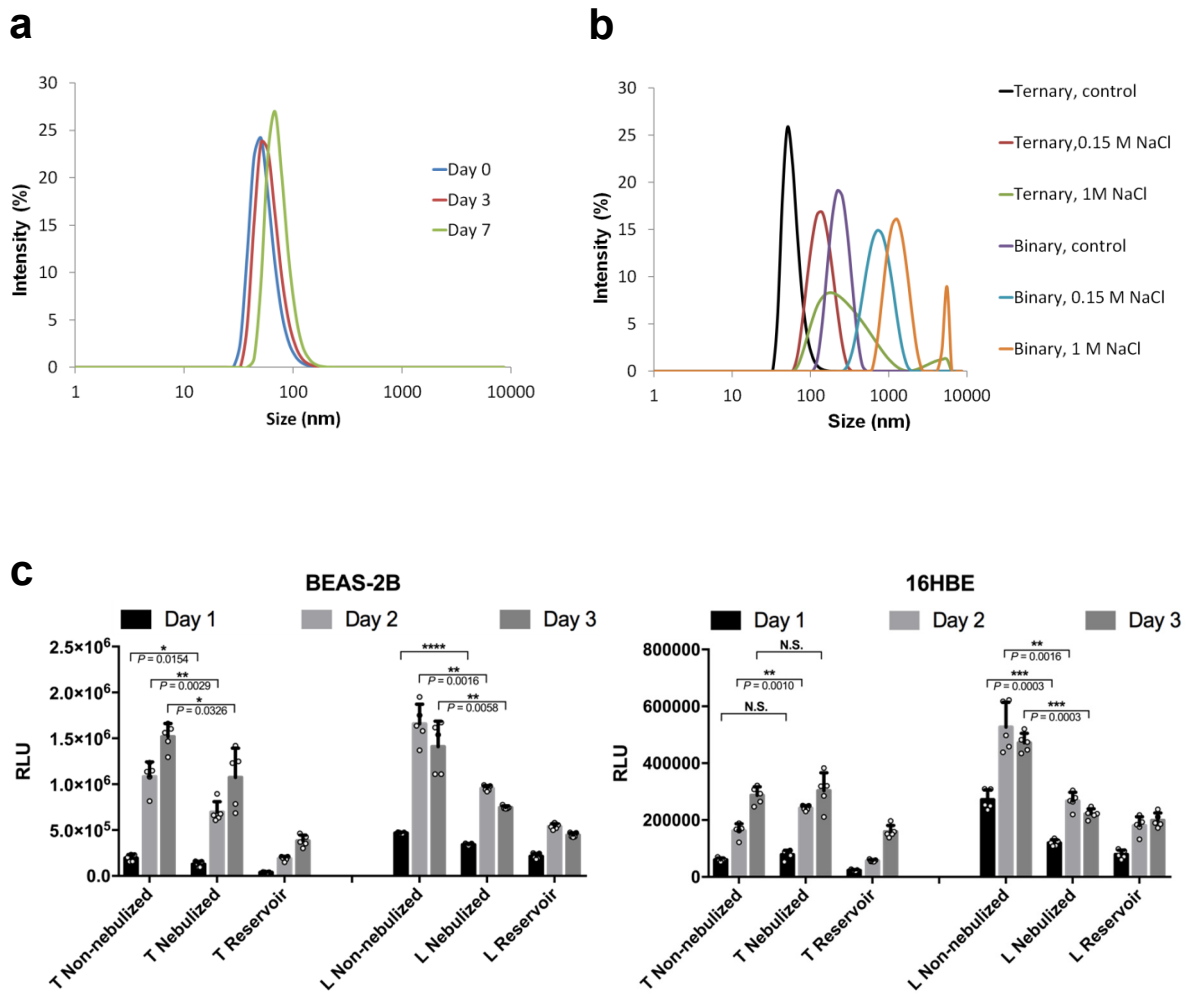
Supplementary Fig. 22 | Integration sites identified within the genomic DNA of (pGM206-fLUC-CFTR/T2+SB100X-mRNA) co-treated mice (n=3 biologically independent animals) eighteen weeks post-dosing. Upper panel: the ratio of transposition events within each chromosome to the total integration events (*SB*-treated), a computer-stimulated random integration profile was applied as a control (Random). Lower panel: a schematic overview of the chromosome distribution of detected integration sites.



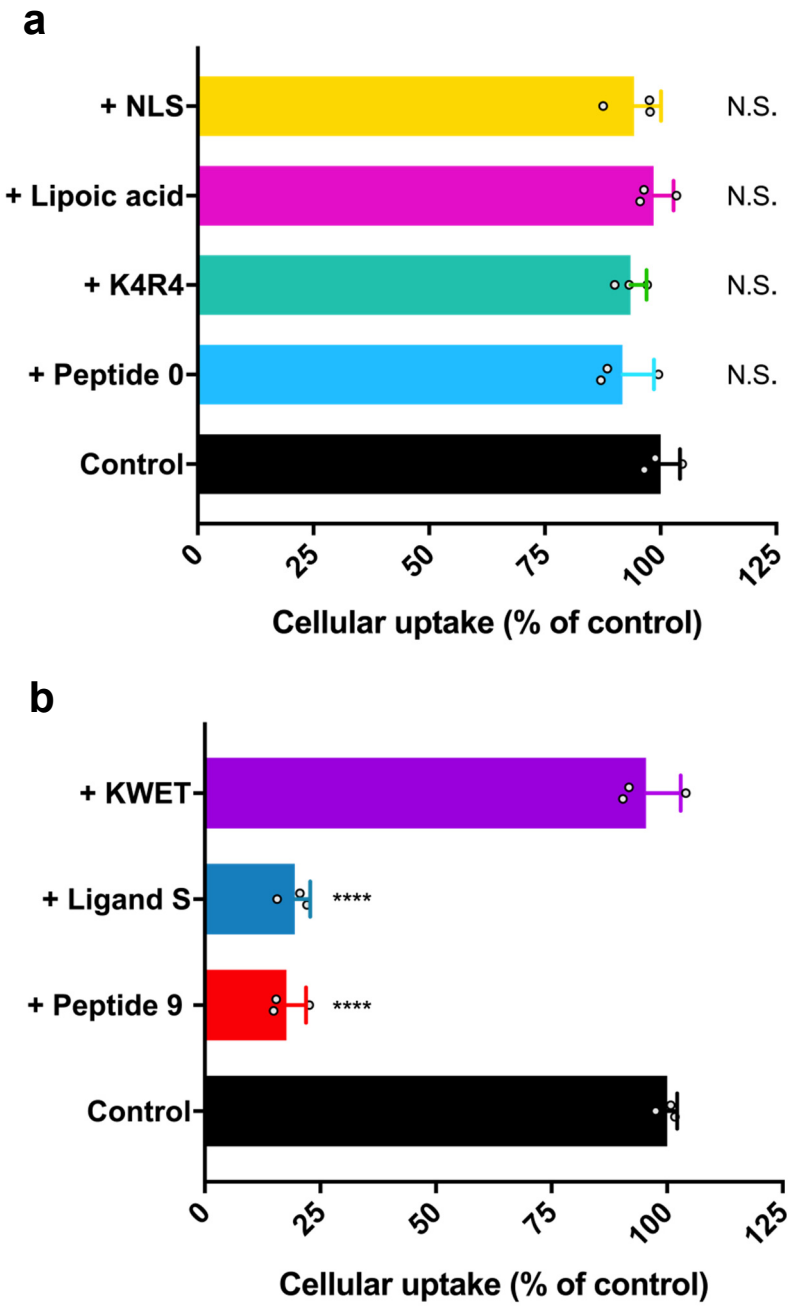
Supplementary Fig. 23 | Representative transfection efficiency of MetLuc-mRNA-based ternary complexes comprising other poloxamines or a poloxamer in 16HBE cells. "T304-binary" represents binary complex prepared by poloxamine 304 (T304) at concentration of 2000 (w/w, relative to mRNA) and MetLuc-mRNA; "T304-ternary" means ternary complex consisting of T304 at concentration of 2000 (w/w), peptide8 at the N/P ratio of 15 and MetLuc-mRNA; "T904-binary" was prepared by poloxamine 904 (T904) at concentration of 100 (w/w) and MetLuc-mRNA; "T904-ternary" represents ternary complex comprising T904 at concentration of 100 (w/w), peptide6 at the N/P ratio of 5 and MetLuc-mRNA; "T90R4-binary" was prepared by poloxamine 90R4 (T90R4) at concentration of 1600 (w/w) and MetLuc-mRNA; "T90R4-ternary" represents ternary complex containing T90R4 at concentration of 1600 (w/w), peptide7 at the N/P ratio of 2.5 and MetLuc-mRNA; "L64-binary" represents binary complex prepared by poloxamer 184 (L64) at concentration of 300 (w/w) and MetLuc-mRNA; "L64-ternary" means ternary complex consisting of L64 at concentration of 300 (w/w), peptide0 at the N/P ratio of 5 and MetLuc-mRNA. All binary complexes were prepared in Tyrode's solution, and all ternary complexes were prepared in NF-water. The concentration of MetLuc-mRNA in all groups was fixed at 400 ng/well (relative to 96 well plate). The luciferase activity was measured 24 h after transfection (4 h incubation) and was expressed in RLU. Data represent the mean ± SD of five independent experiments.



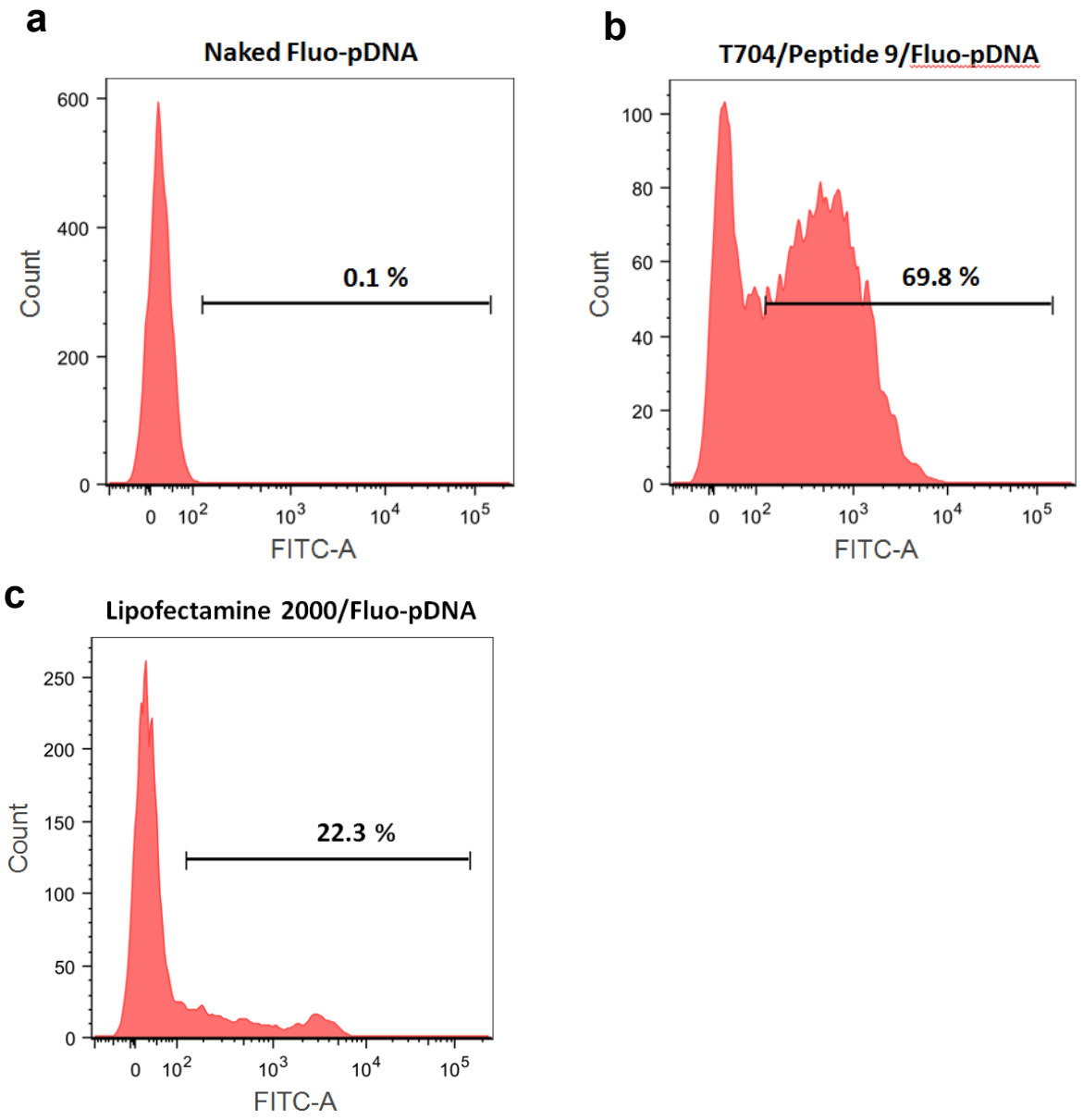
Supplementary Fig. 24 | *In vitro* transfection profile of peptide9-based binary complexes in BEAS-2B and 16HBE cells. (a) 400 ng/well MetLuc-mRNA was incubated with peptide9 at different N/P ratios from 2.5 to 20 in NF-water. After 30 min incubation at RT, samples were added into 96-well plate containing pre-seeded cells and incubated with the cells in Opti-MEM (without serum and antibiotics) for 4 h at 37 ℃ in a humidified atmosphere (95% air, 5% CO_2). The luciferase activity in 50 µl supernatants from transfected BEAS-2B or 16HBE cells was measured after 24 h and its activity was expressed in RLU. **(b)** MetLuc-pDNA/peptide9 binary complexes were prepared in the same way as the mRNA counterpart, the luciferase activity in 50 µl supernatants from transfected cells was measured 24 h, 48 h, 72 h and 96 h after transfection and its activity was expressed in RLU. The data are given as the mean ± SD of five independent experiments.



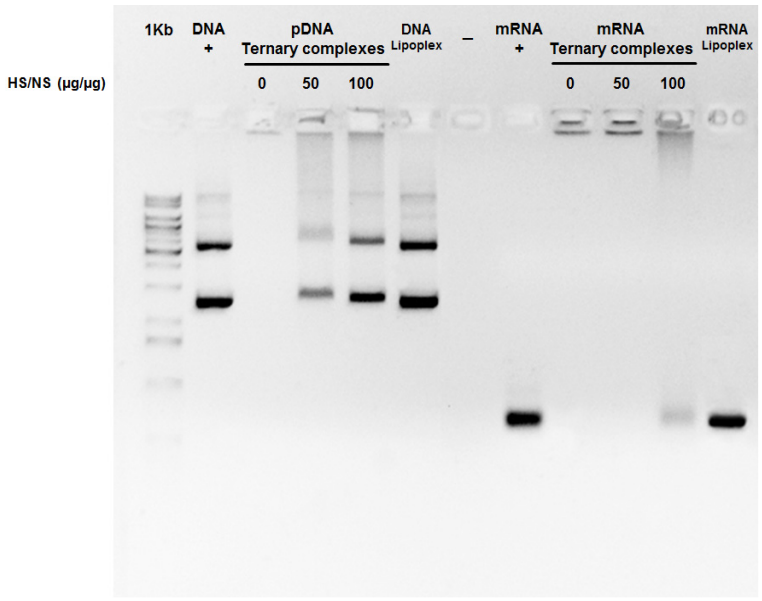
Supplementary Fig. 25 | Supplementary investigations on the stability of ternary complexes and binary counterparts. (a) The hydrodynamic diameter of ternary complex (pDNA/T704/peptide9) in nuclease-free water (NF-water) at room temperature. **(b)** The stability of pDNA/T704 binary complex and pDNA/T704/peptide9 ternary complex against solution with different salt concentrations. **(c)** The influence of nebulization process on the transfection efficiency of pDNA/T704/peptide0 ternary complex and Lipofectamine2000-based formulations. "T" means pDNA/T704/peptide0 ternary complex and "L" means pDNA/Lipofectamine2000 lipoplex. The data are given as the mean ± SD of five biologically independent samples. N. S. meant not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ by two-tailed unpaired *t*-test for the indicated comparisons (where applicable, the exact *P* values are shown in the figures). The results of **a**,**b** were repeated four times independently with similar results.



Supplementary Fig. 26 | Investigations on the receptor mediated cellular uptake of the ternary complex. (a) Non-receptor mediated endocytosis of peptide0-based ternary complex, which cannot be inhibited by an excess amount of free peptide0 and its fragment sequence (i.e., NLS, Lipoic acid and K4R4). K4R4 represents the peptide KKRRRRKK. The non-inhibited cellular uptake of peptide0-based ternary complex was used as the control. **(b)** Receptor-mediated cellular uptake of peptide9-based ternary complex, which was significantly inhibited by an excess amount of free peptide9 and the free ligand S (GACSERSMNFCG) which is contained in peptide9. KWET represents the peptide KETWWETWWTEWWTEW which is the hydrophobic moiety of peptide9. The non-inhibited cellular uptake of peptide9-based ternary complex was used as the control. All data are expressed as the mean ± SD of three biologically independent samples. ****$P < 0.0001$ compared with the control group, N. S. meant not significant compared with the control, using two-tailed unpaired *t*-test.



Supplementary Fig. 27 | Supplementary cellular uptake profiles of peptide9-based ternary complex and Lipofectamine2000-based lipoplex. The cellular uptake of **(a)** naked fluorescein labelled pDNA (Fluo-pDNA), **(b)** Fluo-pDNA/T704/peptide9 ternary complex and **(c)** Fluo-pDNA /Lipofectamine2000 lipoplex in 16HBE cells. All Data were repeated three times independently with similar results.



Supplementary Fig. 28 | Binding and condensation properties of ternary complexes containing pDNA or mRNA. Complexes were incubated with increasing amounts of heparan sulfate (HS) per µg nucleic acid (NS). Naked nucleic acid was used as a positive control (+) and NF-water as a negative control (-). "DNA Lipoplex" represents Lipofectamine2000-based lipoplex containing pDNA with the optimum composition. "mRNA Lipoplex" represents Lipofectamine2000-based lipoplex containing mRNA with the optimum composition. The results were repeated three times independently with similar results.

SUPPLEMENTARY METHODS

Reagents. Poloxamine 304 (T304) and poloxamine 904 (T904) were kindly provided by BASF GmbH (Ludwigshafen, Germany). Poloxamine 90R4 (T90R4), poloxamer 184 (L64) and heparan sulfate were purchased from Sigma-Aldrich (Germany). 2-Chlorotrityl chloride resin, all Fmoc or Boc protected α-amino acids, N,N-diisopropylethylamine (DIPEA), piperidine and trifluoroacetic acid (TFA) were purchased from Iris Biotech (Marktredwitz, Germany). All other solvents and small molecular reagents were obtained in high quality (analytical or HPLC grade) from Sigma-Aldrich (Germany).

Synthesis of peptides. All peptides were produced by Fmoc solid-phase peptide synthesis. Briefly, 2-Chlorotrityl chloride resin was applied as the solid phase. 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) was used as the coupling reagent and N,N-diisopropylethylamine (DIPEA) served as the base. Lipoic acid, myristic acid or cholesterol was coupled to the peptides before deprotection and cleaving. The peptides were subsequently cleaved from the resin by incubation in dichloromethane methylene chloride/trifluoroethanol/acetic acid (70/20/10, v/v/v) at room temperature (RT) for 2 h. The resin was filtrated, and the solvent was removed by evaporation. After deprotection with trifluoroacetic acid/water/phenol/triisopropylethylamine (88:5:5:2) at RT for 2 h, the yield solution was added into diethyl ether on ice for precipitation. The final peptides were purified using reverse-phase HPLC and were confirmed by liquid chromatography-mass spectroscopy (LC/MS).

Cell culture. BEAS-2B (human bronchial epithelial cell line) cells were obtained from the ATCC (American Type Culture Collection, Wesel, Germany) and were cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 1% (v/v) penicillin/streptomycin (Gibco). 16HBE (human bronchial epithelial cell line) cells were generously provided by Prof. Dr. Dieter C. Gruenert (University of California at San Francisco, CA, USA) and were cultured in Eagle's Minimal Essential Medium (Gibco) with 10% heat-inactivated FBS (Gibco) and 1% (v/v) penicillin/streptomycin (Gibco) at 37 ℃ and 5% CO_2. CFBE-WT cells expressing wild-type CFTR and CFBE-delF cells which contain the most common CF phenotype causing mutation (F508del) were obtained from Gregory Fleming James Cystic Fibrosis Research Center (University of Alabama at

Birmingham, AL, USA) and were cultured in the same Eagle's Minimal Essential Medium as described above. All cell lines used in current study were obtained from original providers who authenticated the cell lines using morphlogy, karyotypring and PCR-based approaches. No additional authentication has been performed. All cell lines tested negative for mycoplasma contamination. All experiments were performed on cells in the logarithmic growth phase.

Nebulization study. Nebulization experiments were conducted using a PARI Boy® Nebulizer (PARI GmbH, Germany). Briefly, a fraction of complexes was kept apart and used as a "non-nebulized" control. The rest of the solution was aerosolized for 5 minutes by employing PARI Boy® Nebulizer. The nebulized solution was collected in a separate tube. "Non-nebulized" control, collected solution (nebulized) and those remain in the nebulizer (reservoir) was incubated with pre-seeded BEAS-2B cells and 16HBE cells in 96-well plates for 4 h at 37 ℃ in a humidified atmosphere. The luciferase activity in 50 µl of supernatants was measured 24 h, 48 h, and 72 h after transfection and its activity is expressed in RLU. The cell culture medium was replaced each day after sampling.

Flow cytometry. To quantitatively determine the percentage of transfected cells, EGFP-mRNA or EGFP-pDNA was incubated with T704 at the concentration of 50 (w/w, for BEAS-2B cells) or 63 (w/w, for 16HBE cells) and peptide9 at the N/P ratio of 5 to form ternary complexes. Lipofectamin2000 and brPEI at optimum concentration were used as lipoplex and polyplex controls. The amount of EGFP-mRNA or EGFP-pDNA in each sample was 4 µg/well. Then the complexes were incubated for 4 h with BEAS-2B cells (6×10^5/well) or 16HBE cells (7.5×10^5/well) which were pre-seeded in 6-well plate 24 h before transfection. Cells were harvest 24 h (EGFP-mRNA transfection) or 48 h (EGFP-pDNA transfection) after transfection and suspended in a flow buffer (PBS with 2% FCS, 2mM EDTA, 0.005% NaN_3; Sigma-Aldrich, Germany). Dead cells were excluded based on 7-AAD staining (eBioscience, Germany). 10000 live cells per sample were detected by flow cytometry (FACS LSRFortessa, BD Bioscience). Data were analysed with FLOWJO (Version 9.8.3, FlowJo LLC, USA).

In order to study the cellular uptake of T704-based binary and ternary complexes, 16HBE cells (7.5×10^5/well) were seeded in 6-well plate 24 h before investigation. Fluo-pDNA-based binary complex and ternary complex were prepared as described above. The amount of Fluo-pDNA in each sample was 4 µg/well. The naked Fluo-pDNA-treated sample served as a

negative control. After 4 h incubation, the medium was removed. The cells were harvested and rinsed with cold PBS for three times followed immediately by flow cytometry analysis. The amount of 10000 live cells were collected for each sample.

In order to identify possible internalization mechanism of the ternary complex, 16HBE cells were pre-incubated at 4 ℃ or treated with specific inhibitors at 37 ℃ for 1 h (except sodium chlorate which was 24 h pre-incubation), then incubated with the ternary complex at 4 ℃ or in the presence of inhibitors at 37 ℃ for another 4 h. the concentration of inhibitors was: (I) 10 μg/ml chlorpromazine; (II) 1 μg/ml filipin; (III) 0.5 mM amiloride; (IV) 1 mM protamine sulfate; (V) 35 mM sodium chlorate; (VI) 0.5 mg/ml sodium azide. The cellular uptake of the ternary complex without any treatments was used as the positive control. The results are shown as relative uptake rate of different groups which normalized according to the positive control.

With the purpose of identifying receptor-mediated internalization mechanism of the ternary complex by human bronchial epithelial cells, 16HBE cells were pre-incubated in following conditions at 37 ℃ for 1 h, then incubated with peptide0 or peptide9-based ternary complex in the presence of following samples for another 4 h. (I) For the evaluation of peptide0-based ternary complex, excess amounts of free peptide0 (0.01 μg/μl), "K4R4" peptide (cationic moiety of peptide0, 0.005 μg/μl), lipoic acid (0.0008 μg/μl) or nuclear localization signal (NLS, 0.0035 μg/μl) were used as potential receptor-mediated endocytosis competitors. The cellular uptake of peptide0-based ternary complex without any treatment was used as the control; (II) To study the receptor-mediated endocytosis of peptide9-based ternary complex, excess amount of free peptide9 (0.02 μg/μl), "KWET" peptide (the anchor moiety of peptide9, sequence: KETWWETWWTEWWTEW, 0.009 μg/μl) or "ligand" (the targeting moiety of peptide9, sequence: GACSERSMNFCG, 0.005 μg/μl) were used as receptor-mediated endocytosis competitors. The non-treated cellular uptake of peptide9-based ternary complex was used as the control. After 4 h incubation, medium was removed. The cells were harvested and rinsed with cold PBS for three times followed immediately by flow cytometry analysis. The results are shown as the relative uptake rate of different groups which normalized according to the control group.

Confocal microscopy. 16HBE cells at the density of 3.5×10^5/well were seeded in 6-well plate 24 h before investigation. Fluo-pDNA-based binary complex and ternary complex

(Fluo-pDNA/T704/peptide0) were prepared as described above. The amount of Fluo-pDNA in each sample was 3 μg/well. LysoTracker® Red DND-99 was applied as endocytic vesicles marker according to the manufacturer's instructions. After 4 h incubation, cells were rinsed with cold PBS three times and the nuclei were stained by DAPI via 10 min incubation before imaging on a confocal laser scanning microscope (LSM-510, Carl Zeiss Microscopy GmbH, Germany).

Heparan sulfate competition assay. The binding strength of nucleic acids within the ternary complex was evaluated by heparan sulfate (HS) competition assay. Briefly, pDNA/T704/peptide9 or mRNA/T704/peptide9 ternary complex with the same composition was incubated with different amounts of HS at RT for 45 min, the resultant samples were investigated by electrophoresis on a 1% agarose gel containing 1 μg/ml ethidium bromide. To all samples, 3 μl of 6X loading buffer (ThermoFisher Scientific) was added before loading the samples onto the gel in Tris-acetate-EDTA (TAE) buffer for 60 min electrophoresis at 120 V.

***In vitro* immunofluorescence microscopy.** In order to detect CFTR protein in cultured cells, a immunofluorescence study was performed according to established protocols[1,2]. Briefly, T704/peptide9/pGM206-fLUC-CFTR/T2 ternary complex and T704/peptide9/*SB*100X-mRNA ternary complex were incubated with pre-seeded CFBE-delF cells (3×10^5/well in 6-well plate) for 4 h to obtain *SB*-transposon system transfected CFBE-delF cells. The concentration of T704 was 63 (w/w) and the N/P ratio of peptide9 was 5. The amount of pGM206-fLUC-CFTR/T2 and *SB*100X-mRNA was 2.5 μg/well and 0.25 μg/well respectively. Cells were split at 1:2 ratios when the wells were full. Two weeks after transfection, the cell samples were incubated overnight at 4 °C with a CFTR primary antibody ("570", The University of North Carolina, USA) and a nonimmune mouse IgG (all at 10 μg/ml). For immunodetection, cell samples were incubated for 1 h at RT with an Alexa Fluor®594 labelled anti-Hemagglutinin 16B12 secondary antibody (ThermoFisher Scientific) and Alexa Fluor®488 phalloidin (ThermoFisher Scientific) diluted 1:200 and 1:150, respectively.

PCR analysis.

In vitro study: Real-time quantitative PCR (qPCR) was carried out to analyse the presence and expression levels of *Sleeping Beauty* (*SB*)-transposon system specific human CFTR messenger RNA (*SB*-hCFTR-mRNA, which differs from the wild-type human CFTR

messenger RNA) in (pGM206-fLUC-CFTR/T2+*SB*100X-mRNA) transfected CFBE-delF cells two weeks post-transfection. Untreated CFBE-WT and CFBE-delF cells were applied as controls. Total RNA was extracted using NucleoSpin® RNA Plus kit (MACHEREY-NAGEL GmbH, Germany) according to the manufacturer's protocol. Complementary DNA (cDNA) was obtained from the reverse transcription reaction of 1 µg RNA using Transcriptor First Strand cDNA Synthesis Kit (04379012001, Roche). qPCR was performed in three replicates using ABI StepOnePlus™ Real-Time PCR system (Applied Biosystems). Each reaction mixture contained 10 µl of Fast SYBR® Green Master Mix 2X (4385610, ThermoFisher Scientific), 1.4 µl (10 µM) each primer (SB-CFTR, forward and reverse) and 20 ng cDNA, with the addition of nuclease-free water to a total volume of 20 µl. The default PCR thermal-cycling conditions were used for the amplification procedure. Human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) served as an internal control and was amplified in separate reactions. To verify the specificity of primers, a conventional PCR was performed with 1 µl of reverse transcribed cDNA in a 25 µl mixture composed of 0.5 µl (10 µM) each primer (SB-CFTR, forward and reverse), 10.5 µl nuclease-free water and 12.5 µl One *Taq* 2X Master Mix with standard buffer (M0482, New England Biolabs). PCR conditions were: denaturation at 94 ℃ for 30 seconds; 30 cycles of 94 ℃ for 30 seconds, 60 ℃ for 1 minute, 68 ℃ for 1 minute were performed with a final extension of 68 ℃ for 5 minutes. Experiments using nuclease-free water instead of reverse transcriptase were applied as negative controls. The PCR products were evaluated using 1% agarose gel electrophoresis stained with ethidium bromide.

In vivo study: Both conventional PCR and qPCR were performed to confirm the presence and amount of *SB*-transposon system specific human CFTR gene (i.e. codon optimized human CFTR gene compared with wild-type human CFTR gene) in untreated WT mice, untreated CF mice, (pGM206-fLUC-CFTR/T2+*SB*100X-mRNA) co-treated, pGM206-fLUC-CFTR/T2-treated, *SB*100X-mRNA-treated CF mice eighteen weeks after dosing. Genomic DNA was obtained from mouse lung tissues using NucleoSpin® Tissue kit (MACHEREY-NAGEL GmbH, Germany) according to the manufacturer's instructions. The conventional PCR was carried out in a 30 µl reaction mixture containing 3 µl 10X NH4 reaction buffer (MB-9100001, PAN-BIOTECH), 2 µl (50 mM) $MgCl_2$, 50 ng DNA templates, 0.2 mM of each dNTP, 3 µl (5 µM) each primer (SB-CFTR-2, forward and reverse), 0.4 U *Taq* DNA polymerase (10342020, ThermoFisher Scientific) and 11 µl nuclease-free water. PCR conditions were: denaturation at 94 ℃ for 3 minutes; 35 cycles of 92 ℃ for 30 seconds, 60 ℃

for 1 minute, 72 ℃ for 1 minute were performed with a final extension of 72 ℃ for 2 minutes. The PCR products were evaluated using 1% agarose gel electrophoresis stained with ethidium bromide. qPCR was performed in triplicate using ABI StepOnePlus™ Real-Time PCR system (Applied Biosystems). Reaction mixtures of 20 µl contained 10 µl of Fast SYBR® Green Master Mix 2X (4385610, ThermoFisher Scientific), 0.5 µg DNA templates and 1 µl (10 µM) each primer (SB-CFTR-2, forward and reverse). The default PCR thermal-cycling conditions were used for the amplification procedure. Mouse glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) served as an internal control and was amplified in separate reactions. All primer sequences can be found in "**SUPPLEMENTARY NOTES"**.

SUPPLEMENTARY RESULTS

Each moiety that constitutes the peptide is essential to the transfection efficiency of peptide-poloxamine nanoparticles

To prove those moieties that constitute the peptide play pivotal roles in boosting transfection efficiency of the ternary complex, we utilized three peptide controls to form the ternary complex and compared their activities in transfecting airway epithelial cells with peptide0-based ternary complex. Peptide1 shares the same cationic moiety and targeting moiety with peptide0 but has no anchor moiety. Peptide2 is a cationic moiety negative control of peptide0 (the NLS sequence was simultaneously eliminated from peptide2, as there are also many basic amino acids in the NLS). Peptide3 shares the same anchor moiety and cationic moiety with peptide0 but contains a mutated NLS targeting sequence in which the lysine at position 5 has been replaced by threonine. This mutation is known to be nuclear transport deficient[3]. The optimized peptide1-based, peptide2-based and peptide3-based ternary complexes showing the maximum transfection efficiency was evaluated (Supplementary Fig. 8-11) and compared with the peptide0-based counterpart. Ternary complex comprising pDNA/T704/peptide1 displayed less than 10% (in BEAS-2B cells) or 50% (in 16HBE cells) of the maximum signal intensity of peptide0-based counterpart (Supplementary Fig. 7a). Regarding mRNA transfection, peptide1-based ternary complex could just reach 30% of the transfection efficiency of mRNA/T704/peptide0 complex in both cell lines (Supplementary Fig. 7b). Formulations prepared by pDNA/T704/peptide2 and mRNA/T704/peptide2 showed rather low transfection efficiency that was similar to the T704-based binary counterparts in BEAS-2B and 16HBE cells (Supplementary Fig. 7c-d). In the evaluation of the targeting moiety, pDNA/T704/peptide0 ternary complex expressed significantly higher transgene expression than the pDNA/T704/peptide3 counterpart in BEAS-2B cells (Supplementary Fig. 7e). Even though the transfection efficiency mediated by pDNA/T704/peptide0 and pDNA/T704/peptide3 ternary complex was similar on day 2 in 16HBE cells, the former was significantly higher than the latter on the other time points in this cell line (Supplementary Fig. 7e). These data revealed that all the functional moieties, including the anchor moiety, the cationic moiety and the targeting moiety, are indispensable to the efficient *in vitro* transfection of T704/peptide-based ternary complex. Among all these moieties, the cationic part is particularly important for *in vitro* gene transfection. This was in agreement with previous studies which indicate that positive charge in the carrier system not only is helpful in condensing nucleic acids payload to avoid nuclease degradation but also plays important roles

in overcoming various subcellular barriers[4,5]. Heparan sulfate proteoglycans (HSPG) on the cell surface may aid in the internalization process of arginine-based peptide[6]. The heparan sulfate chains would be degraded once inside the endosome, leading to dissociation of the peptides and consequent interaction of the arginine oligomers with the endosomal membrane, thus promoting its endosomal escape and subsequent release into the cytoplasm[7].

Optimizing the peptide structure with different candidate modules

In order to optimize the peptide structure for more potent transfection, we designed and synthesized another series of peptides via integration of various functional modules (peptide 4-12). Peptide4 and peptide5 kept the same anchor moiety and cationic moiety with peptide0 (same numbers of lysine and arginine in the sequence) but contained different targeting moieties. Ligand SERSMNF (ligand S) in peptide4 and ligand YGLPHKF (ligand Y) in peptide5 were originally developed by Hart et al. (University College London, London, UK) and showed high affinity to the human airway epithelial cells[8]. Ligand S displays close similarity to receptor binding proteins of rhinovirus which binds the intercellular adhesion molecule-1 (ICAM-1)[9]. Ligand Y closely resembles part of a targeting protein expressed by the intracellular pathogen *Legionella pneumophila* although its receptor is currently unknown[10]. The amino acids sequence "GAC…CG" could provide conformational stability for the whole ligand[11]. Peptide6 and peptide7 shared a similar structure with peptide4 and peptide5 but differed in the cationic moiety. Unlike the cationic moiety of peptide4 and peptide5 wherein lysine outnumbered arginine, the cationic moiety of peptide6 and peptide7 contained more arginine than lysine. Peptide8 had the same anchor moiety and targeting moiety with peptide4 but contained a cationic moiety dominated by histidine. Peptide9, peptide10, peptide11 and peptide12 were derived from peptide4 by the substitution of different candidate modules of the anchor moiety, while peptide10 comprised a shortened cationic moiety. The optimum composition of peptide4-based to peptide12-based ternary complexes showing the maximum transfection efficiency was first identified (Supplementary Fig. 8-11). The pDNA or mRNA transfection efficiency of ternary complexes containing these peptides in the optimum conditions was subsequently compared. pDNA expression mediated by peptide4-based, peptide5-based and peptide7-based ternary complexes was similar (that mediated by peptide5-based and peptide7-based ternary complexes was slightly better at day 3) to that mediated by peptide0-based ternary complexes in BEAS-2B cells, while peptide9-based ternary complex showed the highest transfection efficiency from day 1 to day 4 among all the tested groups in this cell line (Supplementary Fig. 12a). In the

transfection of 16HBE cells, both pDNA/T704/peptide6 and pDNA/T704/peptide9 ternary complexes showed overwhelming signals at each time point compared with other ternary complexes (Fig. 3a). In terms of mRNA transfection, peptide9-based ternary complex was more efficient than other counterparts in both BEAS-2B (Supplementary Fig. 12b) and 16HBE cells (Fig. 3b). These results revealed that peptide9-T704 ternary complex was the most promising candidate in all aspects. Apart from the same cationic moiety and targeting moiety inherited from peptide4, peptide9 specifically contained an enlarged hydrophobic area made by a quadruple tryptophan-rich sequence in the anchor moiety[12]. An attractive hypothesis for the superiority of peptide9 would be that the expanded hydrophobic moiety may increase its capacity for docking with the polypropyleneoxide (PPO) groups of T704, thus resulting in a more compact and stable structure than other counterparts. This could be partially confirmed via the size measurement showing that the particle size of peptide9-based ternary complex was slightly smaller than the peptide4-based counterpart (Supplementary Table2). Meanwhile, we also evaluated the gene transfection ability of peptide9 (as a single component to form the binary complex with nucleic acids). Peptide9 alone just mediated low levels of mRNA or pDNA transfection in both investigated cell lines (Supplementary Fig. 24), emphasizing again the superiority of ternary complex. In the evaluation of targeting moiety, different candidates had their own advantages in different aspects, but ligand S exceeded others in transfecting 16HBE cells that are more static compared with fast-dividing BEAS-2B cells, thus providing huger subcellular barriers and better mimicking *in vivo* status. Another advantage of ligand S is that it was completely functional at low concentrations compared with the NLS, thereby decreasing the positive charge and toxicity of nanocomplexes. It is worth to note that ternary complexes formed by peptide8 and peptide10 which possess “weak” cationic moiety (in terms of types and numbers of amino acids) were not capable of mediating efficient gene transfection in both cultured cells, highlighting the importance of cationic moiety. One possible explanation for this phenomenon is that histidine or limited numbers of lysine/arginine might not be sufficient to trigger an efficient intracellular uptake and endosomal escape of nucleic acid payloads[13]. When comparing the transfection efficiency of peptide4-based and peptide6-based ternary complexes in 16HBE cells, arginine residues were more efficient in mediating mRNA and pDNA transfection compared with lysine residues, and there needs to be a certain number of arginine residues (> 8) for them to be effective in the ternary complex.

Supplementary stability studies

As revealed by the TEM study, the morphology alterations between binary complexes and ternary complexes suggested that the peptide component could distinctively improve the size and shape of T704-based complex into a more condensed, uniform spherical shaped and well-distributed status. The peptide component in ternary complex possibly leads to the formation of a compact micelle-like structure consisting of a condensed hydrophobic core, comprising polypropyleneoxide (PPO) and peptide where nucleic acid payloads bind, and a hydrophilic shell that is composed of polyethyleneoxide (PEO) blocks from T704 with higher density compared with the T704-based binary counterpart. This hydrophilic outer corona may exclude nanoparticles from aggregation, thus further increases the stability of ternary complexes. Colloidal stability studies were performed to partially confirm this hypothesis and showed that ternary complex retained its particle size below 100 nm within one week at room temperature (Supplementary Fig. 25a). The ternary complex also exhibited better colloidal stability under physiological and high salt conditions than the binary counterpart (Supplementary Fig. 25b). All these features of ternary complex help to meet the criteria of efficient inhaled gene vectors which must be small enough to diffuse through the mucus mesh (100-200 nm) while possessing a muco-inert surface to avoid adhesion to mucus constituents[14].

For the potential clinical applications of peptide-poloxamine nanoparticles, the aerosol inhalation approach would be preferred when administering formulations to the airway of patients, rather than the invasive intratracheal instillation with low patient compliance. As a result, it is important to know whether ternary complexes could resist the shear force created during nebulization and whether they remain the activity after nebulization. We evaluated the transfection efficiency of non-nebulized and nebulized pDNA/T704/peptide0 ternary complex as well as pDNA/Lipofectamine2000 lipoplex. Nebulized lipoplex showed significantly lower transfection efficiency compared with the non-nebulized one in both cell lines, whereas the nebulization process did not severely affect the pDNA/T704/peptide0 ternary complex (Supplementary Fig. 25c), indicating that peptide-poloxamine nanoparticles were more resistant to the shear force created by jet-nebulizer comparing with the lipid-based counterparts.

Receptor-mediated cellular uptake

Potential receptor-mediated endocytosis of the peptide-poloxamine complexes was examined by the competition of excess free peptides and their fragments. In terms of Fluo-pDNA/T704/peptide0 ternary complex, its cellular uptake was not influenced by free K4R4 peptide (KKRRRRKK, the cationic moiety of peptide0) and free peptide0 (Supplementary Fig. 26a). This might result from the fact that positively charged peptides did not have specific receptors in the cells and could utilize alternative pathways to enhance translocation through the plasma membrane[15]. A similar situation could be observed in the case of free lipoic acid and free NLS (Supplementary Fig. 26a). However, an excess amount of free ligand S (GACSERSMNFCG) and free peptide9 substantially decreased the cellular uptake of Fluo-pDNA/T704/peptide9 ternary complex (Supplementary Fig. 26b), indicating that ligand S played an important role in mediating the cellular uptake of peptide9-based ternary complex into airway epithelial cells.

Why mRNA-based ternary complex was less efficient than the lipofectamine2000 counterpart

Transgene expression mediated by the peptide-poloxamine complexes was more efficient than that mediated by 25 kDa brPEI polyplexes in all aspects. But mRNA transfection mediated by ternary complex was still inferior to the Lipofectamine2000-based counterpart, this observation is consistent with previous studies implying the superiority of lipid-based carriers compared with other nonviral vectors in mRNA delivery[16]. We found this phenomenon did not result from the amount of nucleic acid payloads delivered into cells by these carriers, as ternary complex could efficiently mediate the uptake of nucleic acid payloads by almost 70% 16HBE cells whereas the Lipofectamine2000 counterpart was internalized by only 22% 16HBE cells (Supplementary Fig. 27). The binding strength represents a critical parameter in mRNA transfection because the mRNA molecules should be released from the delivery vehicles to facilitate subsequent translation process[17]. We performed a heparan sulfate (HS) competition assay to see if there is any difference between the binding strength of mRNA within the ternary complex and that of pDNA. Results showed that pDNA could be released from ternary complex when the HS/pDNA ratio (w/w) reached 50, whereas mRNA could not be released from ternary complex at the same HS/mRNA ratio, only partial mRNA release could be observed in ternary complex at the highest HS/mRNA ratio (100 (w/w)) (Supplementary Fig. 28). The study also revealed that mRNA within Lipofectamine2000 lipoplex (without any treatment) could not be retarded (Supplementary Fig. 28, lane “mRNA

Lipoplex"), revealing the weak affinity between mRNA and Lipofectamine2000. Therefore, one possible reason for the inferior mRNA transfection efficiency of the ternary complex compared with that of lipoplex resulted from the strong binding between mRNA molecules and the cationic moiety within the peptide. Further efforts to improve the transfection efficiency of mRNA-based ternary complex will be focused on the adjustments of the cationic moiety within the peptide in order to obtain a balance between the affinity to mRNA molecules and the ability of overcoming intracellular barriers.

SUPPLEMENTARY NOTES

Primers

In vitro study:

PCR_SB-CFTR_Fwd: GCTGAACCTGATGACCCACTCT

PCR_SB-CFTR_Rev: TGGTGGAGGATCTTGCTCACTG

PCR_SB-CFTR_Product Lenth: 545 bp

PCR_hGAPDH_Fwd: GTGGGGCGCCCCAGGCACCA

PCR_hGAPDH_Rev: CTCCTTAATGTCACGCACGATTTC

PCR_hGAPDH_Product Lenth: 515 bp

In vivo study:

PCR_SB-CFTR-2_Fwd: CAGCAGTTCCTGGTGATTGA

PCR_SB-CFTR-2_Rev: TCCTGCACTTCCTCCTCTGT

PCR_SB-CFTR-2_Product Lenth: 200 bp

PCR_mGAPDH_Fwd: GTTGTCTCCTGCGACTTCA

PCR_mGAPDH_Rev: GGTGGTCCAGGGTTTCTTA

PCR_mGAPDH_Product Lenth: 184 bp

DNA sequence

pGM206-fLUC-CFTR/T2

AGATCTTATACAGTTGAAGTCGGAAGTTTACATACACTTAAGTTGGAGTCATTAA
AACTCGTTTTTCAACTACTCCACAAATTTCTTGTTAACAAACAATAGTTTTGGCAA
GTCAGTTAGGACATCTACTTTGTGCATGACACAAGTCATTTTTCCAACAATTGTTT
ACAGACAGATTATTTCACTTATAATTCACTGTATCACAATTCCAGTGGGTCAGAA

GTTTACATACACTAAGTTGACTGTGCCTTTAAACAGCTTGGAAAATTCCAGAAAA
TGATGTCATGGCTTTAGAAGCTTCCCACAATAAGTTGGGTGAATTTTGGCTCATTC
CTCCTTTCTATAGGATTGAGGTCAGAGCTTTGTGATGGAATTCTGTGGAATGTGTG
TCAGTTAGGGTGTGGAGATCTGTTACATAACTTATGGTAAATGGCCTGCCTGGCT
GACTGCCCAATGACCCCTGCCCAATGATGTCAATAATGATGTATGTTCCCATGTA
ATGCCAATAGGGACTTTCCATTGATGTCAATGGGTGGAGTATTTATGGTAACTGC
CCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTATGCCCCCTATTGATGTC
AATGATGGTAAATGGCCTGCCTGGCATTATGCCCAGTACATGACCTTATGGGACT
TTCCTACTTGGCAGTACATCTATGTATTAGTCATTGCTATTACCATGGGAATTCAC
TAGTGGAGAAGAGCATGCTTGAGGGCTGAGTGCCCCTCAGTGGGCAGAGAGCAC
ATGGCCCACAGTCCCTGAGAAGTTGGGGGGAGGGGTGGGCAATTGAACTGGTGC
CTAGAGAAGGTGGGGCTTGGGTAAACTGGGAAAGTGATGTGGTGTACTGGCTCC
ACCTTTTTCCCCAGGGTGGGGGAGAACCATATATAAGTGCAGTAGTCTCTGTGAA
CATTCAAGCTTCTGCCTTCTCCCTCCTGTGAGTTTGGTAAGTCACTGACTGTCTAT
GCCTGGGAAAGGGTGGGCAGGAGATGGGGCAGTGCAGGAAAAGTGGCACTATG
AACCCTGCAGCCCTAGGAATGCATCTAGACAATTGTACTAACCTTCTTCTCTTTCC
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ATATTAAGAAAGGCCCTGCCCCATTCTACCCTCTGGAAGATGGCACTGCTGGTGA
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CACTTACCCCCTGGCTTCAATGAGTATGACTTTGTGCCTGAGAGCTTTGACAGGG
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ATCTTTGGCAACCAGATCATCCCTGACACTGCCATCCTGTCTGTGGTTCCATTCCA
TCATGGCTTTGGCATGTTCACAACACTGGGGTACCTGATCTGTGGCTTCAGAGTG
GTGCTGATGTATAGGTTTGAGGAGGAGCTGTTTCTGAGGAGCCTACAAGACTACA
AGATCCAGTCTGCCCTGCTGGTGCCCACTCTGTTCAGCTTCTTTGCCAAGAGCACC
CTCATTGACAAGTATGACCTGAGCAACCTGCATGAGATTGCCTCTGGAGGAGCAC

CCCTGAGCAAGGAGGTGGGTGAGGCTGTGGCAAAGAGGTTCCATCTCCCAGGAA
TCAGACAGGGCTATGGCCTGACTGAGACCACCTCTGCCATCCTCATCACCCCTGA
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GCCCTATGCCCTGATCAAGGGGATCATCCTGAGAAAGATTTTCACCACCATCAGC
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GCTGCAGAACCTGCAGCCTGACTTCAGCTCTAAGCTGATGGGCTGTGACAGCTTT
GACCAGTTCTCTGCTGAGAGGAGGAACAGCATCCTGACAGAGACCCTGCACAGA
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TTCAAGCAGACAGGGGAGTTTGGGGAGAAGAGGAAGAACTCCATCCTGAACCCC
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RNA sequence

*SB*100X-mRNA

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