

Bioengineering an Ultra-Stable and Cost-Effective FGF2 (NGF2) with Extended Circulatory Half-Life for Next-Generation Therapeutics

Suet-Ying Tam

Genomic Nantijen Diagnostic Limited

Man-Chung Choi

Genomic Nantijen Diagnostic Limited

Nga-King Law

Genomic Nantijen Diagnostic Limited

Chi-Hoi Chau

Genomic Nantijen Diagnostic Limited

Sai-Fung Chung

16900402r@connect.polyu.hk

Genomic Nantijen Diagnostic Limited

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Abstract

Human fibroblast growth factor 2 (FGF2) is one of the potent signaling proteins for various therapeutic applications, especially in cell regeneration. However, the clinical translation of FGF2 has been constrained by two major hurdles, which are inherent instability in solution (< 1 h stability) and an unacceptably short circulation half-life (< 1 day). Compensatory strategies such as dose escalation and frequent dosing are neither a practical nor a viable solution to resolve these fundamental pharmacokinetic deficiencies. Here, we report a rational bioengineering fusion protein (NGF2), which is fused FGF2-STAB with albumin binding domain (ABD) via a soft linker (GGGGS)₅, to overcome those challenges. The fusion construct preserved both functionalities, enabling ABD to bind with human serum albumin (HSA) for extended half-life while FGF2-STAB to bind to the FGF2 receptor for cell signaling. More importantly, the simplified and cost-effective production process makes this fusion protein much easier to scale up and suitable for clinical use, helping to overcome the major barriers that have long limited the development of FGF2-based therapies.

Highlights

- Fusing albumin binding domain to FGF2-STAB, NGF2, was constructed and highly stable
- NGF2 could bind to human serum albumin to overcome instability and short half-life
- NGF2 preserved its function on cell proliferation for practical therapeutic use

Introduction

Human fibroblast growth factor 2 (FGF2) is a well-known one of the strongest signaling proteins that can control cell proliferation, differentiation and angiogenesis in various tissues such as muscle, blood vessels, teeth, skin, nerves, cartilage, bone and tendons. Therefore, recombinant FGF2 is regarded as a multiple-function therapeutic protein showing great market value [1, 2].

Nevertheless, the stability of wild-type FGF2 in solution form is less than one day, which limits its abovementioned medical applications. In contrast to wild-type FGF2, the thermostable form of FGF2, FGF2-STAB, contains nine mutations that extend its stability in solution form to more than 7 days at 37°C without any degradation [3]. Despite the successful development of a thermostable FGF2, the main obstacle to its therapeutic applications remains its small molecular size of 18 kDa [4], which is much smaller than the renal clearance cut-off of 30 – 50 kDa [5]. This indicates that the half-life of both wild-type FGF2 and FGF2-STAB in the human body is less than one hour [6]. To address this limitation, different biotechnology techniques have been adopted to enlarge the molecular size of FGF2. Two research groups have developed site-specific PEGylated FGF2 via PEGylation technology to extend the FGF2 half-life [7, 8]. Apart from them, another research group has developed dextran/PEG nanoparticle coating technology to enhance the FGF2 half-life for the treatment of liver fibrosis [9]. However, those technologies require expensive PEG molecules or nanoparticles and require extra steps for conjugation and purification, leading to a significant increase in the production cost. Unlike them, we have fused an

albumin-binding domain (ABD) with FGF2-STAB to generate a new FGF2 protein, termed NGF2 (23 kDa). The function of ABD is to bind tightly to human serum albumin (67 kDa). As a result, the resulting complex of NGF2 with human serum albumin is approximately 100 kDa, which is well above the renal clearance cut-off, meaning that our NGF2 proteins are not readily eliminated by renal filtration. Furthermore, the interaction between the neonatal Fc receptor (FcRn) and human serum albumin plays a crucial role in extending the half-life of albumin-bound proteins. FcRn binds to human serum albumin in a pH-dependent manner within endosomes, protecting it from lysosomal degradation and recycling it back to the cell surface. This FcRn-mediated salvage pathway significantly prolongs the circulation time of albumin and its associated cargo. Therefore, by binding to human serum albumin, NGF2 not only avoids renal clearance but also benefits from FcRn-driven recycling, which can further enhance its *in vivo* half-life [10].

Our results demonstrated that the fusion protein of NGF2 retained the binding ability between ABD and albumin in native gel electrophoresis. This result was further corroborated through AlphaFold3 structure prediction. Additionally, AlphaFold3 structure prediction rationally simulated the binding of NGF2-albumin complex to both FcRn and FGFR2, implying that our designed soft repeated linker (GGGGS x5) successfully separates ABD from FGF2 without hindering their corresponding binding functions. Moreover, the *in vitro* stability test showed that NGF2 was highly stable at 4°C and 37°C after 7 days. Also, our *in vitro* cell line proliferation experiment gave a positive agreement with AlphaFold3, and this experiment, the 3T3 cell lines were significantly proliferated starting at 0.25 ng/μl. Our newly designed NGF2 is not only stable *in vitro*, but also *in vivo*. Unlike PEGylation and dextran/PEG nanoparticle coating technologies, the fusion protein technology does not require the usage of expensive active PEG molecules. Therefore, our NGF2 provides new insights into FGF2 for medical applications *in vitro* and *in vivo* with low production cost.

Materials and methods

Expression, production and purification of NGF2

The target gene encoding albumin binding domain (ABD) fusion with the N-terminus of FGF2-STAB by a linker (GGGGS)₅, namely NGF2, in the pET21a vector was transformed into BL21(DE3) for expression. A single colony was picked and grown overnight at 30 °C in sterile LB medium that contained 50 μg/ml kanamycin with shaking at 250 rev/min. The overnight culture was inoculated into 200 mL sterile TB medium with 50 μg/ml kanamycin in a ratio of 1: 100 and grown at 30 °C with shaking at 250 rev/min. For the protein expression, 0.5 mM IPTG was added once the culture optical density (OD₆₀₀) reached 0.6–0.8. The cell pellet was collected by centrifugation and lysed by FS-600N ultrasonic processor (600 W, Shanghai Shengxi Supersonic Instrument) in resuspension buffer (20 mM Tris buffer, 100 mM NaCl, pH 7.0). The supernatant was then purified by HiTrap Q HP column (GE Healthcare). The flow through of the Q column was collected and further purified by HiTrap SP HP column (GE Healthcare). NGF2 was

eluted at 60% of elution buffer (20 mM Tris, 1 M NaCl, pH 7.0) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

In silico analysis of the binding between NGF2 and albumin, and FGFR2/FcRn

The structures of three input protein sequences were predicted using AlphaFold3 in multimer mode to model their interactions. The soluble extracellular ligand-binding domain of FGFR2 was selected according to the crystal structure of the FGF–FGFR2 complex (PDB: 1EV2). For FcRn, the soluble domain was selected based on the albumin–FcRn complex crystal structure (PDB: 4N0F). The highest-ranking models were chosen for downstream analysis of the binding domains.

Albumin binding assay of NGF2

The binding between NGF2 and human serum albumin (HSA) was analyzed. A fixed amount of NGF2 (2 µg) was mixed with different mole ratios of human serum albumin (HSA), followed by the incubation at 37 °C for 10 min. Their mixtures were analyzed by native polyacrylamide gel electrophoresis.

Cell proliferation assay of NGF2

NIH-3T3 cells were seeded into 96-well plate at a density of 3000 cells/well and incubated at 37 °C in complete DMEM medium (DMEM 31966, Gibco® + P/S + 10% newborn calf serum) overnight in a humidified 5% CO₂ (HERAccl 150i). After 24 hours, the cell medium was replaced by DMEM medium with low concentration of serum (DMEM 31966, Gibco® + P/S + 0.2% newborn calf serum). After 24 h starvation, different concentrations of NGF2 were added into the cells and incubated for 72 h. Then, ten microliters of 5 mg/mL of MTT reagent were added into each well, followed by incubation at 37 °C for 4 h. Then, the solution in each well was completely removed and 100 µL/well dimethyl sulfoxide (DMSO) was added. With complete solubilization of the formazan crystals, absorbance at 540 nm was detected by microplate reader (Thermo scientific multiskan FC). The IC₅₀ value was calculated using the Prism software (GraphPad Software Inc, La Jolla, CA, USA) with the non-linear regression sigmoidal dose-response curve model. Independent experiments were performed in at least triplicate.

In Vitro study of the stability of NGF2

A fixed amount of NGF2 (2 µg) in PBS solution was incubated at 4 °C and 37 °C. At day 0, NGF2 was collected and loaded with SDS loading dye as a control. After 7 days incubation, the same amount of NGF2 at 4 °C and 37 °C were collected and analyzed by SDS-PAGE.

Results and Discussion

NGF2 expression and purification

The target protein NGF2 was expressed in soluble fraction with a molecular size of < 25 kDa (the theoretical molecular size: 23.1 kDa) (Fig. 1, Lane 2). Owing to its high isoelectric point, NGF2 could not bind to Q column and was eluted in flow-through (Fig. 1, lane 4). Moreover, it could tightly bind to SP column and be eluted at 60% of elution buffer with an ultra-purity as shown in Lane 8. This production process is significantly simpler and more cost-effective than conventional PEGylation, primarily due to the avoidance of expensive PEG molecules and the labor-intensive steps associated with PEG conjugation and subsequent removal of excess PEG.

The binding ability of NGF2 to human serum albumin (HSA)

To evaluate whether our NGF2 could influence the binding between the albumin binding domain (ABD) and human serum albumin (HSA), different mole ratios of HSA were mixed with a fixed amount of NGF2. As shown in Fig. 2, 1 mole of NGF2 could fully bind to 0.5 mole of HSA, suggesting that the fusion of the ABD to FGF2 did not affect the interaction between ABD and HSA. Moreover, the binding between NGF2 and HSA resulted in a higher molecular weight complex (< 146 kDa) (Fig. 2), which exceeds the cutoff size of renal clearance [5], potentially enhancing the half-life of NGF2 by minimizing renal elimination.

Cell proliferation induced by NGF2

After confirming the binding between ABD and HSA, the functionality of NGF2 was also determined. In the presence of albumin binding domain (ABD), NGF2 still could significantly induce the proliferation of NIH-3T3 cell starting at a concentration of 0.25 ng/μl. It could maximumly increase in the cell intensity up to ~ 180% compared to the control.

In silico analysis of the binding between NGF2, human serum albumin (HSA), FGF2 receptor and FcRn

Not only did native gel analysis demonstrate the interaction between NGF2 and HSA, and the cell proliferation assay confirm NGF2 functionality, but AlphaFold3 predictions also provided structural insights (Fig. 4). The AlphaFold3 model of the ternary NGF2–HSA–FGFR2 complex showed moderate confidence (pTM = 0.45, ipTM = 0.43), suggesting possible steric hindrance or flexibility at the interface. Nevertheless, our *in vitro* cell proliferation assay strongly supported that NGF2 retained the ability to bind and activate FGFR2. Our designed (GGGS)₅ linker effectively separated the ABD from FGF2, allowing ABD to engage HSA while FGF2 could still interact with FGFR2 (Fig. 4).

On the other hand, AlphaFold3 predicted a more reliable binding mode for the NGF2–HSA–FcRn ternary complex (pTM = 0.70, ipTM = 0.68). This supports that NGF2 can simultaneously bind HSA and engage FcRn, indicating that our design may benefit from FcRn-mediated recycling and thus achieve a prolonged *in vivo* half-life (Fig. 5).

The stability of NGF2

One of the major challenges to the clinical development of FGF2 as a therapeutic is its inherent instability. Only half of the wild-type FGF2 could retain its biological activity at 37°C for 30 min [11, 12]. Different formulations have been investigated to preserve the biological activity of FGF2 in aqueous form [12]. Herein, even without formulation, our NGF2 in aqueous solution was extremely stable without self-cleaved at 4 °C and 37 °C after 7-day incubation (Fig. 6).

Conclusion

Our NGF2 was successfully produced in an active form capable of binding to human serum albumin for an extended half-life, while simultaneously engaging the FGF2 receptor to induce cell proliferation. This design not only overcomes the intrinsic short half-life of FGF2 but also substantially reduces production costs and complexity, offering a promising route for FGF2-based therapeutic applications.

Declarations

Competing Interests

The authors declare no conflicts of interest.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

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Author Contribution

Author contributions Conceptualization: S.F.C. and C.H.C.; Methodology: S.F.C., S.Y.T. and M.C.C.; Software: S.F.C., S.Y.T. and M.C.C.; Formal analysis: S.F.C., S.Y.T. and M.C.C.; Investigation: S.F.C., S.Y.T. and M.C.C.; Data curation: S.F.C., S.Y.T. and M.C.C.; Writing—original draft preparation: S.F.C. and S.Y.T.; Writing—review and editing: M.C.C., L.N.K. and C.H.C.; Resources: L.N.K. and C.H.C; Supervision: S.F.C., L.N.K. and C.H.C. All authors reviewed the manuscript.

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References

1. Beenken, A., & Mohammadi, M. (2009). The FGF family: biology, pathophysiology and therapy. *Nature reviews Drug discovery*, 8(3), 235–253. <https://doi.org/10.1038/nrd2792>
2. Song, J. A., Koo, B. K., Chong, S. H., Kwak, J., Ryu, H. B., Nguyen, M. T., Vu, T. T. T., Jeong, B., Kim, S. W., & Choe, H. (2013). Expression and purification of biologically active human FGF2 containing the b' a' domains of human PDI in Escherichia coli. *Applied biochemistry and biotechnology*, 170(1), 67–80. <https://doi.org/10.1007/s12010-013-0140-3>
3. Kadlecová, Z., Sevriugina, V., Lysáková, K., Rychetsky, M., Chamradová, I., & Vojtová, L. (2023). Liposomes affect protein release and stability of ITA-Modified PLGA–PEG–PLGA hydrogel carriers for controlled drug delivery. *Biomacromolecules*, 25(1), 67–76. <https://doi.org/10.1021/acs.biomac.3c00736>
4. Liao, S., Bodmer, J., Pietras, D., Azhar, M., Doetschman, T., & Schultz, J. E. J. (2009). Biological functions of the low and high molecular weight protein isoforms of fibroblast growth factor-2 in cardiovascular development and disease. *Developmental Dynamics*, 238(2), 249–264. <https://doi.org/10.1002/dvdy.21677>
5. Ruggiero, A., Villa, C. H., Bander, E., Rey, D. A., Bergkvist, M., Batt, C. A., Manova-Todorova, K., Deen, W. M., Scheinberg, D. A., & McDevitt, M. R. (2010). Paradoxical glomerular filtration of carbon nanotubes. *Proceedings of the National Academy of Sciences*, 107(27), 12369–12374. <https://doi.org/10.1073/pnas.0913667107>
6. Liebman, B., Schwaegler, C., Foote, A. T., Rao, K. S., Marquis, T., Aronshtam, A., Bell, S. P., Gogo, P., LaChapelle, R. R., & Spees, J. L. (2022). Human growth factor/immunoglobulin complexes for treatment of myocardial ischemia-reperfusion injury. *Frontiers in Bioengineering and Biotechnology*, 10, 749787. <https://doi.org/10.3389/fbioe.2022.749787>
7. Zhao, J., Li, Q., Wu, J., Zhou, C., Cao, Y., Li, X., & Niu, J. (2020). Structure-based site-specific pegylation of fibroblast growth factor 2 facilitates rational selection of conjugate sites. *Biotechnology Journal*, 15(2), 1900203. <https://doi.org/10.1002/biot.201900203>
8. Lühmann, T., Gutmann, M., Moscaroli, A., Raschig, M., Béhé, M., & Meinel, L. (2019). Biodistribution of site-specific PEGylated fibroblast growth factor-2. *ACS Biomaterials Science & Engineering*, 6(1),

425–432. <https://doi.org/10.1021/acsbiomaterials.9b01248>

9. Kurniawan, D. W., Booiyink, R., Pater, L., Wols, I., Vrynas, A., Storm, G., Prakash, J., & Bansal, R. (2020). Fibroblast growth factor 2 conjugated superparamagnetic iron oxide nanoparticles (FGF2-SPIONs) ameliorate hepatic stellate cells activation in vitro and acute liver injury in vivo. *Journal of controlled release*, 328, 640–652. <https://doi.org/10.1016/j.jconrel.2020.09.041>
10. Sand, K. M. K., Bern, M., Nilsen, J., Noordzij, H. T., Sandlie, I., & Andersen, J. T. (2015). Unraveling the interaction between FcRn and albumin: opportunities for design of albumin-based therapeutics. *Frontiers in immunology*, 5, 118912. <https://doi.org/10.3389/fimmu.2014.00682>
11. Benington, L. R., Rajan, G., Locher, C., & Lim, L. Y. (2021). Stabilisation of recombinant human basic fibroblast growth factor (FGF-2) against stressors encountered in medicinal product processing and evaluation. *Pharmaceutics*, 13(11), 1762. <https://doi.org/10.3390/pharmaceutics13111762>
12. Chen, G., Gulbranson, D. R., Yu, P., Hou, Z., & Thomson, J. A. (2012). Thermal stability of fibroblast growth factor protein is a determinant factor in regulating self-renewal, differentiation, and reprogramming in human pluripotent stem cells. *Stem cells*, 30(4), 623–630. <https://doi.org/10.1002/stem.1021>

Figures

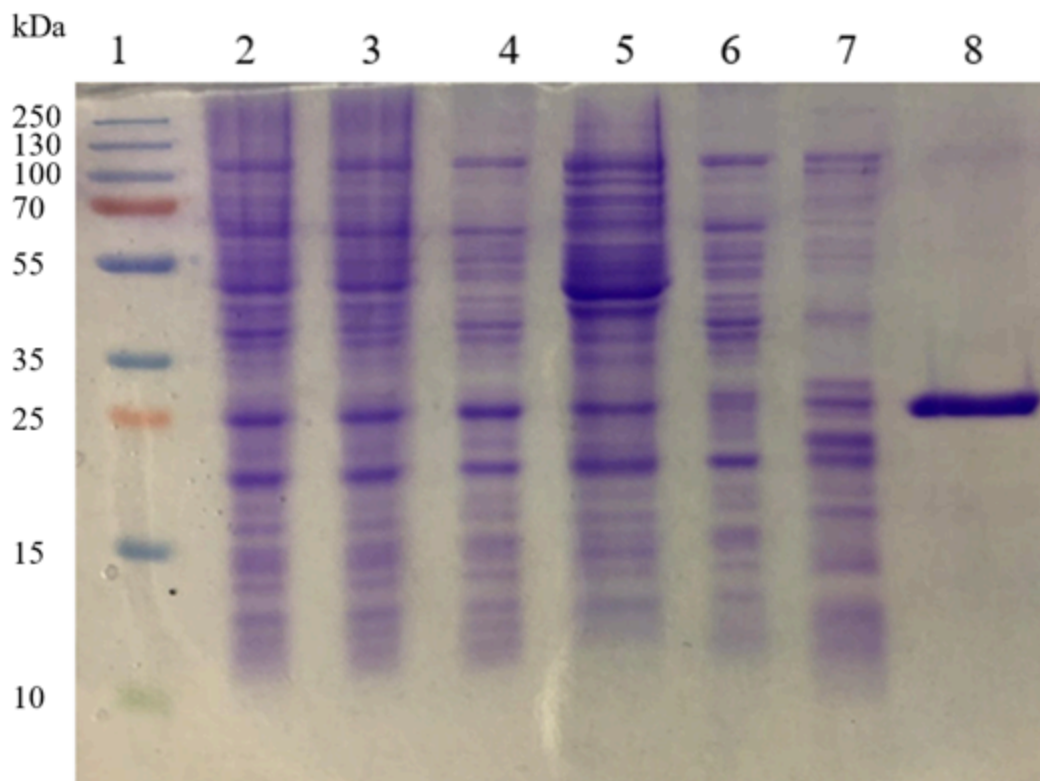


Figure 1

SDS-PAGE analysis of NGF2 expression and purification. Lane 1: Marker; Lane 2: Total lysate; Lane 3: Soluble fraction; Lane 4: Flow through of Q column; Lane 5: 100% elution from Q column; Lane 6: Flow through of SP column; Lane 7: 30% elution from SP column; Lanes 8: NGF2 in 60% elution from SP column

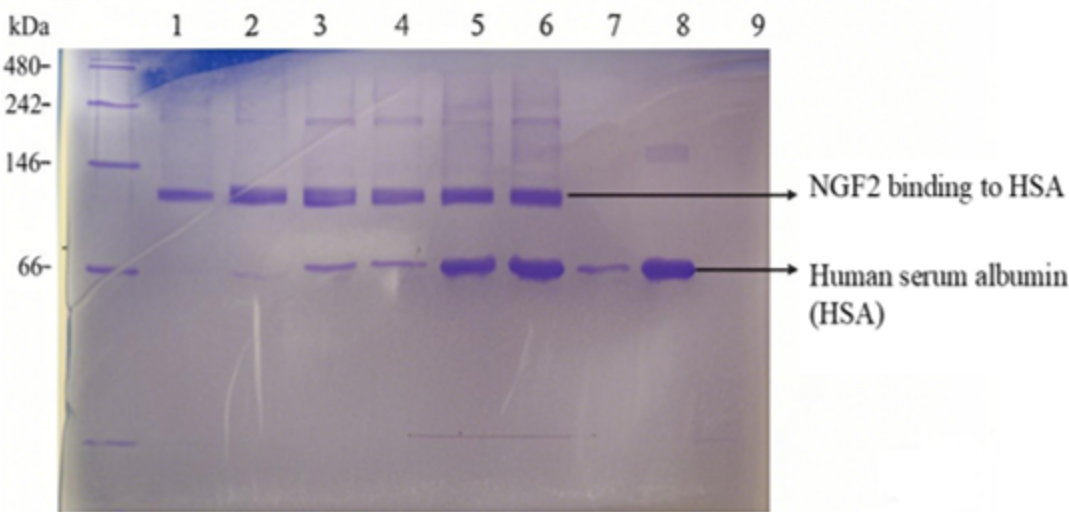


Figure 2

Native polyacrylamide gel assay of a fixed amount of NGF2 mixed with different mole ratios of human serum albumin (HSA). Mole of NGF2: Mole of HSA in lane 1: 1:0.25; lane 2: 1: 0.5; lane 3: 1: 0.75; lane 4: 1:1; lane 5: 1: 1.5; lane 6: 1: 2; lane 7: 0: 0.25; lane 8: 0: 2; lane 9: 1:0.

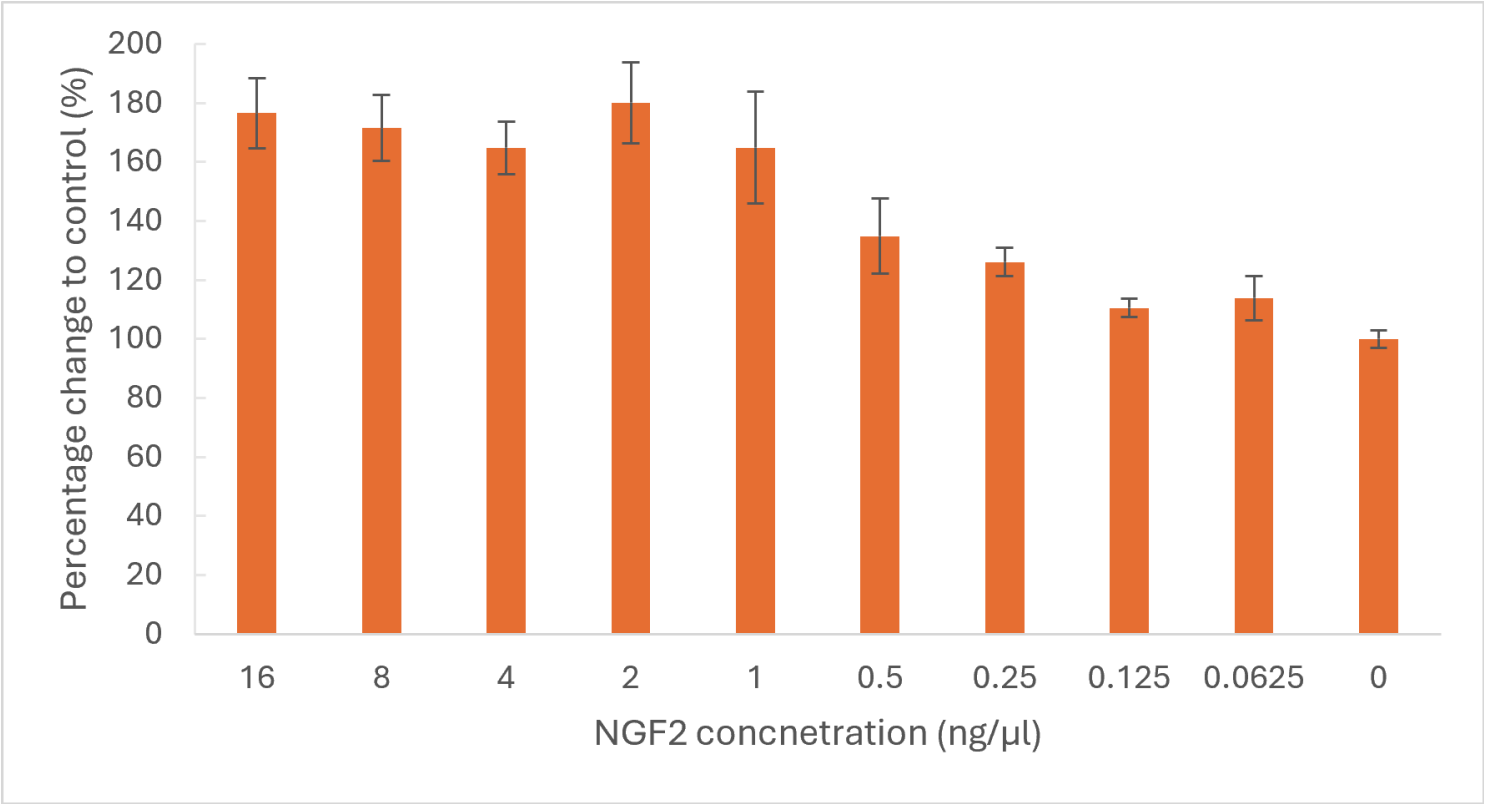


Figure 3

NIH-3T3 cell proliferation induced by different concentrations of NGF2

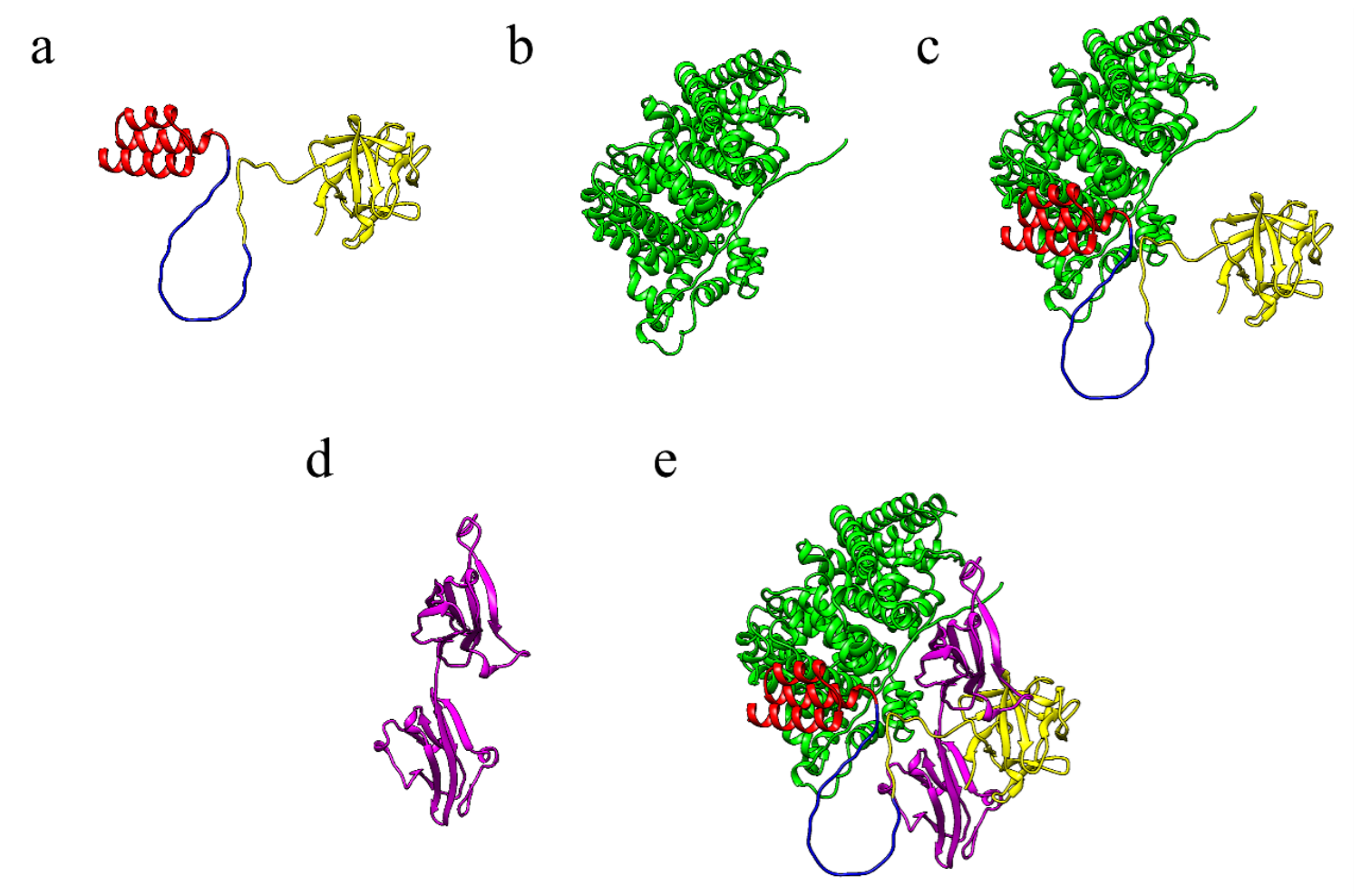


Figure 4

The structural prediction by AlphaFold3. (a) NGF2. Red: albumin binding domain (ABD); Blue: a soft linker (GGGGS)₅; Yellow: FGF2 (b) Human serum albumin (HSA). (c) NGF2-HSA complex. (d) Soluble domain of FGFR2. (e) FGFR2-NGF2-HSA complex.

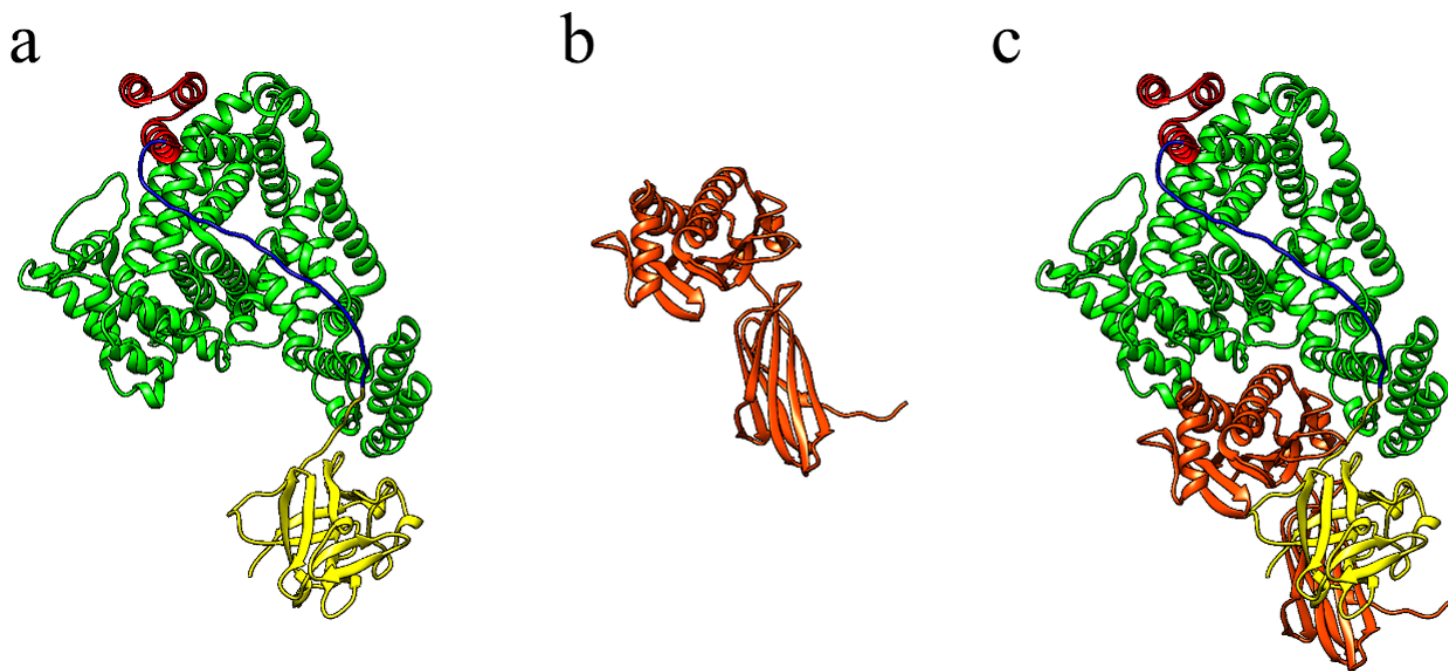


Figure 5

The structural prediction by AlphaFold3. (a) NGF2-HSA complex. (b) Soluble domain of FcRn. (c) NGF2-HSA-FcRn complex.

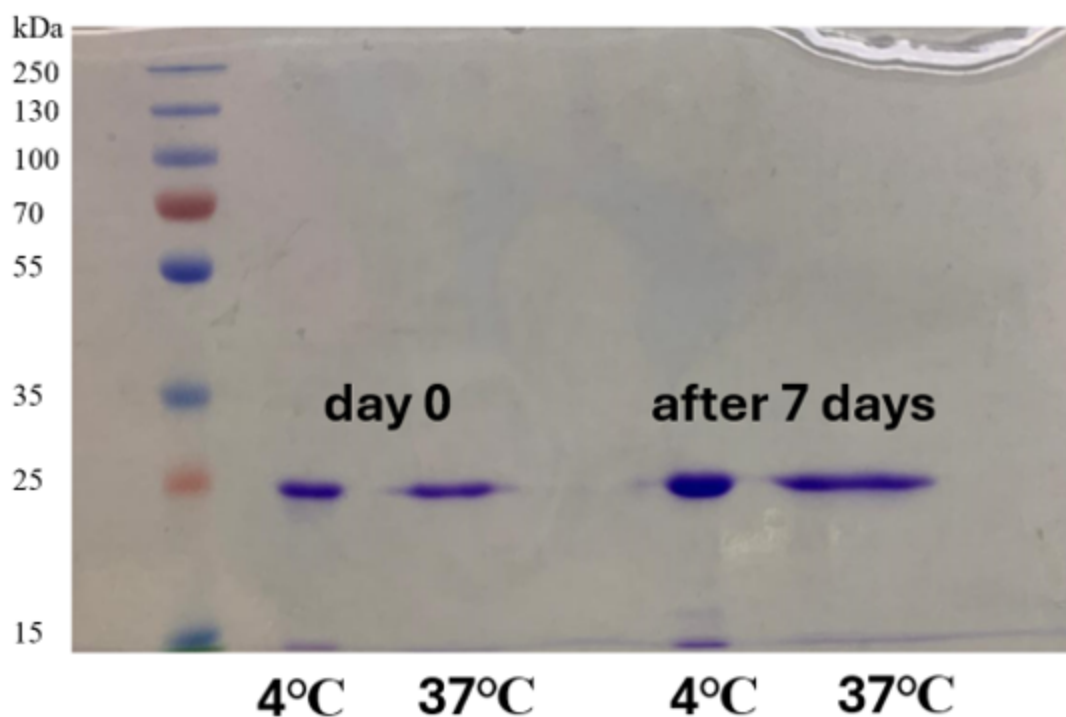


Figure 6

SDS-PAGE analysis of NGF2 in PBS solution at 4 °C and 37 °C for 7 days.