Intracellular Expression of B-Domain-Deleted Factor VIII Using Circular mRNA in Chinese hamster ovary cells

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Abstract

Hemophilia A is an X-linked bleeding disorder caused by mutations in the *F8* gene, leading to deficiency of clotting factor VIII (FVIII). Current therapy relies on frequent recombinant FVIII infusions, which are limited by short protein half-life. Circular mRNA (circRNA) has emerged as a promising expression platform due to its enhanced stability and translational efficiency. This study evaluated expression of full-length FVIII (FL-FVIII) and B-domain-deleted FVIII (BDD-FVIII) derived from circRNA in Chinese hamster ovary (CHO) cells. Plasmids encoding FL-FVIII and BDD-FVIII were first transformed into *E. coli*, validated by restriction digestion, and used for *in vitro* transcription and RNA circularization. CHO cells were transfected with FL-circRNA or BDD-circRNA, and FVIII expression was analyzed at 24-, 48-, 72-, and 96-hour post-transfection. FVIII expression from FL-circRNA remained indistinguishable from endogenous background at all time point in both cell supernatant and lysate. In contrast, a distinct intracellular protein band of approximately 170 kDa, consistent with single-chain BDD-FVIII, was detected at 24 hours in cells transfected with BDD-circRNA , clearly exceeding the endogenous background signal. These findings demonstrate that BDD-circRNA supports efficient intracellular FVIII translation but not secretion, highlighting its potential for further optimization of circRNA-based FVIII expression systems.

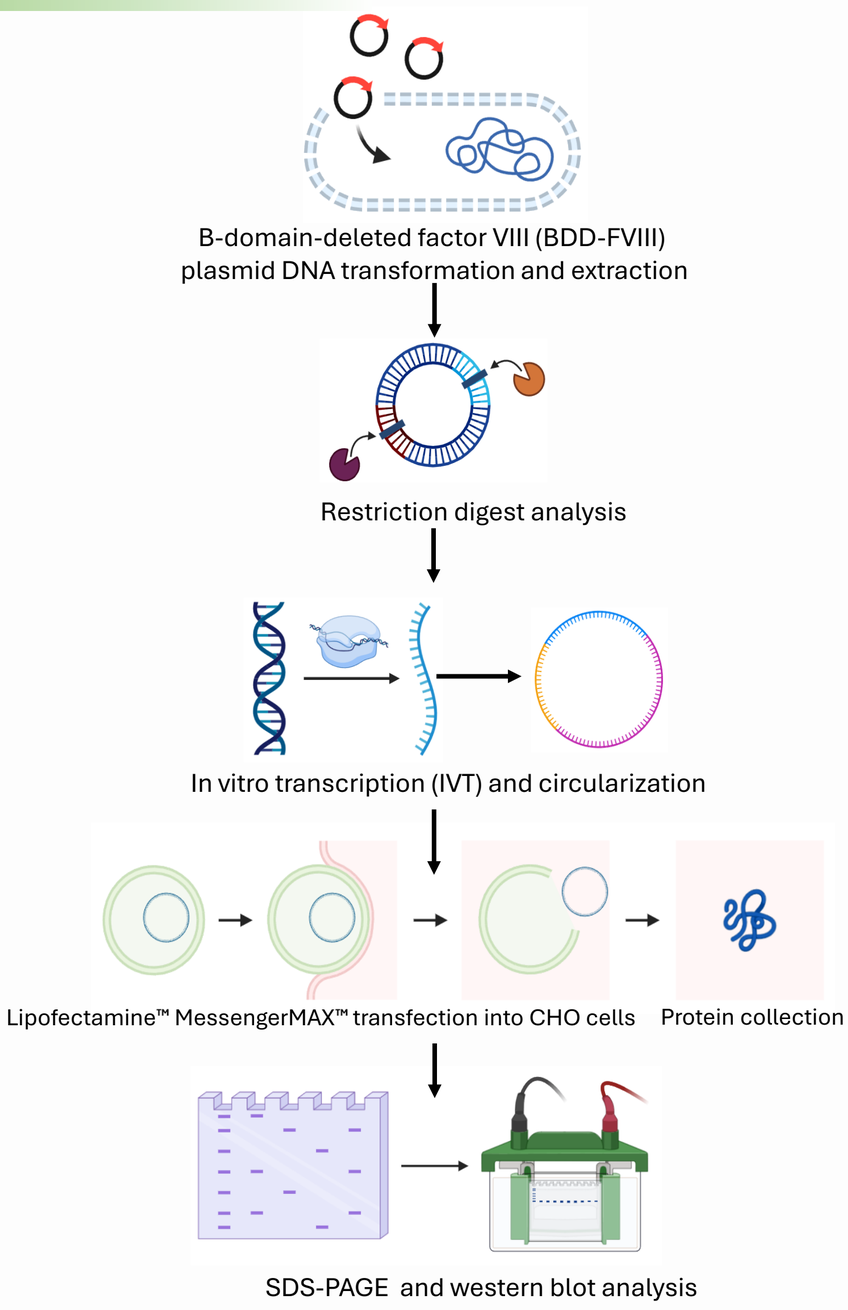
Introduction

Hemophilia A is an X-linked disorder caused by *F8* mutations that lead to Factor VIII (FVIII) deficiency and bleeding of variable severity. Conventional recombinant FVIII therapy is limited by its short half-life and frequent dosing requirements (Chernyi et al., 2024). Although B-domain-deleted FVIII (BDD-FVIII) improves biosynthesis, linear mRNA approaches remain hindered by instability and inefficient protein secretion (Pipe, 2009).

FVIII is mainly produced in liver sinusoidal endothelial cells and circulates bound to von Willebrand factor for stabilization. Following vascular injury, thrombin activates FVIII, enabling its function in the intrinsic coagulation pathway and clot formation (O'Donnell et al., 2019).

Circular mRNA (circRNA), with its covalently closed structure, offers greater stability and prolonged protein translation compared with linear mRNA (Wang et al., 2023; Chen et al., 2023). This study therefore evaluates full-length and BDD circRNA constructs to determine their potential to enhance FVIII expression for improved mRNA-based therapy in Hemophilia A.

Materials and Methods



Results and discussion

The integrity of the pUC57_101(+)_circBDD-FVIII plasmid (8,419 bp) was verified by restriction digestion with AscI and EcoRV-HF, yielding the expected fragments of 5,257 bp and 3,162 bp, thereby confirming correct plasmid architecture and its suitability for in vitro transcription and circRNA production. A faint additional band observed in both digested and undigested samples likely represented the open circular plasmid form caused by single-strand nicking. Subsequent agarose gel electrophoresis of IVT-generated RNA demonstrated successful synthesis and circularization of the BDD-FVIII construct: the linear precursor and auto-circularized RNA were initially visible as separate bands, and after circularization, a single band at the expected size (~5,257 bp) confirmed the formation of BDD-circRNA and the disappearance of the linear precursor.

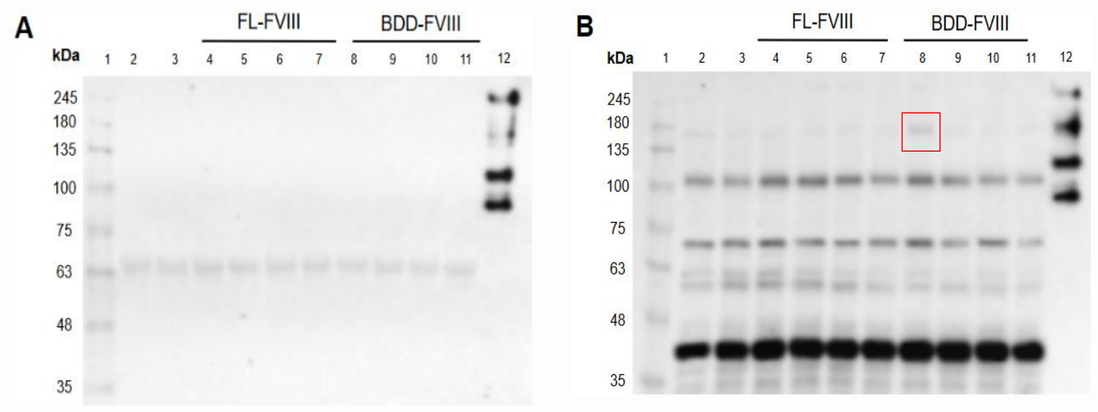


Fig. 1 Stability investigation of the full-length and BDD-circRNA. (A) Cell supernatant. Lane 2: negative control, Lane 3: CHO cells treated with Lipofectamine™ MessengerMAX™, lane 4-7: CHO cells transfected with FL-circRNA, lane 8-11: CHO cells transfected with BDD-circRNA, lane 12: Kovaltry (positive control). (B) Cell lysate. lane 2: negative control, lane 3: CHO cells treated with Lipofectamine™ MessengerMAX™, lane 4-7: CHO cells transfected with FL-circRNA. lane 8-11: CHO cells transfected with BDD-circRNA, lane 12: Kovaltry (positive control). Both cell supernatant and cell lysate were collected at 24-,48-,72-, and 96-hour post-transfection.

Although Factor VIII (FVIII) is primarily synthesized in liver sinusoidal endothelial cells, low-level endogenous *F8* expression has been reported in various cell lines (Campos-da-Paz et al., 2008). To identify a suitable host for recombinant production, endogenous FVIII expression was evaluated in CHO, HEK293T, and Vero cells, where low-intensity FVIII-immunoreactive bands (~65–75 kDa) were detected in both lysates and supernatants, likely reflecting minimal endogenous expression or nonspecific antibody binding. CHO cells exhibited the lowest background and were selected for further studies. Following transfection with full-length circRNA (FL-circRNA) or B-domain-deleted circRNA (BDD-circRNA), Western blot analysis showed persistent ~65 kDa bands in supernatants across all conditions, including controls, suggesting background reactivity (Fig. 1A). In contrast, a distinct ~170 kDa intracellular band corresponding to single-chain BDD-FVIII (Grushin et al., 2014) was observed at 24 hours post-transfection with BDD-circRNA (Fig. 1B), confirming successful intracellular translation, though no corresponding secreted protein was detected. FL-circRNA did not produce detectable FVIII above background levels. The intracellular accumulation of BDD-FVIII likely reflects impaired post-translational processing and secretion, as efficient FVIII export depends on proper folding, glycosylation, and interactions with ER chaperones such as BiP, calnexin, and calreticulin (Kaufman et al., 1997), indicating a bottleneck at the level of ER–Golgi trafficking.

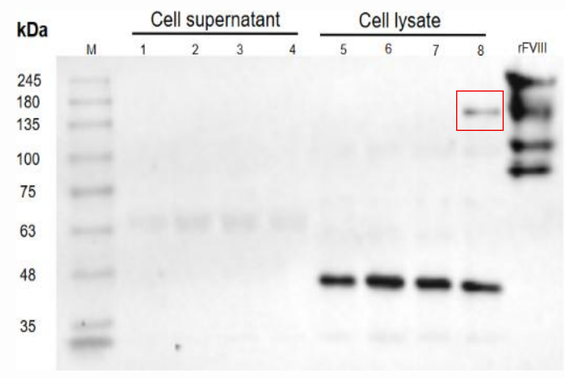


Fig. 2 24-hour post-transfection of FL-circRNA and BDD-circRNA. lane 1-4: Cell supernatant, lane 1: negative control, lane 2: Lipofectamine™ MessengerMAX™, lane 3: FL-circRNA, lane 4: BDD-circRNA, lane 5-8: Cell lysate, lane 5: negative control, lane 6: Lipofectamine™ MessengerMAX™, lane 7: FL-circRNA, lane 8: BDD- circRNA, Lane 9: Kovaltry (positive control).

A replication experiment in CHO cells transfected with FL-circRNA or BDD-circRNA confirmed the previously observed expression pattern (Fig. 2). A distinct ~170 kDa single-chain BDD-FVIII band was detected in BDD-circRNA–transfected cell lysates at 24 hours (Fig. 2, lane 8), while no intact FVIII was observed in culture supernatants, indicating intracellular retention due to B-domain deletion impairing ER–Golgi trafficking. These findings reinforce circRNA as an effective platform for intracellular FVIII translation but highlight limited secretion efficiency, suggesting that further optimization of BDD-circRNA, including B-domain sequence engineering, is required to enhance extracellular release.

Conclusion

In conclusion, our initial assessment confirmed that CHO cells exhibited the lowest endogenous FVIII expression background when compared to HEK293T and imHC cell lines, establishing their suitability for recombinant expression studies. Following transfection with the BDD-circRNA, FVIII distinguish from an endogenous background was undetectable in the cell supernatant, a result attributed to the removal of the B-domain, which is critical for efficient FVIII secretion. Critically, however, analysis of the cell lysate revealed a promising, distinct single-chain FVIII protein band in the BDD-circRNA group, demonstrating superior intracellular production compared to the FL-circRNA construct at 24-hour post-transfection.

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