METHODS AND APPLICATIONS

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The production of recombinant platelet-derived growth factor D using the second generation modified vaccinia Ankara viral system

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Abstract

The vaccinia virus expression system is known for the efficient production of recombinant proteins with "appropriate" posttranslational modification using desired mammalian cell lines. However, being a replication competent virus, vaccinia virus poses a health threat to immunocompromised individuals and requires biosafety level 2 (BSL2) laboratory precautions, thereby restricting its use by the scientific community. Development of the host range restricted modified vaccinia Ankara (MVA) system has allowed researchers to work with a safer virus even at BSL1. Here, we report on the use of an improved second generation MVA viral system incorporating two selective markers and fluorescent proteins for easier recombinant virus identification. Notably, we demonstrate that this novel system is capable of producing secreted recombinant proteins, a finding not previously reported. Through purification and characterization of wild type and mutant platelet-derived growth factor D (PDGF D) dimer species, we demonstrate this system is capable of producing the latent full-length PDGF D dimer, partially processed intermediate dimer (hemidimer), as well as fully processed growth factor domain dimer that show chemical integrity and biological activity. Importantly, this system is amenable to scaling up for the mass production of recombinant PDGF D (rPDGF D) dimer species.

KEYWORDS
full-length dimer, growth factor domain dimer, hemidimer, MVA, PDGF D, recombinant protein

1 | INTRODUCTION

The platelet-derived growth factor (PDGF) family of ligands has been extensively studied since the 1980s.[1-11] PDGF ligands are produced by a variety of cell types including platelets, macrophages, endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells, while their cognate receptors are highly expressed in cells of mesenchymal origin. Epithelial or endothelial cell-derived PDGF ligands activate their receptors expressed

in surrounding stromal cells, thereby mediating cell–cell interactions.[12] The involvement of the PDGF family in a myriad of physiological and pathological processes including organ development, angiogenesis, bone remodeling, cardiovascular disease, inflammatory responses, and cancer provides a great impetus for producing recombinant proteins of these ligands to permit scientists to study the mechanisms of PDGF biology and to test their therapeutic efficacies.[13–15] The classic PDGF ligands, PDGF A and B, are secreted as active homodimeric or heterodimeric ligands.[4] Advancement in recombinant protein technology has allowed for the production of these homo- and heterodimeric ligands.[16] In contrast to PDGF A and B, PDGF C and D are secreted as inactive homodimeric ligands.[4,17,18] In addition to the growth factor domain (GFD) which they share sequence homology with PDGF A and B, PDGF C and D possess an inhibitory N-terminal CUB domain which can be removed by extracellular serine proteases. The removal of one CUB domain from the inactive full-length (FL) homodimer generates a hemidimer (HD) and the subsequent removal of the other CUB domain results in an active GFD dimer (GFD-D).[19,20] Once activated, the GFD-D of PDGF C and D bind their cognate receptor α-PDGFR and β-PDGFR, respectively, resulting in activation of intracellular signal transduction leading to many different cellular responses including cellular proliferation, survival, migration, and differentiation.[4,20–25]

Although both PDGF B and GFD-D of PDGF D are potent activators of β-PDGFR, increasing evidence indicates functional differences between PDGF B and D. The CUB domain of PDGF D exerts inhibitory activity not only for its binding to β-PDGFR but also for PDGF D deposition to the extracellular matrix (ECM).[26] Our previous study suggested that differential ECM binding affinity among PDGF D dimer species (GFD-D > HD > FL dimer) may allow the formation of growth factor gradient in vivo as the latent FL dimer travels further within the tissues.[26] These properties may mediate more sophisticated signal transduction by PDGF D *in vivo* compared to PDGF B which is secreted as an active growth factor with a high ECM binding affinity, therefore acting as a local growth factor. Besides this, a novel function of HD,[27] although little is known at present, may also contribute to functional differences between PDGF B vs. PDGF D. To investigate complex functions of PDGF D, it is critical to produce recombinant proteins of PDGF D dimer species which can be utilized both *in vitro* and *in vivo*. Currently, only GFD-D protein of PDGF D is commercially available and its activity has been validated.

Using wild-type Western Reserve vaccinia virus, our group was able to produce FL PDGF C and PDGF D proteins which can be processed into the active form as evidenced by PDGFR activation and subsequent signal transduction.[19,25,28] However, Western Reserve vaccinia virus is a replication competent virus requiring BSL2 precautions and poses a health risk.[29] An improved and safer variant of vaccinia virus was developed through serial passages of the Chorioallantois vaccinia virus Ankara strain in chicken embryonic fibroblasts.[30] The modified vaccinia Ankara (MVA) strain is replication-incompetent in most mammalian cells with the exception of hamster BHK-21 cells which are fully permissive while several African green monkey cell lines are semi-permissive. Furthermore, MVA is unable to propagate in all healthy or immunocompromised mammalian species examined so far and in particular humans.[30] The safety of the MVA virus prompted research to develop its use as a viral expression vector for vaccination purposes.[31] Moreover, MVA has proved to be an attractive tool for recombinant protein production with an excellent protein production efficiency particularly after integration of the genes encoding the bacteriophage T7 RNA polymerase and regulatory elements of the *Escherichia coli* lac operon.[32]

Within this report, we describe the use of a second generation MVA viral system encoding the T7 RNA polymerase (MVA-T7g) that allows for easy and convenient selection of recombinant MVA virus and their recognition by fluorescence microscopic analysis. Importantly, we demonstrate the efficiency of this new system in producing secreted FL dimer (FL-D), HD, and GFD-D of PDGF D. We also successfully produced mutant FL dimer and HD which are resistant to serine protease-mediated proteolytic processing. Finally, we demonstrate that this system can be scaled up for production and purification of chemically stable and biologically active PDGF D proteins.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The Syrian hamster baby kidney cells (BHK21) were purchased from ATCC and grown in G-MEM (ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, ThermoFisher Scientific), 2 mg/ml tryptose phosphate broth (ThermoFisher Scientific) and 40ug/ml gentamycin (ThermoFisher Scientific). The murine fibroblast NIH3T3 cell line was grown in DMEM/F12 (ThermoFisher) supplemented 10% FBS. All cell cultures were maintained at 37°C with 5% CO_2.

2.2 | Reagents

Xanthine, hypoxanthine, mycophenolic acid (MPA), coumermycin, and isopropyl β-ᴅ-1-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich (St. Louis,

MO). Anti-PDGF D antibody was generated in house against the GFD of PDGF D as described in Reference 25. Antibodies against phospho-β-PDGFR, total β-PDGFD, phospho-Akt, and total Akt were purchased from Cell Signaling Technology (Danvers, MA). The anti-Penta-His antibody was purchased from Qiagen (Germantown, MD).

2.3 | Cloning of PDGF D:His variants in pVOTE2

His-tagged FLD, cleavage-resistant FLD (CRD) and triple mutant (TM) PDGF D were cloned into the NdeI and SacI restriction enzyme sites of pVOTE2,[33] while His-tagged or untagged GFD2 were cloned into the NdeI and XhoI restriction enzyme sites of pVOTE2. The PfuTurbo Cx HotStart DNA Polymerase amplification kit (Agilent, Santa Clara, CA) was used to amplify His-tagged FLD, CRD, and TM PDGF D variants from the pTF7 vector (described in Reference 26) with the aforementioned flanking restriction enzyme sites using the forward cloning primer: 5-CGATAATCATATGTCGCACCGGC-3′ and reverse cloning primer: 5-TGGCAGCAGCGAGCTCAGC TTCCTTT-3′ under the following PCR conditions; denaturation 95°C for 30 s, annealing 55°C for 30 s, and extension 72°C for 90 s. His-tagged and untagged GFD2, described in Reference 26, were amplified from the pSecTag2 vector with the aforementioned flanking restriction enzyme sites using the forward cloning primer: 5-GGC TAGCCCATATGGAGACAGAC-3′ and the reverse cloning primer: 5-GCCCTCCTCGAGCGGCCGCC-3′ under the following PCR conditions; denaturation 95°C for 30 s, annealing 55°C for 30 s, and extension 72°C for 60 s. The PCR products for PDGF D variants and pVOTE2 vector were digested with the respective restriction enzymes, treated with CIP (New England Biolabs, Ipswich, MA) then ligated using the Takara DNA ligation kit (Takara Bio, San Jose, CA). Vectors were then transformed into competent DH5α *E. coli* (ThermoFisher Scientific), isolated using the Qiagen HiSpeed Plasmid Maxi kit (Qiagen) and authenticated using Sanger sequencing (Genewiz, South Plainfield, NJ).

2.4 | Establishment of modified virus Ankara recombinant virus encoding PDGF D variants

To establish modified virus Ankara (MVA) recombinant virus to produce PDGF D proteins, BHK21 cells were plated at the cell density of 1 x 10⁶ cells in a six-well culture plate and grown for 24 hr. Confluent cells were then infected with 1 ml of MVA-T7g virus in cell culture medium at about 0.1 plaque forming units then incubated at 37°C for 1 hr to allow for viral infection. Residual virus in the media was removed by aspiration and cells gently washed with 1 ml OptiMEM (ThermoFisher Scientific). Cells were then transfected with pVOTE2-PDGF D:His expression plasmids described above using Lipofectamine 2000 (ThermoFisher Scientific) in 1 ml OptiMEM overnight at 37°C then fed with 1 ml of growth media. For the CRD:His/GFD2 MVA recombinant virus, BHK21 cells infected with MVA-T7g virus were transfected with a 1:1 ratio of pVOTE2-CR FLD:His and pVOTE2-GFD2 plasmids. Once cytopathic events (c.p.e.) were observed, characterized by a cell shape change (rounding up), the entire cell culture plate was frozen at −20°C. Three freeze/thaw cycles were then performed on the infected/transfected cell cultures to release the recombinant virus from the cells. Serially diluted recombinant virus was then used to infect a new six-well plate culture of BHK21 cells in 2% serum G-MEM supplemented with 0.5 mg/ml xanthine, 0.05 mg/ml MPA, and 0.03 mg/ml hypoxanthine. BHK21 cells exhibiting both green fluorescent protein (GFP) and red fluorescent protein (RFP)-positive viral plaques were used to perform three freeze/thaw cycles. The released virus was once more serially diluted and used for the second round of viral selection by infecting BHK21 cells grown in 5% FBS G-MEM supplemented with 0.5 mg/ml xanthine, 0.05 mg/ml MPA, 0.03 mg/ml hypoxanthine, and 100 ng/ml coumermycin. Additional two cycles of selection with MPA and coumermycin were performed until GFP positive and RFP negative viral plaques were observed. The desired PDGF D-MVA-T7g virus was then amplified in the absence of MPA and coumermycin and stored at −20°C.

2.5 | Isolation of recombinant PDGF D (rPDGF D)

BHK21 cells were grown up to confluence in 100 mm plates then infected with the respective PDGF D-MVA-T7g virus for 1 hr. The remaining virus in media was removed, and cells gently washed twice with PBS to remove any FBS remnants. Cells were then cultured in 12.5 ml serum free G-MEM supplemented with 1 mM IPTG for 36–48 hr. Conditioned media (CM) was collected and cleared by centrifuging at 3,000 rpm for 5 min. This CM was used to assess the production of secreted PDGF D using SDS-PAGE followed by immunoblot analysis.

To isolate rPDGF D, the cleared CM was equilibrated with binding buffer containing 20 mM sodium phosphate pH 7.4, 500 mM sodium chloride, and 20 mM imidazole. The equilibrated CM was then pumped through a HisTrap HP (Sigma-Aldrich) column at a rate of 1 ml/min using a BioRad BioLogic LP (BioRad, Hercules, CA) at

4°C. The HisTrap column was then washed with binding buffer and eluted using an elution buffer containing 20 mM sodium phosphate pH 7.4, 500 mM sodium chloride, and 200 mM imidazole. For PDGF D HD isolation, in addition to the binding buffer wash, the HisTrap column was washed with 20 mM sodium phosphate pH 7.4, 300 mM sodium chloride and 40 mM imidazole, then eluted with 20 mM sodium phosphate pH 7.4, 500 mM sodium chloride, and 75 mM imidazole. The starting material (equilibrated CM) and eluted fractions were loaded on a reducing 10% SDS-PAGE gel and subjected to immunoblot analysis using Penta-His or anti-PDGF D antibodies. Fractions demonstrating high levels of rPDGF D proteins were pooled and desalted with PBS using a 5% Tween-20 blocked Amicon Ultra concentrator (Millipore Sigma, St. Louis, MO).

2.6 | Quantitation of rPDGF D proteins

Known concentrations of bovine serum albumin (BSA, ThermoFisher Scientific), the starting material (equilibrated CM) and the desalted recombinant proteins were loaded on a reducing 10% SDS-PAGE gel. The gel was then stained with the Pierce Silver Stain Kit (ThermoFisher Scientific). Band intensity was measured using NIH ImageJ and a concentration curve was generated for the BSA standards. The concentration of rPDGF D was estimated using this concentration curve.

2.7 | β-PDGFR transactivation assay in NIH3T3

To test the activity of rPDGF D proteins within the CM or after purification, NIH3T3 cells were plated in a six-well dish and grown for 24 hr. These cells were serum starved for 24 hr before treatments with 0.5 ml of PDGF D CM for 15 min or 1 nM of recombinant GFD2 dimer for 5 or 15 min. To assess the biological activity of FLD, 1 nM FLD was digested with 7.5 nM recombinant matriptase (rMat, R&D Systems, Minneapolis, MN) for 2 hr at 37°C. The reaction mixture containing matriptase-processed GFD-D was used to activate β-PDGFR in NIH3T3 cells for 15 min. Immunoblot analysis was performed with anti-phospho-β-PDGFR and anti-β-PDGFR antibodies using whole cell lysates prepared by lysing cells for 30 min in 1X RIPA lysis buffer (Millipore Sigma) supplemented with 100 mM PMSF, 200 mM $NaVO_3$, 1 M NaF, and 8% 50X protease inhibitor cocktail (Roche, Indianapolis, IN). Protein concentration was determined using the Pierce BCA protein quantitation assay (ThermoFisher Scientific).

3 | RESULTS

3.1 | A novel second generation MVA viral system for the production of recombinant proteins of interest

The first generation MVA system incorporated the lac operon-regulated T7 RNA polymerase and GFP at the thymidine kinase (TK) locus of the vaccinia genome and the E. coli gpt (xanthine–guanine phosphoribosyltransferase) gene at the hemagglutinin (HA) locus for positive recombinant virus selection using MPA.[32] A novel second generation MVA viral system (MVA-T7g) was established with an eGFP gene driven by a synthetic early/late vaccinia virus promoter so that GFP fluorescence is stronger than the first generation MVA-T7. Moreover, using G418 selection, a NeoR-mCherry fusion gene is inserted adjacent to a fusion gene encoding the dimerization domain of the E. coli gyrase B and the catalytic domain of the dsRNA-dependent protein kinase (PKR) at the HA locus (Figure 1a). Expression of this Gyr-PKR fusion protein is used as negative selection by preventing MVA-T7g multiplication in the presence of the drug coumermycin which induces Gyr-PKR dimerization entailing phosphorylation of eIF2, resulting in inhibition of protein synthesis. MVA-T7g virus could then be used as a parental virus to isolate recombinant virus encoding genes of interest as well as the E. coli xanthine–guanine phosphoribosyltransferase (gpt) gene inserted between the HA right (HA_R) and HA left (HA_L) arms of an expression plasmid through homologous recombination in the HA locus thereby replacing both the Gyr-PKR and the mCherry-Neo fusion genes (Figure 1a). Such recombinant viruses can be selected after one round of culture in the presence of MPA for positive selection of virus with a gene of interest and gpt expression and several rounds of negative selection through inhibition of parental virus MVA-T7g multiplication in the presence of coumermycin. Compared to the parental MVA-T7g virus that expresses both eGFP and RFP, these recombinant viruses are easily tracked by the loss of red fluorescence and can be amplified without a plaque purification step (Figure 1b).

3.2 | Generation of recombinant viruses encoding wild-type and mutant PDGF D dimer species

Serine proteases including matriptase cleave PDGF D at its cleavage site ($PRYR_{247}GR_{249}/SY$) within the hinge region between the CUB and GFD of FL PDGF D (FLD) (Figure 2a and References 25 and 28). Removal of one of the inhibitory CUB domains generates an HD composed

FIGURE 1 Description of the second generation modified vaccinia Ankara (MVA)-T7g virus. (a) Diagrammatic representation of the second generation MVA-T7g virus and the pVOTE2 expression plasmid. (b) Fluorescence microscopic analysis of green fluorescent protein (GFP) and red fluorescent protein (RFP) in BHK21 cells transduced with parental MVA-T7g virus
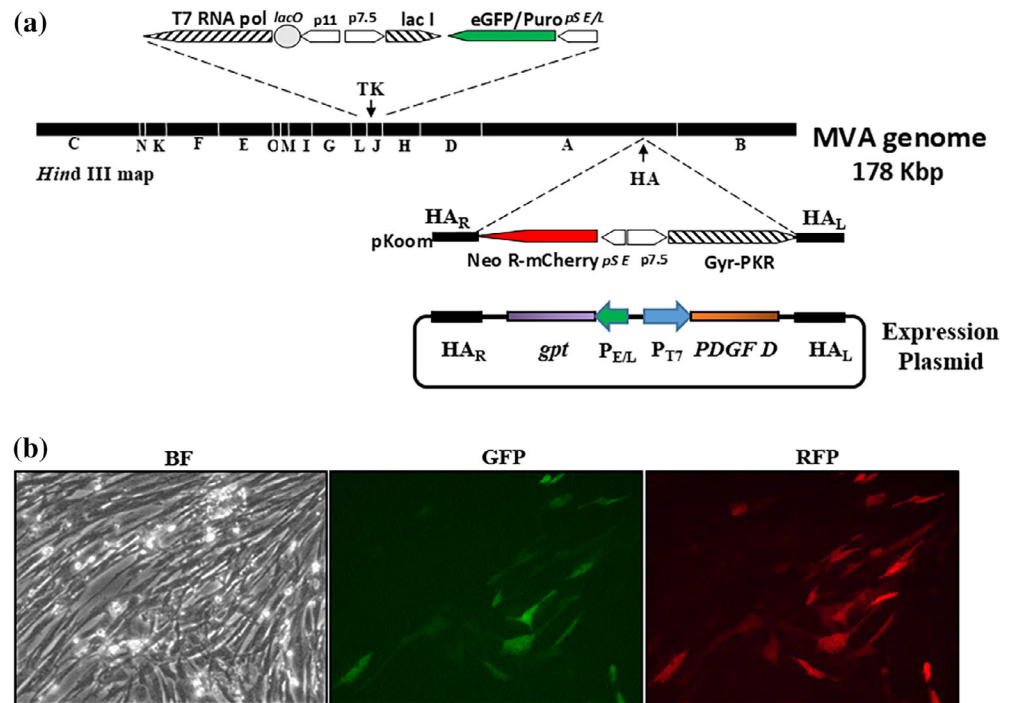

of an FLD and a GFD subunit and further removal of the second CUB domain yields the active GFD-D (Figure 2a and Reference 20). To generate a stable FLD resistant to proteolytic processing, the arginine residues (R_{247}/R_{249}) in the serine protease cleavage site within the linker region of PDGF D were mutated to alanines as previously described,[26] and this PDGF D variant was termed as cleavage-resistant PDGF D (CRD). To investigate whether PDGF D has any biological functions independent of its ability to activate β-PDGFR possibly via the CUB domain, three arginine residues in the $R_{340}R_{341}GR_{343}$ motif within the GFD, known to be critical for its binding to β-PDGFR, were mutated to alanines (TM).[26] To purify recombinant GFD-D proteins, the CUB domain was removed and an immunoglobulin kappa chain leader sequence was added to allow for secretion of GFD-D (GFD2). PDGF D variants with or without His-tag were cloned into the pVOTE2 expression plasmid as depicted in Figure 2b.

To generate recombinant MVA-T7g virus encoding PDGF D variants, BHK21 cells were infected with the MVA-T7g virus for 1 hr followed by transfection with control pVOTE2 (empty vector) or pVOTE2 containing FLD, CRD, TM, or GFD2 of PDGF D. For the generation of HD, pVOTE2-CRD (His tagged) and pVOTE2-GFD2 (non-His tagged) vectors at a 1:1 ratio were simultaneously transfected. Cells were selected with coumermycin as a negative selection drug against parental virus expressing the fusion gene Gyr-PKR. In addition, MPA was used as a positive selection drug selecting for recombinant virus which expresses gpt to metabolize MPA.[32]

Moreover, GFP$^+$ foci confirmed viral infection while RFP$^-$ foci indicated homologous recombination at the HA locus replacing the RFP-Gyr-PKR with gpt-PDGF D variant as illustrated in Figure 1a.

3.3 | Production of rPDGF D dimer species using the second generation MVA-T7g viral system

Through monitoring of PDGF D-MVA recombinant viral production, we observed that cells infected with FLD- or CRD-MVA-T7g virus develop GFP$^+$ and RFP$^-$ foci as expected (Figure 2c), whereas the parental MVA-T7g express both GFP and RFP (Figure 1b). This was also observed for other PDGF D variant-MVA-T7g constructs indicating successful recombinant virus productions. While the second generation MVA-T7g virus has been previously used to produce intracellular protein, its ability to produce secreted protein has not been demonstrated. Here, we tested whether the second generation MVA-T7 viral system can be utilized for the production of secreted PDGF D dimer species. To this end, CM was generated from BHK21 cells infected with different PDGF D variant-MVA viruses in the absence or presence of IPTG that triggers transcription of the lac operon, therefore inducing production of recombinant proteins. Immunoblot analysis of the CM show efficient PDGF D production compared to control recombinant virus established using an empty pVOTE2 vector (EV) (Figure 3a). In control experiments in the absence of IPTG, rPDGF D
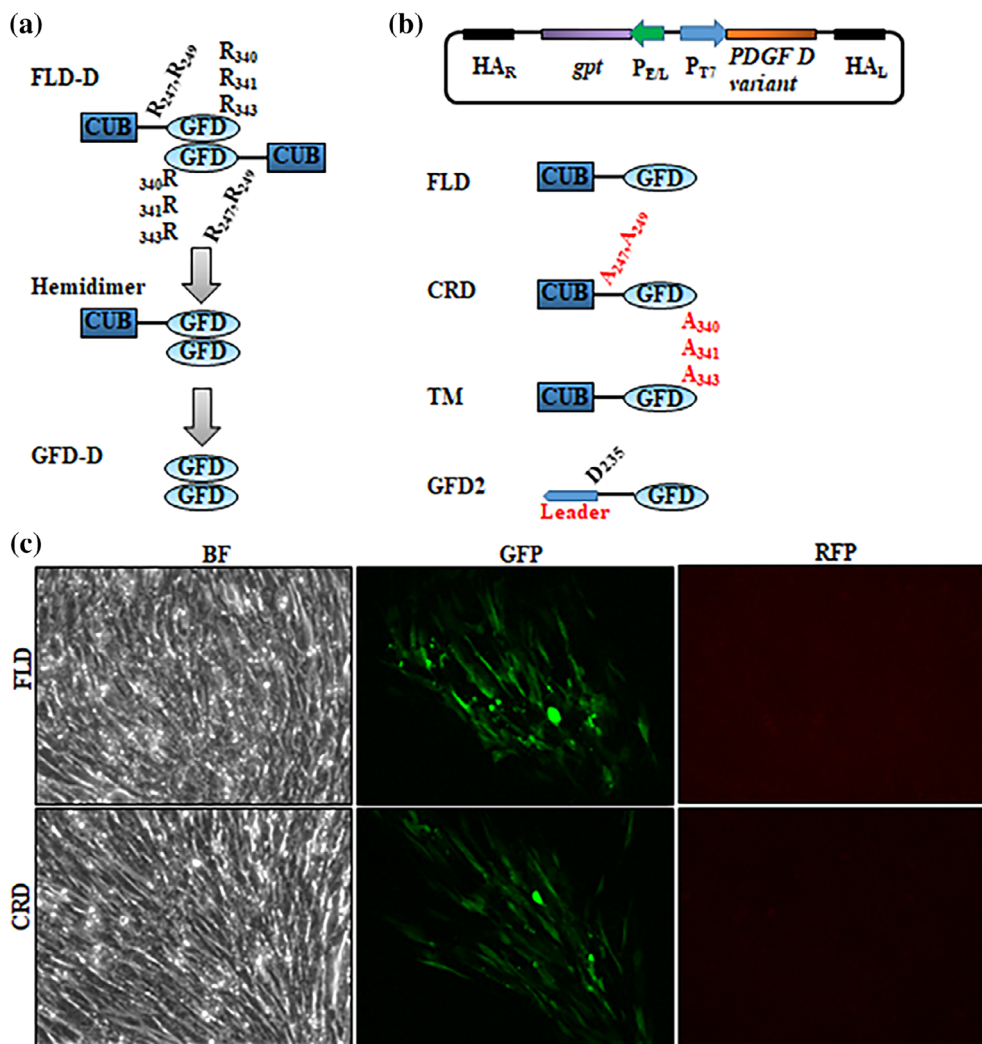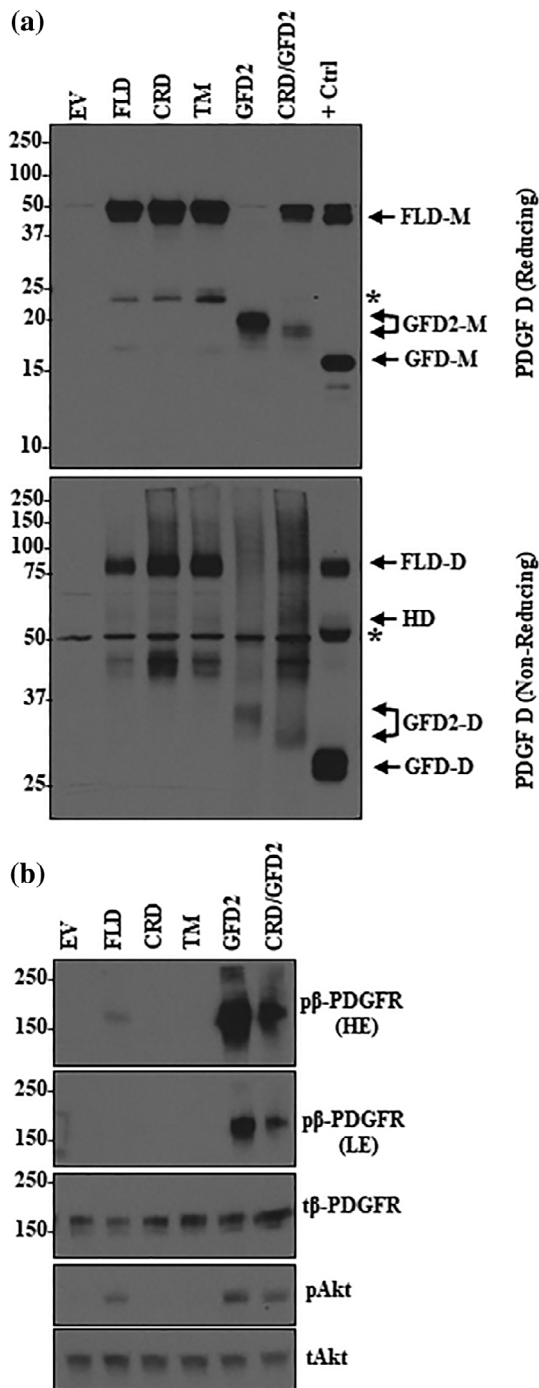
FIGURE 2 Establishment of recombinant modified vaccinia Ankara (MVA)-T7g viruses expressing platelet-derived growth factor (PDGF) D variants. (a) A schematic diagram depicting a two-step proteolytic process of latent FLD-D into the active growth factor domain dimer (GFD-D): The removal of one CUB domain by serine proteinase-mediated cleavage at its cleavage site ($PRYR_{247}GR_{249}/SY$) in the linker region generates the hemidimer (HD) and the removal of the second CUB domain generates GFD-D. Arginine residues R_{340}, R_{341}, and R_{342} are critical for GFD binding to β-PDGFR are indicated. (b) Diagrammatic representation of PDGF D variants used to generate recombinant MVA-T7g viruses. FLD, wild-type PDGF D; CRD, cleavage-resistant PDGF D mutant, where $R_{247}R_{249}$ in the serine protease cleavage site are mutated to alanines; TM, triple mutant PDGF D, where the R_{340}, R_{341}, and R_{343} in the β-PDGFR binding region are mutated to alanines; GFD2, GFD expression plasmid (D_{235}–R_{370}) under the immunoglobulin kappa chain leader sequence mediating GFD secretion. (c) Microscopic analysis of green fluorescent protein (GFP) and red fluorescent protein (RFP) as well as bright field (BF) of BHK21, infected with MVA-T7g virus, transfected with FLD- or CRD-pVOTE2, and selected in the presence of coumermycin

proteins were not detectable, indicating a tightly regulated inducible system for protein production (data not shown).

Immunoblot analysis in reducing condition detected ~50 kDa FLD, CRD, and TM as well as ~20 kDa GFD2 monomers (Figure 3a, upper panel). The molecular weight of recombinant GFD2 protein is slightly bigger than the GFD monomer (GFD-M) observed in the positive control lane since GFD2 contains an additional linker sequence. The CRD/GFD2 MVA virus produced both the 50 kDa full length monomer and the GFD2

protein. It should be noted that the MVA viral system produced rPDGF D proteins which are readily detectable in CM, whereas ~50-fold concentration of CM was required to detect PDGF D proteins from PDGF D over-expressing cells where PDGF D is being driven by a CMV promoter of an expression plasmid.[24]

To examine whether these PDGF D variants are properly dimerized, the same samples were analyzed on a nonreducing SDS-PAGE followed by immunoblot analysis. As shown in the lower panel of Figure 3a, ~90 kDa FLD-D, CRD-D, and TM-D as well as ~35 kDa GFD2-D

(a)

(b)

assay in NIH3T3 cells as we previously described[23] using CM collected from cells infected with control, FLD, CRD, TM, GFD2 or CRD/GFD2 recombinant virus. Wild-type FLD, which can spontaneously undergo proteolytic activation by trace amounts of serine proteases present in CM, was able to activate β-PDGFR and its downstream signal transducer Akt at a low level (Figure 3b). In contrast, CRD, resistant to serine-protease-mediated proteolytic activation, failed to activate β-PDGFR. TM dimers with mutations in the β-PDGFR binding site also failed to activate β-PDGFR as expected. Importantly, CM containing GFD2 dimer effectively activated β-PDGFR signaling (Figure 3b). CM produced by the mixture of CRD/GFD2 recombinant viruses also resulted in β-PDGFR activation, likely by GFD2 dimer. Taken together, these data demonstrate that the MVA-T7g system efficiently produces properly dimerized and secreted PDGF D variants that maintain biological properties.

3.4 | Purification of recombinant FLD, CRD, and TM dimers of PDGF D

Our small-scale proof-of-concept experiments supported the use of the MVA-T7g second generation system for rPDGF D production (Figures 1–3). Next we scaled up to a large culture to purify rPDGF D by infecting BHK21 cells with FLD-, CRD-, or TM-MVA-T7g recombinant viruses as well as EV-MVA-T7g virus as a control. To purify PDGF D dimer species containing the C-terminal histidine tag, we used the His-Trap immobilized-metal affinity chromatography (IMAC).[36] While CM generated from control virus (EV) infected cells did not have any recombinant proteins (data not shown), anti-PDGF D and anti-His antibodies detected ~90 kDa PDGF D dimer

FIGURE 3 The second generation modified vaccinia Ankara (MVA)-T7g system effectively produces structurally stable and biologically active platelet-derived growth factor (PDGF) D dimer species. (a) Immunoblot analysis of PDGF D in reducing (top panel) and nonreducing (bottom panel) conditions using conditioned media (CM) collected from BHK21 cells infected with recombinant FLD-MVA-T7g, CRD-MVA-T7g, TM-MVA-T7g, growth factor domain (GFD)2-MVA-T7g, or mixture of CRD/GFD2-MVA-T7g viruses. Positive control (+Ctrl) proteins were prepared using LNCaP overexpressing PDGF D described in Reference 28. FLD-M and FLD-D, full-length PDGF D monomer and dimer, respectively; GFD-M and GFD-D, growth factor domain monomer and dimer, respectively; HD, hemidimer; *, nonspecific band. (b) Immunoblot analysis of indicated markers using cell lysates from NIH3T3 cells treated with CM from BHK21 cells expressing indicated PDGF D variants. HE, high exposure; LE, light exposure

were detected. Importantly, the mixture of CRD- and GFD2-recombinant viruses led to dimerization of CRD-D (90 kDa) and GFD2-D (proteolytically processed ~32 kDa) dimers as well as the 58 kDa HD containing both CRD and GFD subunits (Figure 3a, lower panel and also see Figure 6).

Maintaining the proper tertiary structure and biological activity is key to the manufacturing of recombinant proteins.[34,35] Thus, we performed a β-PDGFR transactivation

in CM (starting material before purification) prepared using FLD-, CRD-, or TM-MVA-T7g virus infected cells (Figure 4a–c). Analysis of the flow through and wash fractions demonstrate efficient binding of recombinant proteins to the His-Trap column (Figure 4a–c, second and third lanes, respectively). Imidazole competes with the His-tag and thus elutes recombinant FLD, CRD, and TM proteins (Figure 4a–c, fractions 5 and 10).

The eluted fractions containing recombinant proteins were pooled and desalinated by diafiltration using PBS.[37] Silver staining of the desalted recombinant protein showed a single ~90 kDa band suggesting clean recombinant protein preparation (Figure 5a). Quantitation of the silver stain showed a range of 12–36 μg protein yield produced from the 50 ml starting CM (Table 1).

Next, we tested the structural integrity and activity of rPDGF D. To this end, 1 nM of purified rFLD and rCRD dimers were digested with increasing concentrations of recombinant matriptase. In agreement with our previous report,[20] a dose dependent, two-step processing of full length 90 kDa FLD dimer into the 58 kDa HD and eventually the 18 kDa (GFD-D), was detected as shown by immunoblot analysis of PDGDF D in nonreducing condition (Figure 5b, top panel). Immunoblot analysis under reducing conditions detected 50 kDa FLD and 18 kDa GFD monomer proteins (Figure 5b, bottom panel). As

expected, rCRD was not proteolytically activated by recombinant matriptase due to the mutations at the serine protease cleavage site. Next, we tested the activity of the purified recombinant FLD-D by β-PDGFR transactivation assay. As shown in Figure 5c, rFLD dimer digested with matriptase activated β-PDGFR and its secondary signal transducer Akt. These results demonstrated that structurally and biologically stable FL PDGF D variants can be purified using the MVA-T7g second generation MVA system.

3.5 | Purification of recombinant HD of PDGF D

Although the yield appears to be low, Figure 3 indicates the possibility of purifying HD of PDGF D using the second generation MVA-T7g system. Thus, we scaled up the BHK21 cell culture and infected those cells with the mixture of His-tagged CRD/untagged GFD2 recombinant MVA viruses. Analysis of the starting CM showed both the 58 kDa HD and the 90 kDa CRD-D (Figure 6b, lane 1). Therefore, we developed a strategy to purify serine protease cleavage-resistant, stable HD as follows: CRD-D and HD, but not GFD2-D, are captured by the His-Trap Column, and HD is differentially eluted using increasing
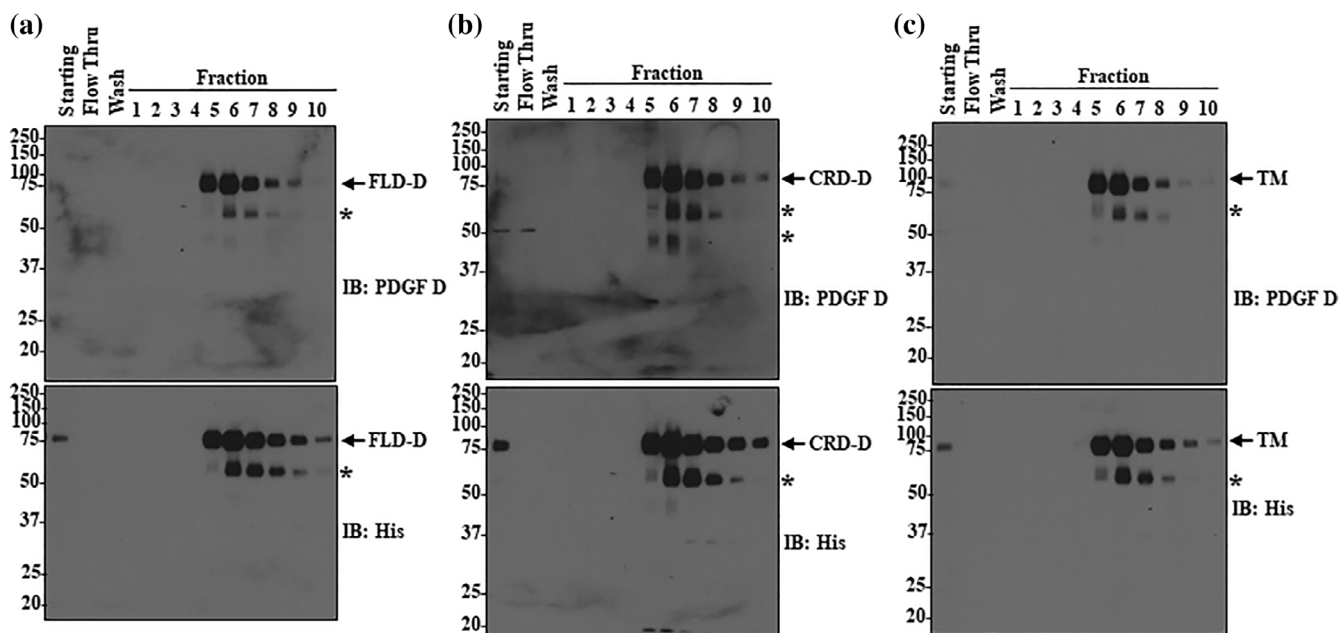
FIGURE 4 Purification of wild-type and mutant platelet-derived growth factor (PDGF) D dimer proteins using high-performance immobilized metal affinity chromatography (IMAC) column. His-tagged proteins in 50 ml conditioned media (CM) from BHK21 cells infected with FLD-modified vaccinia Ankara (MVA)-T7g (a), CRD-MVA-T7g (b), or TM-MVA-T7g (c) virus were purified using His-Trap and subjected to immunoblot analysis using anti-PDGF D (top panel) or anti-His antibody (bottom panel). CM before His-tag protein purification (starting), flow-through, wash fraction (wash), and elution fractions were analyzed. FLD-D, full-length PDGF D dimer; CRD-D, cleavage-resistant PDGF D dimer; TM, triple mutant full-length PDGF D. *, nonspecific band
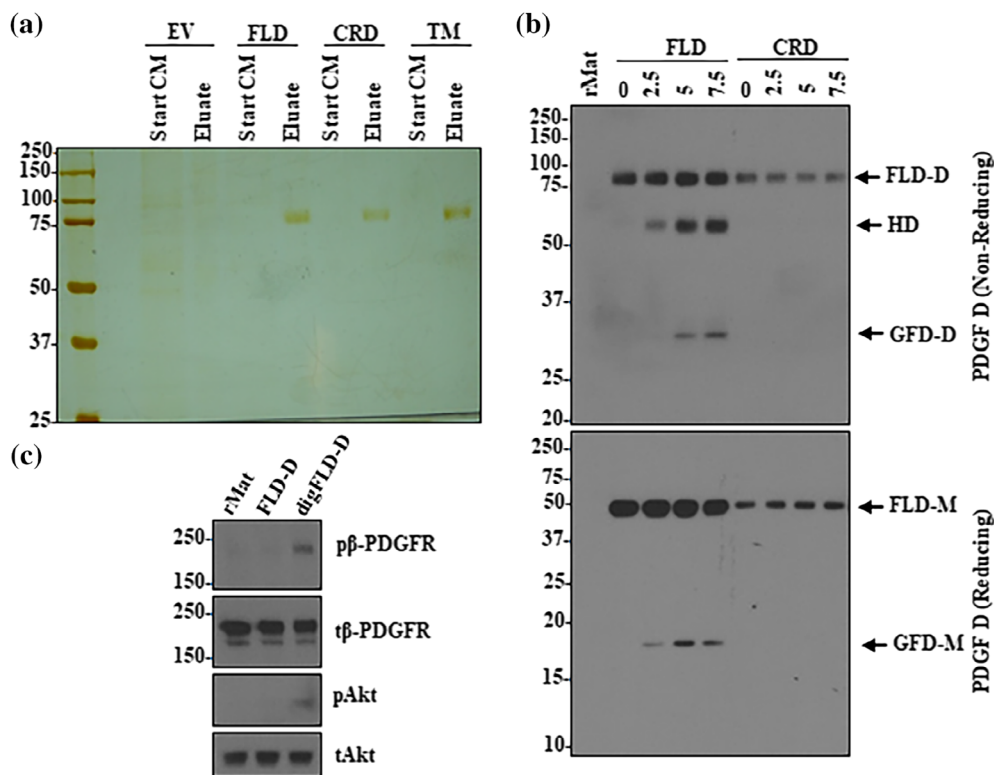
FIGURE 5 The modified vaccinia Ankara (MVA)-T7g viral system produces structurally stable and biologically active recombinant platelet-derived growth factor (rPDGF) D. (a) A representative Silver Stain of the rPDGF D produced in Figure 4. (b) 1 nM of FLD or cleavage-resistant FLD (CRD) were digested with increasing concentrations of recombinant matriptase (0–7.5 nM rMat) and subjected to immunoblot analysis in nonreducing (top panel) and reducing (bottom) panel using anti-PDGF D antibody. FLD-M and FLD-D, full-length PDGF D monomer and dimer, respectively; growth factor domain (GFD)-M and GFD-D, growth factor domain monomer and dimer, respectively; HD, hemidimer. (c) Serum-starved NIH3T3 fibroblasts were treated with undigested (FLD-D) or matriptase digested FLD-D (digFLD-D), followed by immunoblot analysis of indicated markers. Recombinant matriptase (rMat) alone included as a control

TABLE 1 Summary of rPDGF D protein production using the second generation MVA-T7g system

| PDGF D variant	Starting conditioned media volume (ml)	Pooled fraction volume (ml)	Desalted volume (µl)	Concentration (ng/µl)	Total protein produced (ng)
FLD	50	4	310	38.8	12,028
CRD	50	4	200	181	36,200
TM	50	4	230	112.1	25,783
GFD	100	5	170	48.4	8,228
HD	50	9	240	12.35	2,964

Abbreviations: GFD, growth factor domain; MVA, modified vaccinia Ankara; PDGF, platelet-derived growth factor; rPDGF, recombinant PDGF.

concentrations of imidazole as depicted in Figure 6a[26]. As shown in Figure 6b, we successfully separated HD from CRD-D by first eluting CRD-D from the His-Trap column with a lower concentration (40 mM) of imidazole over a longer period of time (26 fractions) compared to a higher concentration of imidazole used for CRD-D elution in 10 fractions in Figure 3. Subsequently, HD was eluted with (75 mM) of imidazole. Immunoblot analysis of purified HD in both nonreducing and reducing

conditions confirmed that the 58 kDa dimer contains both CRD and GFD2 subunits (Figure 6c).

3.6 | Purification of recombinant GFD-D of PDGF D

Although recombinant GFD-D of PDGFD is commercially available, silver staining of those revealed a myriad of

(a)

(b)

(c)
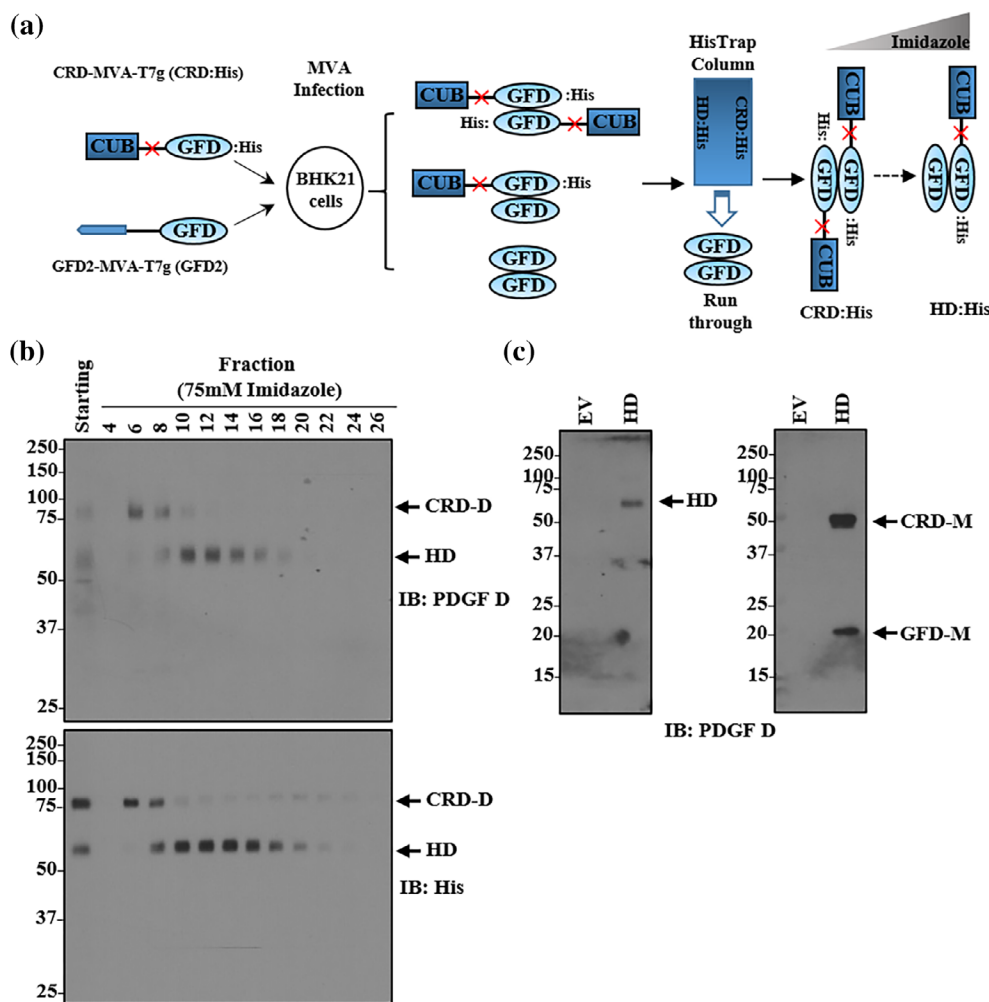
FIGURE 6 Production and purification of cleavage-resistant hemidimer using the modified vaccinia Ankara (MVA)-T7g viral system. (a) Diagrammatic representation of the strategy to purify serine protease cleavage-resistant platelet-derived growth factor (PDGF) D hemidimer (HD). (b) CRD-D and CRD/GFD2 hemidimer were differentially eluted from the His-Trap column using increasing concentrations of imidazole and subjected to immunoblot analysis in nonreducing condition using anti-PDGF D (top panel) and anti-His (bottom panel) antibodies. CRD-M and CRD-D, cleavage-resistant PDGF D monomer and dimer, respectively. (c) Purified rHD was reanalyzed by immunoblot analysis in both nonreducing (left panel) and reducing (right panel) conditions using a PDGF D specific antibody. CRD-M, cleavage-resistant PDGF D monomer; GFD-M, growth factor domain monomer

protein bands suggesting less than optimal purification of this recombinant protein (data not shown). To purify GFD-D, we infected BHK21 cells with His-tagged GFD2-MVA-T7g virus, described in Figure 2. rGFD2-D under the IgG kappa chain leader sequence was efficiently secreted and we successfully purified this protein from the CM using a His-Trap column (Figure 7a). To test whether the purified rGFD2-D is biologically active, NIH3T3 were treated with 1 nM of GFD2-D for 5 or 15 min. Both β-PDGFR and its secondary messenger Akt were readily activated by recombinant GFD2-D (Figure 7b), whereas the control (veh) solution, prepared from CM of control virus-infected BHK21cell and gone through the same His-Trap purification procedure, had no effect on β-PDGFR signaling.

Taken together, the present study reports on the production and purification protocols for rPDGF D dimer species utilizing the second generation MVA viral system. These PDGF D dimer species will serve as valuable reagents to unveil the complex roles of PDGF D for the regulation of many different cellular processes in vitro as well as in vivo.

4 | DISCUSSION

Despite the advantages of the vaccinia virus expression system that allows protein production in the desired mammalian cells for proper posttranslational modification, the use of replication-competent vaccinia virus strains such as the Western Reserve strain has been limited due to health risks. In this regard, great strides have been made to improve the safety of the vaccinia virus expression system. The host range restricted MVA strain has been engineered to encode the bacteriophage T7 RNA polymerase for enhanced safety either upon transfection of MVA infected cells with the foreign gene to be expressed[29,38] or, as carried out herein, after incorporation of the desired foreign gene into the viral genome.[32] While the original MVA-T7 expression system relies on MPA for recombinant virus selection, the second generation MVA system employs the mCherry marker and a Gyr-PKR fusion gene in the viral HA locus whereby resistance to coumermycin toxicity selects against parental virus and loss of RFP fluorescence serves as a visual

(a)

(b)
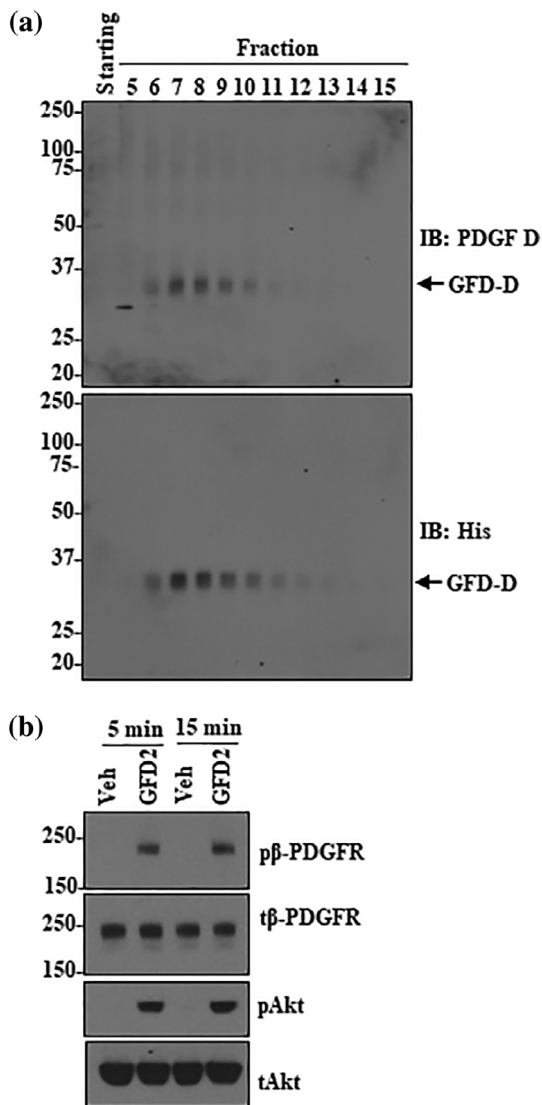
FIGURE 7 Purification of active growth factor domain dimer (GFD-D) of platelet-derived growth factor (PDGF) D. (a) Immunoblot analysis of purified rGFD-D proteins using anti-PDGF D (top) and anti-His (bottom) antibodies in a nonreducing condition. (b) NIH3T3 cells were stimulated with 1 nM rGFD-D for 5 or 15 min then β-PDGFR activation and subjected to immunoblot analysis for the indicated markers. The control (veh) solution, prepared from conditioned media (CM) of control virus-infected BHK21cell and gone through the same His-Trap purification procedure, was included as a negative control

confirmation of the production of the desired recombinant virus.

In this report, we demonstrate that the second generation MVA virus expression system is very effective in producing secreted recombinant proteins in particular many variants of PDGF D. The PDGF family members undergo sophisticated posttranslational modifications including dimerization, glycosylation, and proteolytic processing, essential for their activities.[4,13,18,39] Unlike the classic PDGF ligands PDGF A and B dimers, PDGF D is secreted as a latent growth factor dimer containing the inhibitory CUB domain. In addition to the intracellular posttranslational modifications, the latent PDGF D dimer undergoes extracellular proteolytic processing which removes the CUB domains for the generation of active GFD-D in a two-step manner via the generation of HD. As a member of the PDGF family of ligands, PDGF D has been shown to play diverse roles in the pathophysiology of the eye, kidney, vasculature as well as a myriad of malignancies including prostate cancer.[4,18,40–42] Increasing evidence demonstrate the functional differences between the two β-PDGFR ligands, PDGF B and D, indicating potential roles of the CUB domain in the modulation of PDGF D signaling and/or PDGF D interactions with ECM, thereby regulating the PDGF D distribution within tissues.

To dissect the roles of different PDGF D dimer species in a wide array of physiological and pathological processes, it is essential to produce structurally stable and biologically active recombinant PDGF D variants. Currently, GFD-D of PDGF D is commercially available from "R&D Systems," produced in NS0-derived murine myeloma cells; however, our analysis raises a concern as to its purity (data not shown). Using a germ wheat expression system, "Abnova" has generated full length PDGF D, FLD. However, the biological activity of this recombinant protein is not demonstrated by the vendor. Since the proper posttranslational modifications such as the formation of disulfide bonds, glycosylation, and phosphorylation are not warranted in the cell-free wheat germ protein expression system,[43] this cell-free protein expression system does not appear to be ideal for the production of PDGF D dimer species which require proper posttranslational modifications. In the current study, using the second generation MVA virus protein expression system combined with high performance HisTrap IMAC, we successfully produced and purified recombinant PDGF D dimer species which main biological activity and chemical integrity.

A challenge to recombinant protein production is the generation of heterodimeric proteins.[16] The classic PDGF ligands, PDGF A and B, are secreted as homo- or heterodimers.[1,4] To produce the PDGF AB heterodimer, PDGF A and B genes can be expressed in two separate constructs or in one construct as a bicistronic message.[16] Although endogenous HD is produced by extracellular proteolytic cleavage of one CUB domain in the FL PDGF D homodimer, our approach to produce stable HD, resistant to undergo further proteolytic cleavage by serine protease is the formation of heterodimerization of CRD and GFD2 (Figure 6a). Through co-infection and transfection of BHK21 cells with MVA-T7g virus and two separate

expression plasmids one containing the His-tagged CRD and untagged GFD2 genes, respectively, we successfully established a mixture of His-tagged CRD-MVA-T7g and GFD2-MVA-T7g viruses for the generation of stable HD.

We expect that recombinant PDGF D dimer proteins produced in this study will be valuable not only for investigating the complex roles of PDGF D in vitro as well as in vivo, but also for potential clinical applications. Receptor tyrosine kinase signaling induced by growth factors such as PDGF B, was shown to enhance osteogenic activity of BMP or TGF-β.[44,45] However, its efficacy depends on the optimal concentrations of PDGF B relative to BMP-2 or TGF-β and the sequential schedule of PDGF delivery to BMP-2 rather than simultaneous administration,[46,47] making it difficult to use as a therapeutic intervention. Importantly, our group has shown a critical role of PDGF D in bone biology. PDGF D initiates bone remodeling through osteoclast activation involving HD of PDGF D, resulting in overall osteoblastic responses.[27] Our recent study further demonstrates the direct role of GFD-D of PDGF D in promoting osteoblast differentiation of human bone marrow mesenchymal cells.[48] In the future, we envision the clinical use of rPDGF D dimer proteins to control physiological or pathological processes including bone healing at the injury sites. The second generation MVA viral system lends itself as an easy scalable system and the His-tag used to purify rPDGF D could either be removed proteolytically or alternatively would display very low immunogenicity and would not hinder preclinical or clinical use of the produced recombinant proteins.[49]

Taken together, the current work reports on a new MVA viral system that improves the first generation MVA system allowing for easier recombinant virus isolation. This novel system was used to produce homo- and heterodimeric variants of PDGF D that are shown to preserve their chemical integrity and biological activity.

AUTHOR CONTRIBUTIONS

Abdo J. Najy: Conceptualization (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (equal); writing – original draft (equal); writing – review and editing (equal). **Tri Pham:** Conceptualization (equal); formal analysis (equal); methodology (equal); writing – review and editing (equal). **Karine Pradeau-Aubreton:** Methodology (equal). **Robert Drillien:** Methodology (equal); resources (equal); writing – review and editing (equal). **Hyeong-Reh Choi Kim:** Conceptualization (equal); formal analysis (equal); funding acquisition (equal); methodology (equal); supervision (equal); writing – original draft (equal); writing – review and editing (equal).

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